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Effet thérapeutique des vésicules extracellulaires isolées à partir de cellules stromales mésenchymateuses dans la sclérodermie systémique

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UNIVERSITÉ DE MONTPELLIER

Effet thérapeutique des vésicules extracellulaires isolées à partir de cellules stromales mésenchymateuses dans la sclérodermie systémique

La sclérodermie systémique (ScS) est une maladie rare et sévère, caractérisée par une dérégulation des cellules immunitaires, une fibrose et une vasculopathie généralisées. Aucun traitement curatif n'est validé à ce jour. Les cellules stromales mésenchymateuses (CSMs), dont l'efficacité a été démontrée dans plusieurs modèles précliniques de ScS, sont actuellement évaluées dans des essais cliniques. Elles exercent leurs propriétés régénératrices au travers de la sécrétion de médiateurs libérés dans le milieu extracellulaire ou contenus dans des vésicules extracellulaires (EVs). Nous nous intéressons ici au rôle des EVs libérées par les CSMs dans leur effet systémique au cours de la ScS.

Dans un modèle murin de ScS induit par des injections d'acide hypochloreux, nous démontrons que les EVs stoppent l'évolution de la maladie au niveau cutané et pulmonaire sur le plan clinique, histologique et moléculaire. Nous identifions miR-29a-3p au sein des CSMs et de leurs EVs et prouvons qu'il est garant de leur effet thérapeutique, en ciblant *Dnmt3a* et *Pdgfr-bb,* deux nouvelles cibles identifiées dans la ScS.

Nous améliorons l'efficacité thérapeutique des EVs sur les anomalies pulmonaires en pré-activant les CSMs à l'aide d'IFNγ alors que l'augmentation de la quantité d'EVs injectées n'a aucun effet. L'IFNγ module l'expression de molécules immunosuppressives dans les CSMs et leurs EVs, potentialisant ainsi leurs propriétés thérapeutiques.

Alors que le rôle des CSMs de patients (CSMs-ScS) dans le développement de la maladie est sujet à débat, nous montrons que les CSMs-ScS inhibent *in vitro* la prolifération des lymphocytes T de manière similaire à des CSMs saines. En outre, elles sont capables d'améliorer le phénotype myofibroblastique de fibroblastes stimulés par du TGFβ1 qui présentent un comportement similaire à celui de fibroblastes de patients dans le modèle *in vitro* mis en place.

En conclusion, les EVs sont aussi efficaces que les CSMs pour traiter la ScS murine et le pré-conditionnement des CSMs par l'IFNγ améliore leur effet au niveau pulmonaire. Les CSMs de patients conservent leurs fonctions anti-fibrotique et immunosuppressive *in vitro* et une de nos perspectives est d'évaluer leur efficacité thérapeutique *in vivo*.

Mots clés : sclérodermie systémique, cellules stromales mésenchymateuses, vésicules extracellulaires, miR-29a-3p, TGFβ1, fibrose

Therapeutic effect of extracellular vesicles derived from mesenchymal stromal cells in systemic sclerosis

Systemic sclerosis (SSc) is a rare and severe disease characterized by generalized fibrosis, vasculopathy and deregulation of immune cells. No curative treatment is validated to date. Mesenchymal stromal cells (MSCs) are being evaluated in clinical trials as they are effective in several preclinical models of SSc. They exert their anti-fibrotic, pro-angiogenic and immunomodulatory properties through the secretion of mediators contained in extracellular vesicles (EVs). We investigate here the role of EVs released by MSCs in their systemic effect during SSc.

In a murine model of hypochlorous acid-induced SSc, we demonstrate that EVs can stop the disease course and improve histological and molecular parameters in skin and lung samples. We identify miR-29a-3p expression in both MSCs and their EVs and prove that miR-29a-3p is responsible for a large part of their therapeutic effect, targeting *Dnmt3a* and *Pdgfr-bb,* two novel targets identified in SSc.

We improve the therapeutic efficacy of EVs on lung abnormalities in SSc mice by stimulating MSCs with IFNγ before EVs production whereas increasing the amount of injected EVs did not have any benefit. IFNγ modulates the expression of known immunosuppressive factors in both MSCs and EVs, potentiating their therapeutic properties.

While the role of MSCs from SSc patients (SSc MSCs) in the development of the disease is debated, we show that SSc MSCs inhibit *in vitro* T lymphocyte proliferation and improve the myofibroblastic phenotype obtained after fibroblast stimulation using TGFβ1 which display a similar profile as fibroblasts from SSc patients in the *in vitro* model used here.

Overall, MSC-EVs are as effective as MSCs to stop the progression of murine SSc and EVs from IFNγ-preconditioned MSCs improve their beneficial effect in the lungs. SSc MSCs maintain their anti-fibrotic and immunosuppressive properties *in vitro* and one of our perspectives is to evaluate their therapeutic efficacy *in vivo*.

Key words: systemic sclerosis, mesenchymal stromal cells, extracellular vesicles, miR-29a-3p, TGFβ1, fibrosis

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LISTE DES ABREVIATIONS

(a)Ac	(auto) anticorps
AAN	auto-anticorps antinucléaires
ADAM	métalloprotéinase comprenant un domaine désintegrine
ADN	acide désoxyribonucléique
ARN	acide ribonucléique
ASC(s)	cellule(s) stromale(s) mésenchymateuse(s) isolée(s) du tissu adipeux
BAX	protéine Bcl-2–associated X
BCL2	B-cell lymphoma 2
CD	cluster de différentiation
CE	cellules endothéliales
CMH	complexe majeur d'histocompatiblité
CMV	cytomégalovirus
CS	cellules souches
CSH	cellules souches hématopoïétiques
CSM(s)	cellule(s) stromale(s) mésenchymateuse(s)
CTGF	facteur de croissance du tissu conjonctif
CXCL	ligand de chimiokines (motif C-X-C)
DNMT	ADN méthyltransférases
d-ScS	sclérodermie systémique de forme cutanée diffuse
endoMT	transition endothélio-mésenchymateuse
EPO	érythropoïétine
ESCRT	Endosomal Sorting Complex Required for Transport
ET	endothéline
EVs	vésicules extracellulaires
FGF	facteur de croissance des fibroblastes
Fli-1	Friend Leukemia Integration 1
FN	fibronectine
FOX	Forkhead box
FRO	formes réactives de l'oxygène
FSV	fraction stroma vasculaire
GFP	protéine fluorescente verte
GVH	greffon contre l'hôte
HGF	facteur de croissance des hépatocytes
HLA	antigènes des leucocytes humains
HOCl	acide hypochloreux
HP	hélicobacter pylori
HTAP	hypertension artérielle pulmonaire
ICST	société internationale de thérapie cellulaire
IDO	Indoléamine 2,3-dioxygenase
IFN	interféron
IGF	facteur de croissance ressemblant à l'insuline
IL	interleukine
iNOS	oxyde nitrique synthase induite
IRF	facteur régulateur de l'interféron
IRMB	Institut de Médecine Régénérative et de Biothérapies
ISEV	société internationale des vésicules extra-cellulaires
LB	lymphocytes B
lncRNA	ARN longs non codants
l-ScS	sclérodermie systémique de forme cutanée limitée
LT	lymphocytes T
MCP	protéine chimio attractante des monocytes
MEC	matrice extracellulaire

MECP2	methyl CpG binding protein 2
MMP	métalloprotéinases
MO	moelle osseuse
MV	microvésicules
NLRP	récepteur de type NOD, contenant un domaine pyrin en N-terminal
NO	oxyde nitrique
PBMCs	cellules mononuclées du sang périphérique
PBS	tampon phosphate salin
PDGF	facteur de croissance dérivé des plaquettes (R : récepteur ; -BB/-DD : isoforme du PDGF de type BB ou DD)
PGE2	prostaglandine E2
PID	pneumopathie interstitielle diffuse
PPARγ	récepteurs γ activés par les prolifératéurs de peroxysomes
Pr	Professeur
RR	risque relatif
ScS	sclérodermie systémique
SDF	facteur dérivé des cellules stromales
SDRA	syndrome de détresse respiratoire aiguë
SGS	syndrome de Gougerot Sjögren
SMAD	Sma Mother Against Decapentaplegic
SOCS	suppresseur des protéines de la signalisation des cytokines
TA	tissu adipeux
TAM	transition adipocytes à myofibroblastes
TEM	transition épithélio-mésenchymateuse
TET1	Tet méthylcytosine dioxygénase de type 1
TGF	facteur de croissance transformant
THBS	thrombospondine
TIMP	inhibiteur tissulaire des métalloprotéinases
TLR	récepteur de type Toll
TNF	facteur de nécrose tumorale
TSG6	gène stimulé par le TNF de type 6
VEGF	facteur de croissance de l'endothélium vasculaire

AVANT-PROPOS

La prise en charge des patients atteints de maladies systémiques auto-immunes rares figure parmi les différentes missions du service clinique de Médecine Interne et Maladies Multi-Organiques (MIMMO) dirigé par le Professeur (Pr) Philippe Guilpain, au sein du CHU de Montpellier. Le service est d'ailleurs Centre de Compétences pour les Maladies Auto-Immunes Systémiques Rares de l'Adulte. Ainsi, les patients atteints de sclérodermie systémique (ScS) y sont orientés pour la prise en charge diagnostique et thérapeutique, cette dernière se limitant actuellement à une approche palliative et symptomatique. Les enjeux de la recherche sur la ScS sont importants afin de pouvoir proposer dans les prochaines années un traitement curatif à ces personnes, souvent jeunes, atteints d'une maladie incurable et potentiellement mortelle.

Dans cette perspective, depuis 2011, le service clinique de MIMMO travaille en étroite collaboration avec l'équipe du Pr Christian Jorgensen de l'Institut de Médecine Régénératrice et de Biothérapies (IRMB). Le Pr Philippe Guilpain puis le Dr Alexandre Maria ont rejoint le groupe du Pr Danièle Noël, intitulé « Organoïdes, cellules souches mésenchymateuses (CSMs) et vésicules extracellulaires pour la thérapie des maladies ostéo-articulaires ». Le Pr Guilpain a instauré le modèle murin de ScS induit par l'acide hypochloreux (HOCl) au sein de l'IRMB, après avoir participé à son élaboration dans l'équipe du Pr Frédéric Batteux quelques années plus tôt. Puis, le Dr Maria, au cours de son travail de thèse, a prouvé l'efficacité thérapeutique des CSMs, au sein de ce modèle. Il a montré qu'un traitement par CSMs murines ou par CSMs humaines isolées à partir de tissu adipeux ou de moelle osseuse, dans une approche préventive ou curative, permet de stopper l'évolution de la maladie chez les souris. Au cours de la même période, une autre doctorante du groupe, Stella Cosenza, a mis au point les méthodes d'isolement et de caractérisation des vésicules extracellulaires (EVs) produites par les CSMs murines, et a montré que ces EVs sont un traitement aussi efficace que les CSMs dans des modèles pré-cliniques d'arthrose et d'arthrite.

Mon travail de thèse s'inscrit dans la continuité de ces projets et se base sur l'observation suivante : l'injection intraveineuse de CSMs dans le modèle murin de ScS entraîne une diminution des symptômes cliniques et des anomalies histologiques et moléculaires dans les prélèvements de peaux et de poumons, alors que les cellules restaient piégées au niveau pulmonaire. Cet effet semblait donc lié à la libération systémique de différents facteurs par les CSMs. Ainsi, mon projet de thèse a pris naissance autour de cette question : les EVs libérées par les CSMs étaient-elles responsables de leur effet systémique dans la ScS ?

Mon premier axe de travail a été dédié à l'évaluation de l'effet thérapeutique d'EVs isolées à partir de CSMs dans ce modèle murin de ScS et l'identification d'un ou plusieurs médiateurs responsables de leur effet thérapeutique. Nous nous sommes intéressés à miR-29a, connu pour ses propriétés anti-fibrosantes et sa diminution d'expression dans les tissus fibrotiques de patients sclérodermiques. Enfin, sachant que le sécrétome et les fonctions des CSMs sont modulés par l'environnement dans lequel elles évoluent, nous avons étudié l'effet d'une pré-activation des CSMs par l'IFNγ sur la fonctionnalité des EVs produites.

Un second axe de travail a été consacré à l'étude du rôle physiopathologique des CSMs dans la ScS. En effet, dans une perspective de thérapie cellulaire, il est important de prendre en considération l'effet de l'environnement sclérodermique sur les propriétés biologiques des CSMs et de leurs EVs. Le rôle physiopathologique des CSMs au cours de la ScS est un sujet à controverses dans la littérature et l'environnement notamment riche en Transforming Growth Factor β1 (TGFβ1), pourrait influer la fonction des CSMs présentes dans de nombreux tissus. Alors que les essais cliniques ont déjà débuté, il s'avère important de s'assurer que des approches auto- ou allogéniques seraient tout aussi efficaces. C'est pourquoi, nous avons évalué l'efficacité thérapeutique des CSMs de patients ScS et de leurs EVs dans des modèles *in vitro* de ScS.

L'introduction bibliographique de ce manuscrit va être divisée en trois parties afin d'entrapercevoir une synthèse de la littérature concernant : 1- la ScS et tout particulièrement sa physiopathologie ; 2- les CSMs et leur effet thérapeutique notamment au cours de la ScS ; 3 - les EVs et le potentiel thérapeutique des EVs produites par les CSMs. Puis, nous présenterons les résultats de mes travaux originaux autour de trois articles que nous discuterons dans la dernière partie, tout en présentant nos perspectives.

INTRODUCTION BIBLIOGRAPHIQUE

1ère Partie : La sclérodermie systémique

La sclérodermie systémique est, par définition, une maladie systémique auto-immune rare, intégrant le groupe des connectivites. Elle est caractérisée principalement par une vasculopathie et une fibrose, affectant la peau et pouvant s'étendre à différents organes tels que les poumons, le cœur, le rein ou encore l'appareil digestif. La sclérodermie systémique (ScS) est à distinguer des formes de sclérodermies localisées, appelée aussi Morphée, au cours desquelles la maladie est strictement limitée à la peau, ou tout au plus aux structures sous-jacentes (1). Dans cette première partie de l'introduction, nous allons décrire successivement les différentes classifications, les manifestations cliniques et immunologiques, l'épidémiologie et le pronostic, la physiopathologie, les modèles d'études et enfin les différents traitements disponibles ou émergeants, de la ScS.

I) Les différentes classifications de la ScS

Historiquement, la première classification établie par Leroy *et al* (2,3) distinguait deux formes principales de ScS dépendant de l'étendue topographique de l'atteinte cutanée :

- La ScS cutanée limitée (l-ScS), au cours de laquelle la sclérose cutanée ne touche que les extrémités distales des membres supérieurs et inférieurs (en deçà des coudes et des genoux) ainsi que le pourtour de la bouche et le visage ;
- La ScS cutanée diffuse (d-ScS), au cours de laquelle la sclérose cutanée peut concerner l'ensemble du tégument.

Moins de 5 % des patients présentent une ScS *sine scleroderma*, qui est une forme rare de ScS où aucun épaississement cutané n'est palpable mais au cours de laquelle les phénomènes de fibrose impliquent différents organes.

En 2013, la Ligue Européenne Contre les Rhumatismes (EULAR) et le Collège Américain de Rhumatologie (ACR) ont établi de nouveaux critères qui présentent une meilleure sensibilité et une meilleure spécificité pour la classification des patients atteints de ScS. Ces critères sont actuellement un outil d'aide au diagnostic dans la pratique clinique quotidienne et servent principalement à homogénéiser les populations incluses dans les essais cliniques en recherche (4). D'après ces recommandations, le diagnostic de ScS peut être retenu si le patient présente un ou plusieurs des critères présentés dans le Tableau 1 et que la somme des points obtenus est supérieure ou égale à 9.

Tableau 1 – Critères de classification de la ScS, selon l'EULAR et l'ACR (2013)

domaine	critères($)	score(*)
épaississement cutané (ne tenir compte que du score le plus élevé)	sclérodactylie s'étendant au-delà des articulations MCP	9
	sclérodactylie ne dépassant pas les articulations MCP	4
	doigts boudinés	2
lésions pulpaires (ne tenir compte que du score le plus élevé)	cicatrices déprimés	3
	ulcères pulpaires digitaux	2
phénomène de Raynaud		3
atteintes pulmonaires	PID et/ou HTAP	2
télangiectasies		2
anomalies capillaroscopiques		2
Ac spécifiques de la ScS	Ac anti-centromères Ac anti-Scl70 Ac anti-RNA polymérase III	3

MCP : métacarpophalangiennes ; PID : pneumopathie interstielle diffuse ; HTAP : hypertension artérielle pulmonaire, Ac : auto-anticorps ; ScS : sclérodermie systémique
$: le critère est retenu, s'il a été mis en évidence au moins une fois au cours de l'histoire de la maladie
* le score de chaque item présent est additionné pour définir un score total. Si celui-ci est supérieur ou égale à 9, le diagnostic de ScS peut être retenu.

Adapté de Van Den Hoogen F et al, Arthritis & Rheumatism (2013)

Aucune classification à ce jour ne permet de couvrir l'ensemble des patients atteints de ScS devant la très grande hétérogénéité de cette maladie, tant sur le plan des atteintes cliniques (cutanées et viscérales) que sur les données immunologiques (panel d'auto-anticorps retrouvés chez les patients). Une grande hétérogénéité est également à souligner en termes d'évolution naturelle de la maladie et de réponse clinique aux traitements, notamment en fonction de la phase de la maladie où le patient est pris en charge. En effet, une autre « classe » de patients a été décrite : les patients de diagnostic très précoce. Ces malades ne rentrent pas forcément dans les critères de 2013 qui ne seraient pas assez sensibles pour les détecter (5). Leur maladie pourrait être encore réversible à ce stade après la mise en place d'un traitement, immunosuppresseur notamment (6,7). Un diagnostic et une prise en charge précoces sont donc primordiaux.

II) Les manifestations cliniques de la ScS

1) La sclérose cutanée

En dehors de la forme de ScS *sine scleroderma*, par définition, la fibrose ou sclérose cutanée est un signe constamment retrouvé chez les patients. Parfois, la sclérose cutanée apparaît seulement au cours de l'évolution de la maladie. Elle touche souvent en premier lieu les doigts : on parle alors de sclérodactylie. Lorsque la sclérodactylie s'étend au-delà des articulations métacarpo-phalangiennes, le diagnostic de ScS peut être posé selon les critères de 2013 (4).

La sclérose cutanée peut se présenter sous différents aspects cliniques et évoluer en plusieurs phases. Au début de la maladie, on parle d'une phase œdémateuse au cours de laquelle les doigts (parfois les mains ou même les membres) paraîtront principalement boudinés, gonflés et douloureux, pouvant conduire à tort vers un diagnostic d'arthrite. La palpation de la peau donne un aspect plus infiltré qu'épaissi. Cette phase, inconstante, s'observe généralement dans les formes de d-ScS. Ensuite, l'inflammation régresse et laisse place, petit à petit, au phénomène de fibrose, responsable d'un épaississement cutané que l'on palpe cliniquement. La peau est indurée et ne peut plus se plisser entre les doigts ; parfois la peau adhère même aux plans profonds. Cet épaississement cutané est classiquement quantifié à l'examen clinique par le score de Rodnan modifié. Ce score permet notamment de suivre l'évolution de la maladie chez les patients (8,9). Il a également été validé pour être un outil pronostic dans les formes de d-ScS. Parfois, l'atteinte cutanée peut évoluer vers une 3ème phase au cours de laquelle elle semble spontanément régresser. Cette phase n'est pour autant pas synonyme de guérison.

Ces atteintes cutanées sont responsables de limitations fonctionnelles telles que des difficultés de préhension, d'ouverture de la bouche (Figure 1) mais aussi de restrictions dans la capacité respiratoire lorsque l'atteinte cutanée du tronc est sévère. Ainsi, le handicap des patients commence déjà par l'atteinte princeps cutané.

Figure 1 – Photographie d'une sclérose cutanée



Sclérose cutanée de la face,
Limitation de l'ouverture buccale et télangiectasies

D'après Sticherling M, Journal of the German Society of Dermatology (2019)

2) Le phénomène de Raynaud

Le phénomène de Raynaud concerne plus de 95% des patients atteints de ScS. Il est souvent le premier signe de la maladie et reste isolé pendant plusieurs années sans laisser présager la survenue d'une ScS. Le phénomène de Raynaud un acrosyndrome secondaire à un trouble vasomoteur entraînant l'arrêt brutal et transitoire de la circulation artérielle des doigts. Il est principalement favorisé par le froid, l'humidité mais aussi les émotions et le stress. C'est un phénomène fréquent en population générale.

Cette ischémie paroxystique des extrémités se manifeste par une crise se déroulant typiquement en trois phases, même si la première peut parfois être isolée. La 1ère phase dite syncopale se manifeste par des doigts blancs et froids : elle dure quelques minutes. Puis, une 2ème phase dite asphyxique apparaît : le tégument devient alors de couleur bleue et le patient ressent des dysesthésies douloureuses (Figure 2). Enfin, au cours de la 3ème phase dite de récupération, les doigts deviennent rouges, tuméfiés et douloureux (10).

Figure 2 – Photographie d'un phénomène de Raynaud



A gauche : phase syncopale ; à droite : phase asphyxique.

D'après Legendre P et Mouthon L, La revue du praticien (2017)

Lorsque la ScS est installée, le phénomène de Raynaud est souvent associé à des troubles trophiques digitaux. En effet, les patients peuvent être victimes de véritables ulcérations laissant une cicatrice pulpaire persistante *ad vitam aeternam* lorsque les plaies arrivent à se refermer, ce qui n'est pas toujours le cas. Ces ulcères digitaux sont soit secondaires à une ischémie de l'extrémité d'un doigt et peut alors évoluer vers la nécrose, soit secondaire à un traumatisme dont la cicatrisation est difficile. L'ensemble de ces atteintes est dû à une vasculopathie, dont nous détaillerons les mécanismes dans le chapitre [V)3)], qui touche le système macro vasculaire (11) et notamment les artères digitales (12), mais aussi et avant tout, la microcirculation sanguine. D'ailleurs, le diagnostic de ScS repose en partie sur la visualisation d'anomalies vasculaires à la capillaroscopie unguéale, qui est l'examen de référence pour le dépistage de la microangiopathie organique. Cet examen permet notamment de visualiser la présence de méga capillaires et une raréfaction des capillaires : ces anomalies ne sont pas spécifiques à 100% de la ScS, mais sont très caractéristiques (13). De fait, les patients souffrant d'un phénomène de Raynaud isolé mais qui présentent des anomalies à la capillaroscopie unguéale, sont plus à risque d'évoluer vers un véritable tableau clinique de ScS (14).

3) Les autres manifestations cutanéo-muqueuses

Le phénomène de Raynaud et la sclérose cutanée sont les symptômes cliniques les plus fréquents au niveau du tégument, mais ce ne sont pas les seuls (15). Tout d'abord, les patients peuvent développer des calcinoses qui sont liées à des dépôts de sels phosphocalciques insolubles dans la peau et les tissus sous-cutanés (16). Lorsqu'elles sont présentes, les calcinoses siègent principalement aux extrémités des doigts des mains, aux faces d'extension des avant-bras et aux faces antérieures des genoux. Elles peuvent également toucher des articulations, des muscles ou encore des tendons, responsables alors d'un lourd handicap fonctionnel. De plus, lorsqu'elles se fistulisent au niveau de la peau, elles peuvent entraîner des infections et sont très difficiles à cicatriser.

Ensuite, les patients peuvent souffrir de télangiectasies, localisées préférentiellement sur le visage (au pourtour des lèvres et sur les lèvres), dans la cavité buccale ainsi que sur les mains (Figure 1). Les télangiectasies sont secondaires à une dilatation des petits vaisseaux sanguins de la peau, rendant le trajet vasculaire visible à l'œil nu, souvent en forme d'étoile. Elles s'effacent à la vitropression. Les patients peuvent également avoir des plages d'hypo- ou d'hyper-pigmentation et dans de rares cas, un aspect mélanodermique.

Enfin, la ScS peut être authentiquement associée à d'autres maladies auto-immunes, dont le syndrome de Gougerot-Sjögren (SGS) au cours duquel le syndrome sec occulo-buccal est constamment présent. Sans répondre pour autant aux critères de classification du SGS (17), 20 % des patients sclérodermiques souffrent de sécheresse buccale (18). Elle est favorisée par l'hyposialie secondaire à l'infiltration fibreuse des glandes salivaires ainsi qu'à la respiration buccale fréquente chez ces malades présentant une incontinence labiale. Les patients peuvent ressentir très tôt un tiraillement de la peau et du prurit, favorisés par une sécheresse cutanée (19).

4) Les atteintes ostéo-articulaires de la ScS

La ScS entraîne différentes atteintes musculosquelettiques (20). Tout d'abord, les patients souffrent fréquemment de douleurs articulaires et péri-articulaires, douleurs le plus souvent d'horaire inflammatoire qui concernent les mains, les doigts et les poignets. Cependant, de réelles synovites ne sont observées que dans 10 à 20% des cas. Ensuite, la ScS affecte également les tendons, entraînant des frictions tendineuses ainsi que des rétractions, pouvant conduire dans les formes les plus graves, à des rétractions irréductibles des doigts responsables d'un lourd handicap fonctionnel. Les atteintes ténosynoviales sont un marqueur de sévérité de la maladie. De plus, la ScS peut également impacter le compartiment musculaire, se manifestant généralement par des myalgies.

Dans les formes sévères, la myopathie est alors responsable d'un déficit moteur proximal. Malgré une prévalence difficilement estimable dans la littérature, ce phénomène semble heureusement rare. Lorsque la ScS est associée à une véritable myopathie inflammatoire, les patients sont porteurs d'auto-anticorps (aAc) de spécificité variable : anti-Pm-Scl (50% des cas), anti-Ku, anti-U1-RNP ou encore anti-RuvBL-1/2 (21). Enfin, les patients peuvent souffrir d'ostéolyses, retrouvées principalement au niveau des phalanges distales des mains ou des pieds mais aussi au niveau de la mandibule et de l'articulation temporo-mandibulaire. Les patients atteints d'ostéolyses sont également à haut risque d'ostéoporose.

5) Les atteintes pulmonaires de la ScS

Les atteintes pulmonaires au cours de la ScS sont secondaires d'une part, à la fibrose du parenchyme pulmonaire responsable d'une pneumopathie interstitielle diffuse (PID) et d'autre part, à une hypertension artérielle pulmonaire dont l'étiologie est multifactorielle. Ces atteintes grèvent lourdement le pronostic vital et fonctionnel des patients, car elles altèrent les échanges gazeux et donc les capacités respiratoires (22).

a) *La pneumopathie interstitielle diffuse*

La prévalence de la PID est variable selon les études en fonction de la définition utilisée par les auteurs mais également de la forme clinique de PID déclarée. En effet, jusqu'à 80% des patients ont une atteinte interstitielle du parenchyme pulmonaire à l'imagerie scannographique (23) alors que seulement 40% des patients ont une anomalie cliniquement parlante (24). La maladie pulmonaire interstitielle est plus fréquente chez les patients d'origine Afro-Américaine, chez les personnes atteintes de la forme de d-ScS et chez les malades ayant certains aAc parmi lesquels on retrouve classiquement les aAc anti-topoisomérase 1.

De la simple toux sèche à la dyspnée hypoxémiante requérant une oxygénothérapie au long cours, la fibrose du parenchyme pulmonaire se manifeste cliniquement alors qu'elle est installée depuis de nombreuses années et souvent irréversible. Un dépistage et un diagnostic précoces sont primordiaux car la mise en place de certains traitements actuellement disponibles pourrait éviter l'installation d'une fibrose irréversible et destructrice du parenchyme.

Classiquement, la PID se localise initialement au niveau des bases des poumons ainsi qu'à la partie postérieure des lobes, pour s'étendre progressivement vers les hiles pulmonaires puis vers les sommets. Mais les formes cliniques de PID ainsi que leur progression naturelle sont variables en fonction des patients. Tout l'enjeu actuel est d'arriver à discriminer rapidement les formes d'évolution sévère des formes les plus bénignes, notamment à l'aide de biomarqueurs, afin de proposer une prise en charge la plus adaptée (25). L'évolution de l'atteinte parenchymateuse concerne surtout les 5 premières années d'évolution de la maladie

après l'apparition du premier symptôme (en dehors du phénomène de Raynaud) et rarement après 15 ans d'évolution (26).

b) *L'hypertension artérielle pulmonaire*

La dyspnée des patients peut également être induite par une hypertension artérielle pulmonaire (HTAP) isolée ou associée à la PID. Cette atteinte sévère concerne près de 10% des patients. L'HTAP peut être secondaire à la PID et/ou à l'atteinte cardiaque ainsi qu'à une vasculopathie des petites artères ou plus rarement à une maladie veno-occlusive (27). Tout comme la PID, l'HTAP reste longtemps asymptomatique. Lorsqu'elle se manifeste par de la dyspnée et/ou des signes d'insuffisance cardiaque droite, il est déjà trop tard. En effet, le temps de survie médian des malades atteints d'HTAP est de 3 ans (28). Les patients atteints de ScS répondent moins bien aux traitements classiques de l'HTAP que des patients atteints d'HTAP sans ScS (29). Finalement, un dépistage et une prise en charge précoces sont, ici aussi, indispensables.

6) Les atteintes cardiaques de la ScS

L'atteinte sclérodermique du cœur est secondaire à une fibrose du tissu cardiaque ainsi qu'à des anomalies de la microcirculation (30). Elle peut entraîner une insuffisance cardiaque mais aussi des troubles du rythme lorsque la fibrose touche le réseau électrique myocardique. La prévalence de l'atteinte cardiaque d'expression clinique est faible, mais lorsqu'elle est présente le pronostic vital est engagé. Cependant, il semblerait que 70% des patients auraient une fibrose myocardique infra-clinique (31). L'insuffisance cardiaque peut aussi être aggravée par l'HTAP. Le péricarde est rarement atteint, le cas échéant réalisant un tableau de péricardite. Les atteintes des valves cardiaques sont exceptionnelles, touchant alors principalement la valve mitrale mais quelques cas de sténose de la valve aortique ont également été rapportés dans la littérature (32–35).

Concernant les gros vaisseaux, on retrouve une description historique d'aortite en 1964 (36). Une étude récente portant sur 139 patients a montré qu'environ 6% des malades avait une dilatation de la racine de l'aorte, alors que cette pathologie est plus rare en population générale (0,05%) (37). Cette anomalie est corrélée à la raréfaction capillaire en capillaroscopie, suggérant que la micro-angiopathie contribuerait à la détérioration de la paroi aortique.

7) Les atteintes rénales de la ScS

Comme les autres organes, la fibrose et la vasculopathie peuvent toucher le rein. La forme clinique la plus sévère, redoutée par tous les cliniciens, est appelée « la crise rénale sclérodermique » (1). Elle est secondaire à l'atteinte vasculaire de la maladie et se manifeste par les signes cliniques d'une hypertension artérielle sévère, d'une insuffisance rénale aiguë voire de micro-angiopathie thrombotique. Heureusement, elle concerne un peu moins de 5% des patients. Différents facteurs prédictifs de survenue de cette crise rénale sclérodermique ont été identifiés et permettent ainsi de surveiller plus étroitement ces malades (38).

8) Les atteintes digestives de la ScS

Tout le système digestif peut être sujet à la maladie. Les lésions sont principalement secondaires au processus de fibrose qui engaine les fibres musculaires lisses entraînant ainsi des troubles de la motilité digestive. Mais, la vasculopathie contribue également à certaines lésions. La quasi-totalité des patients est concernée par une ou plusieurs atteintes digestives (39), qui sont responsables de troubles nutritionnels majeurs aggravant le pronostic de ces patients, déjà altéré. De plus, la dénutrition retarde les processus de cicatrisation des troubles trophiques, déjà difficiles du fait de l'atteinte vasculaire.

Dans la partie haute du tube digestif, l'œsophage et l'estomac sont les plus fréquemment atteints. Le reflux gastro-œsophagien en est la manifestation la plus précoce et concerne plus de 90% des patients : il peut se compliquer d'œsophagite. Dans les formes sévères de ScS, les patients souffrent d'une dysphagie à la suite des troubles moteurs et/ou de l'œsophagite. L'atteinte de l'estomac entraîne un syndrome dyspeptique pouvant conduire à une véritable anorexie par satiété précoce. En effet, la gastroparésie peut évoluer jusqu'à une atonie complète de la paroi musculaire. L'atteinte vasculaire de la muqueuse gastrique est responsable de télangiectasies réalisant un « estomac pastèque », pouvant se compliquer d'évènements hémorragiques potentiellement graves.

Les manifestations cliniques secondaires aux atteintes de la partie basse du tube digestif sont très polymorphes, allant d'une constipation simple (le plus fréquent) à de véritables tableaux d'occlusion intestinale (pseudo-obstruction intestinale chronique ou POIC). De plus, certains patients présentent des diarrhées, dans le cadre de syndrome de pullulation microbienne et/ou syndrome de malabsorption. Enfin, l'atteinte ano-rectale se manifestera par une incontinence anale et/ou un prolapsus rectal.

9) Les atteintes neuropsychologiques de la ScS

Des calcifications vasculaires du système nerveux central ainsi que des lésions de leucoaraïose sont plus fréquemment visibles chez les patients atteints de ScS qu'en population générale, mais leur expression clinique est exceptionnelle. La dépression, d'étiologie plurifactorielle, est répandue chez les patients ScS (17 à 65%) (40).

III) Les auto-anticorps retrouvés au cours de la ScS

La ScS est une maladie dite « auto-immune ». En effet, on retrouve des aAc anti-nucléaires (AAN), chez plus de 90% des malades. Cependant, l'antigène cible de ces aAc n'est pas déterminé dans 20 à 30% des cas. Lorsqu'on retrouve la spécificité des AAN, ils sont généralement mutuellement exclusifs. Le tableau 2 fait la synthèse des aAc actuellement recensés chez les patients atteints de ScS, sachant que les AAN les plus fréquemment retrouvés sont :

- les Ac anti-centromères (plus fréquemment associés aux formes l-ScS),
- les Ac anti-Scl70 (plus fréquemment associés aux formes d-ScS)
- ou encore les Ac anti-ARN polymérase III.

La présence de ces aAc permet d'orienter le diagnostic vers des formes de d-ScS ou de l-ScS, ou encore d'identifier des formes de ScS plus à risque de l'une ou l'autre des complications viscérales sus-citées (PID, HTAP, crise rénale sclérodermique). Cependant, ces associations « AAN-manifestations cliniques » ne sont ni systématiques, ni mutuellement exclusives. A ce jour, le rôle de chacun de ces aAc dans la physiopathologie de la maladie n'a pas été démontré : ils constituent donc, pour l'instant, une aide au diagnostic.

Aussi, les patients atteints de ScS souffrent plus fréquemment d'autres pathologies auto-immunes qu'en population générale et peuvent donc, à ce titre, développer des aAc spécifiques d'autres maladies associées. On peut citer par exemple les aAc anti-SSA(Ro) et les aAc anti-SSB(La) du SGS, les aAc anti-mitochondries de la cirrhose biliaire primitive, les aAc anti-peptides citrullinés de la polyarthrite rhumatoïde ou encore les aAc anti-thyroperoxydase de la thyroïdite d'Hashimoto. Enfin, la séroprévalence des aAc anti-phospholipides chez ces malades serait de 14% selon une méta-analyse publiée en 2018 (41).

Pour terminer, on peut doser d'autres aAc sériques chez les patients, qui ne sont pas recherchés en diagnostic clinique de routine. Ces aAc sont dirigés vers les fibroblastes ou les cellules endothéliales par exemple et leur implication physiopathologique n'est pas démontrée, une fois encore. Nous reviendrons sur ce point dans le paragraphe [V-5)e)].

Tableau 2 – Spécificité des auto-anticorps anti-nucléaires associés à la ScS

Spécificités des anticorps antinucléaires	Aspect de la fluorescence	% de patients	Forme de la maladie	Phénotype Clinique
Ac anti-centromère	Centromérique	20 à 40 %	ScS cutanée limitée	HTAP Atteinte vasculaire périphérique sévère
anti-Scl-70 ou Ac anti-topoisomérase	Homogène et nucléolaire	20 à 30 %	ScS cutanée diffuse	PID sévère Atteinte vasculaire périphérique sévère
anti-ARN polymérase de type III	Mouchetée (± nucléolaire)	20 à 25 % chez les caucasiens d'Amérique du Nord, au Royaume-Uni et environ 5 % en France, en Allemagne, en Italie et au Japon	ScS cutanée diffuse	Atteinte cutanée rapidement progressive Crise rénale sclérodermique Cancer
anti-U3-RNP (anti-fibrillarine)	Nucléolaire	Dans 4 à 10 %	ScS cutanée diffuse > ScS cutanée limitée	PID HTAP Crise rénale Atteinte digestive du grêle
Anti-Th/To	Nucléolaire	2 à 5 %	ScS cutanée limitée	PID HTAP
Anti-Pm-Scl	Moucheté et nucléolaire	2%	ScS cutanée limitée (chevauchement avec myosite)	Myosite
Anti-U1 RNP	Mouchetée		ScS cutanée limitée (chevauchement, MCTD)	Arthrite Myosite HTAP
Anti U11/U12 RNP	Mouchetée	1 à 3 %	ScS cutanée diffuse / ScS cutanée limitée	PID sévère
Anti-Ku	Mouchetée		ScS cutanée limitée (syndrome de chevauchement avec myosite)	Myosite
Anti-RuvBL 1/2	Mouchetée	1 à 3 %	ScS cutanée diffuse (chevauchement myosite)	Myosite

Ac : anticorps ; HTAP : hypertension artérielle pulmonaire ; PID : pneumopathie infiltrante diffuse ; ARN : acide ribonucléique ; RNP : ribonucléoprotéine ; MCTD : mixed connective tissue disease.

D'après le Protocole National de Soins de la ScS (version 2017, révision 2020)

IV) Epidémiologie, maladies associées et pronostic de la ScS

1) Prévalence et incidence de la ScS

La prévalence et l'incidence de la ScS sont variables selon la région du monde étudiée. La prévalence varie entre 30 cas par million d'habitants au Japon à 150 cas par million d'habitants en Europe du Nord, ou encore entre 246 et 443 cas par million d'habitants en Europe du Sud et en Amérique du Nord (42–44). L'incidence oscille entre 10, 14 et 21 cas par million d'habitants et par an pour les mêmes régions, respectivement (45). Ces données de la littérature sont parfois controversées pour un même pays, comme en témoigne une étude portant sur les Etats-Unis (43). En France, la prévalence varie également selon les régions concernées avec une incidence par million d'habitants de 132 cas en Lorraine (46), 228 cas en Alsace ou encore 158 cas en Seine St Denis (47). Ainsi, le nombre de cas total estimé en France est compris entre 6000 et 9000 personnes.

2) Sex-ratio et âge moyen au diagnostic

La ScS concerne généralement 6 fois plus de femmes que d'hommes, même si le sex-ratio varie selon les études entre 3 et 8. Par exemple, en Suède il était de 5.84 en 2014 (48) alors que dans une étude menée en France, il serait de 6.7 (46).

La ScS touche généralement des femmes d'âge moyen, avec un pic d'incidence compris entre 45 et 64 ans, sauf pour les malades d'origine africaine qui seraient affectées plus précocement (49). Les cas sont rares chez l'enfant puisqu'ils représentent moins de 5% des cas de ScS. Une étude menée en Angleterre recensait 0.27 cas de ScS pour 1 million d'enfants, avec un sex-ratio de 3.6 filles pour 1 garçon (50). Les sclérodermies localisées (Morphée) semblent plus fréquentes que les ScS chez l'enfant : le pronostic est donc meilleur.

3) Maladies associées à la ScS

Comme nous l'avons évoqué précédemment, les patients atteints de ScS peuvent souffrir d'autres maladies auto-immunes (51), parmi lesquelles on trouve le SGS, le lupus érythémateux disséminé, le syndrome des anti-phopholipides, la polyarthrite rhumatoïde, les myopathies inflammatoires (polymyosites et dermatopolymyosites), les thyroïdites auto-immunes et la cirrhose biliaire primitive. Ces deux dernières sont les moins fréquemment associées à la ScS (moins de 5%).

L'association « ScS et cancer » n'est pas rare puisque 3.6 à 10% des patients atteints de ScS développent au moins un cancer au cours de leur vie (52). Les cancers du sein, du poumon et de l'œsophage semblent particulièrement fréquents mais ils ne sont pas les seuls à rechercher. Les signes d'alertes sont l'altération de l'état général, un âge au diagnostic de la ScS supérieur à 60 ans et les formes de d-ScS associées aux aAc anti-ARN polymérase III. Ce sujet a fait l'objet d'une revue publiée en 2018 que je vous joins en Annexe 1 (53).

4) Pronostic de la ScS

Bien que la sclérose cutanée soit l'atteinte princeps de la ScS, c'est l'extension de la fibrose aux différents organes qui conditionne le pronostic de la maladie (54). Très souvent, le pronostic vital est engagé du fait d'une atteinte pulmonaire sévère : la PID et l'HTAP sont en effet responsables de, respectivement, 14 et 10% des décès. La défaillance cardiaque est également une des principales causes de mortalité (55). Ces atteintes sont donc à dépister et à prendre en charge le plus précocement possible, même en l'absence de symptômes puisqu'elles peuvent rester longtemps silencieuses tout en faisant des ravages tissulaires à bas bruit. Dans les formes de d-ScS, ces manifestations viscérales vont apparaître, le plus souvent, dans les 3 à 5 ans suivant le 1er symptôme (hors phénomène de Raynaud), en dehors de l'HTAP qui peut se développer bien plus tard. De son côté, lorsqu'elle s'aggrave, la sclérose cutanée évoluera principalement sur les 5 premières années de la maladie. Cette progression est alors un facteur prédictif d'évolution péjorative de la maladie au niveau systémique, et

notamment de déclin de la fonction pulmonaire (56,57). Les patients atteints de l-ScS seront moins touchés par les manifestations viscérales, en dehors de l'HTAP et des complications digestives qui restent toujours menaçantes. Finalement, les taux de survie à 10 ans des malades atteints de d-ScS et de l-ScS sont, respectivement, de 60 et de 80%.

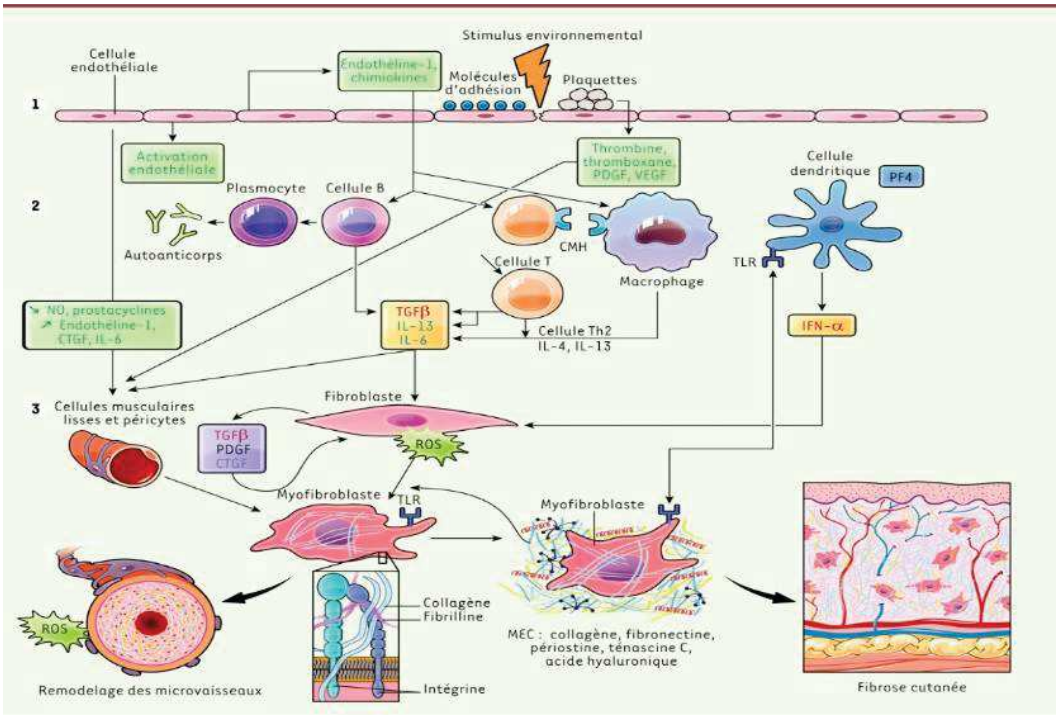
En dehors des atteintes d'organes nobles sus-citées, d'autres facteurs semblent également influer le pronostic, telle que l'origine ethnique. En effet, un travail portant sur plus de 9000 malades a montré que les patients d'origine asiatique présentent des formes plus sévères de ScS que les patients d'origine caucasienne (58). De même, une autre étude plus ancienne a montré que les patients d'origine africaine ont un moins bon pronostic (59). Au total, le taux de mortalité standardisé est probablement le moins bon de toutes les maladies auto-immunes systémiques et il ne semble pas s'améliorer depuis plusieurs années (60), soulignant l'absence de thérapies curatives à ce jour (54). En sus de l'importante mortalité, la ScS est également responsable d'une lourde morbidité et les handicaps sont grands. Ces malades ont très souvent une qualité de vie altérée du fait de douleurs (61) et de troubles fonctionnels secondaires aux phénomènes de fibrose (62).

En résumé, la ScS est une maladie sévère pour laquelle les besoins en traitements efficaces sont élevés ; nous en reparlerons plus tard dans un chapitre dédié aux traitements [VII]. Pour trouver et proposer un traitement curatif et spécifique de la ScS, il faut avant tout en comprendre la physiopathologie, sujet que nous allons détailler dans le prochain chapitre.

V) La physiopathologie de la ScS

La ScS est une pathologie complexe. Des facteurs environnementaux pourraient précipiter la survenue de la maladie qui semble se développer sur un terrain génétique prédisposant. L'ensemble de ces facteurs n'est cependant pas connu à ce jour. Quoiqu'il en soit, la pathologie se développe autour d'un dysfonctionnement conjoint des cellules endothéliales, des fibroblastes et du système immunitaire. Ces trois axes de dysfonctionnement aboutissent finalement au développement d'une fibrose tissulaire et d'une vasculopathie généralisée, qui se traduisent par les différents symptômes cliniques précédemment décrits. D'ailleurs, nous avons pu voir que la ScS est une maladie particulièrement hétérogène au niveau clinique et biologique : cette hétérogénéité rend d'autant plus complexe la compréhension des différents mécanismes physiopathologiques. En 2016, le Pr Yannick Allanore a proposé une synthèse de la physiopathologie de la ScS et des interconnexions entre ces 3 axes de dysfonctionnement (63) (Figure 3).

Figure 3 – Synthèse des connaissances sur la physiopathologie de la ScS



PDGF : facteur de croissance dérivé des plaquettes ; VEGF : facteur de croissance de l'endothélium vasculaire ; CMH : complexe majeur d'histocompatibilité ; CTGF facteur de croissance du tissu conjonctif; TGF : facteur de croissance transformant ; TLR : récepteur de type Toll ; MEC : matrice extracellulaire ; PF4 : facteur plaquettaire 4 ; IFN : interféron ; ROS : espèces réactives de l'oxygène ; IL : interleukine ; NO : monoxyde d'azote

D'après Allanore Y et al, revue Médecine Sciences (2016)

Dans ce chapitre, après avoir évoqué le rôle des facteurs génétiques et des facteurs environnementaux, nous allons synthétiser les connaissances disponibles à ce jour concernant les mécanismes impliqués dans la physiopathologie de la ScS, en les regroupant autour des trois axes de dysfonctionnement sus-cités par souci d'être didactique, même si nous ne le répèterons pas assez, ils peuvent être liés et interconnectés.

1) Rôle des facteurs génétiques

La ScS n'est pas une maladie héréditaire à transmission monogénique. Cependant, les cas familiaux sont nombreux et le risque relatif (RR) de développer une ScS lorsque son frère ou sa sœur est atteint de ScS est compris entre 15 et 19, comparé à la population générale. Pour les autres apparentés du 1er degré (parent, enfant) ce RR est de 13 à 15 (64). Une étude portant sur 1037 patients dans la population de l'Utah a confirmé cet excès de risque pour la ScS mais aussi pour d'autres maladies auto-immunes chez les parents au premier et au deuxième degré du cas index (65). Cette observation est, entre autres, rapportée également en France et dans d'autres états américains (65–67).

Dans un travail réalisé en 2003, sur les 42 paires de jumeaux étudiés où au moins l'un des deux jumeaux était atteint, la ScS affectait les deux jumeaux d'une même paire dans un seul cas de monozygotie (sur 24 paires) et un seul cas de dizygotie (sur 18 paires) : cette étude n'a donc pas révélé de différence statistiquement significative dans les taux de concordance entre les jumeaux monozygotes ou dizygotes (68). La prédisposition génétique ne semble donc pas suffire pour développer une ScS. Différents facteurs épigénétiques, modulés notamment par l'environnement, pourraient intervenir (pour revue, (69)).

De nombreux gènes de susceptibilité à développer la ScS ont été rapportés dans la littérature. Ces descriptions de polymorphisme génétique sont principalement établies à partir d'études d'association et non d'études de liaison génétique du fait de la rareté des familles avec plus de deux personnes atteintes. Plusieurs de ces polymorphismes ont été décrits dans des études isolées. Un exemple du manque de reproductibilité est l'étude du polymorphisme de la fibrilline-1 qui semblait prédisposer à la ScS dans une population de patients japonais et d'indiens Choctaw. Cette association n'a finalement pas été retrouvée dans une population caucasienne européenne (70,71). Globalement, parmi toutes ces études, les données les plus robustes à ce jour concernent les polymorphismes de gènes codant pour des cibles impliquées dans l'immunité cellulaire, tels que STAT4 (72,73), CD247 (74,75), TNFSF4 (4ème membre de la superfamille du TNF) (76,77), Tbx21 (facteur de transcription T-box 21) (78), NLRP1 (récepteur de type NOD, contenant un domaine pyrin en N-terminal 1) (79), ou encore dans l'immunité humorale tels que BLK (la tyrosine kinase lymphoïd B) (80), BANK1 (Protéine d'échafaudage spécifique des cellules B avec répétitions d'ankyrine 1) (81) et IRAK1 (kinase 1 associée au récepteur de l'interleukine-1). Ce dernier gène soulève un intérêt particulier par sa localisation sur le chromosome X puisque la prévalence de la ScS est bien plus grande chez les femmes que chez les hommes (82). Enfin, l'immunité innée et notamment les gènes impliqués dans les voies de l'interféron (IFN) sont particulièrement étudiés : une signature interféron a d'ailleurs été décrite. Dans ce domaine, c'est un polymorphisme du gène IRF5 (facteur régulateur de l'interféron) qui serait un gène de susceptibilité à la ScS (73). De plus, la combinaison de deux polymorphismes de gènes de susceptibilité, celui de STAT4 et celui d'IRF5, majorerait le RR pour développer une ScS et tout particulièrement une forme clinique grave de ScS avec PID (83,84). De même un polymorphisme rare de BANK1 est un facteur de susceptibilité génétique à développer une d-ScS et sa présence conjointe avec celles des polymorphismes de STAT4 et d'IRF5 majorerait ce risque (85).

2) Rôle des facteurs environnementaux

Différents facteurs environnementaux pourraient contribuer au développement de la maladie : des agents infectieux et des toxiques environnementaux ont été incriminés à ce jour, mais ce ne sont probablement pas les seuls impliqués.

a) *Agents infectieux*

Parmi ces facteurs environnementaux, l'infection par le cytomégalovirus (CMV), le virus d'Epstein-Barr (EBV) ou encore l'Helicobacter Pylori (HP) a été proposée. En effet, les aAc anti-topoisomérase (fréquemment retrouvés chez les patients) sont capables de reconnaître un épitope de la protéine UL94 du CMV, laissant supposer qu'une infection à CMV serait à l'origine de la production de ces immunoglobulines G reconnaissant ainsi de façon croisée la protéine UL94 et des antigènes du soi (86). Concernant l'EBV, c'est un virus universel connu pour infecter la quasi-totalité de la population adulte. Il a effectivement été retrouvé dans la plupart des cellules endothéliales et des fibroblastes de peaux de patients atteints de ScS. Or, l'EBV pourrait activer de façon aberrante les récepteurs de type Toll (TLR) et induire l'expression de la voie de signalisation de l'IFN mais aussi du TGFβ, particulièrement impliquée dans le processus de fibrose et dans la vasculopathie (87). Concernant l'HP, une méta-analyse récente a confirmé la sur-représentation des infections antérieures à HP dans une population de patients ScS comparée à la population générale, suggérant un rôle potentiel d'HP dans le déclenchement de la maladie (88).

b) *Toxiques environnementaux*

Différents toxiques environnementaux tels que la silice, les métaux lourds et les solvants organiques ont fait l'objet d'études quant à leur rôle physiopathologique (89), puisque la proportion de patients atteints de ScS est plus grande chez des personnes exposées professionnellement à ces facteurs environnementaux qu'en population générale. Il semble que ces toxiques, particulièrement la silice ou les solvants organiques, entraîneraient un stress oxydant, notamment par la production de formes réactives de l'oxygène (FRO) (90,91).

D'ailleurs, une maladie proche de la ScS peut être déclenchée chez des animaux à l'aide de toxiques environnementaux : c'est ce qui a été décrit dans les modèles induits par la bléomycine (92), par l'injection de chlorure de vinyle (93) ou encore par l'injection d'HOCl (94). Dans ces modèles, le facteur déclenchant de la ScS est un toxique environnemental induisant du stress oxydant, lui-même responsable du déclenchement de la cascade d'évènements aboutissant aux phénomènes de fibrose. L'agression du tissu par l'agent extérieur est donc bien essentielle pour le déclenchement de la pathologie mais probablement aussi pour son

maintien. En effet, l'arrêt des injections d'HOCL dans le modèle de souris sclérodermiques entraîne une régression partielle des lésions (95,96).

Ces facteurs environnementaux, infectieux ou toxiques, interviennent dans le déclenchement de la pathologie par différents mécanismes : stimulation d'une réponse immunitaire, induction d'un stress oxydant, activation de la voie pro-fibrosante du TGFβ1… Ils donnent ainsi naissance à une cascade d'évènements, notamment pro-inflammatoires, responsables du développement de la fibrose et de la vasculopathie, dont nous allons détailler les dysfonctionnements dans les chapitres suivants.

3) Vasculopathie

Au cours de la ScS, différents symptômes cliniques témoignent d'une atteinte des vaisseaux, et tout particulièrement des petits vaisseaux tels que les artérioles et les capillaires. En effet, le phénomène de Raynaud - symptôme clinique très souvent le plus précoce de la maladie, ou encore l'HTAP et la crise rénale sclérodermique - atteintes responsables d'une lourde morbi-mortalité, en sont le reflet par exemple. Les mécanismes physiopathologiques à l'origine de cette vasculopathie ne sont pas totalement compris à ce jour, mais l'endothélium semble largement impliqué dans ce processus pathologique (97).

a) *Rôle de l'endothélium*

L'endothélium est constitué, physiologiquement, d'une monocouche continue de cellules endothéliales (CE) reposant sur une lame basale constituée notamment de collagènes. Le pôle apical de ces cellules est directement en contact avec le sang contenu dans la lumière des vaisseaux. En dehors du fait qu'il contient le sang circulant, l'endothélium contribue à la régulation de la coagulation mais aussi de la vasomotricité ou encore de la perméabilité vasculaire, notamment pour assurer la nutrition des tissus. Au cours de la ScS, l'endothélium des petits vaisseaux est sévèrement atteint : la lame basale est souvent fine et dupliquée, le nombre de capillaires est diminué et il n'y a pas de néoformation vasculaire compensatrice. Il semble que les CE fassent l'objet de lésions sévères et d'une apoptose précoce, et soient ainsi à l'origine des dommages microvasculaires sous-jacents (98). Une hyperméthylation de l'ADN des CE des patients atteints de ScS semble bloquer l'expression de BMPR2 (récepteur de protéine morphogénétique osseuse de type 2), rendant les CE plus sensibles à l'apoptose (99).

De plus, les capacités de néo-angiogenèse à partir de progéniteurs endothéliaux de patients sclérodermiques seraient altérés (100), ne pouvant donc pas compenser cet endothélium endommagé.

On observe également un déséquilibre dans la production des médiateurs régulateurs de la fonction endothéliale. Tout d'abord, la sécrétion d'endothéline 1 (ET1), puissant vasoconstricteur, est majorée. Les taux sériques d'ET1 sont corrélés aux atteintes cliniques vasculaires graves telles que l'HTAP ou les ischémies digitales sévères (101). Ce médiateur est si important dans la physiopathologie que l'antagonisme du récepteur à l'ET1 par le bosentan permet d'améliorer l'HTAP des patients (102). Ensuite, une dérégulation des vasodilatateurs naturels que sont l'oxide nitrique (NO) et la prostacycline est observée. Les taux plasmatiques de la diméthylarginine, qui est un inhibiteur de la voie du NO, sont augmentés chez les patients atteints de ScS comparés à des patients atteints de phénomène de Raynaud sans ScS (103).

Finalement, l'ensemble de ces anomalies entraîne un vasospasme et un remaniement de la paroi des vaisseaux dans laquelle on peut observer une prolifération de l'intima, une hypertrophie de la média et une fibrose de l'adventice. De ce fait, la lumière des vaisseaux est réduite entraînant une ischémie distale et à terme, une oblitération des vaisseaux concernés. Il en résulte des lésions tissulaires secondaires à l'hypoxie induite par ces phénomènes d'ischémie mais aussi, des lésions tissulaires secondaires aux phénomènes d'« ischémie/reperfusion ». En effet, la reperfusion de tissus ischémiés entraîne la formation de FRO. Les FRO induisent des changements dans le métabolisme cellulaire et favorisent notamment l'apoptose des CE, contribuant ainsi aux lésions tissulaires d'aval (104). De plus, un déséquilibre dans la balance coagulation/fibrinolyse résultant de la souffrance cellulaire favorise les dépôts de fibrine et contribue à la prolifération de l'intima ainsi qu'à la perte d'intégrité de la barrière endothéliale (105). Cette perte d'intégrité de la barrière endothéliale entraîne le recrutement de cellules immunitaires, telles que les monocytes et les macrophages, aggravant à leur tour les lésions tissulaires : nous reviendrons sur ce point dans le sous-chapitre consacré au système immunitaire [V)5)].

b) *Facteurs déclenchants*

L'inducteur initial des lésions des CE n'est pas connu ni univoque à ce jour même si différentes hypothèses ont été soulevées. Tout d'abord, le CMV pourrait être en cause. En effet, les aAc anti-Scl70 sont capables de reconnaître d'une part l'épitope -VTLGGAGIWLPP- contenu dans la protéine UL94 du CMV, et d'autre part, la protéine transmembranaire NAG-2 (novel antigen 2) des CE. De plus, un Ac dirigé contre l'UL94 est capable d'induire l'apoptose

de CE (86). Une autre hypothèse soutient le rôle d'aAc spécifiques dirigés contre les CE, dont la prévalence est de 40 à 50% dans la population de patients atteints de ScS. Ces aAc anti-CE sont capables d'induire l'apoptose de CE *in vitro* (106) mais aussi *in vivo* (107). D'ailleurs, leur présence chez les patients est souvent corrélée à une atteinte clinique vasculaire (HTAP, ulcères digitaux), ce qui renforce l'hypothèse de leur rôle physiopathologique. Ni l'origine, ni la cible antigénique précise de ces aAc ne sont validées à ce jour.

c) *Lien entre vasculopathie et fibrose*

Le rôle de l'endothélium, au cours de la physiopathologie de la ScS, ne se limite pas aux lésions ischémiques et tissulaires précédemment décrites. En effet, cet endothélium endommagé va également agir par plusieurs mécanismes, sur le développement de la fibrose.

Comme nous le verrons plus en détail dans la partie suivante, les fibroblastes de patients atteints de ScS (fibroblastes-ScS) sont résistants à l'apoptose : ce phénomène contribue notamment à la persistance de leur phénotype pathologique et des lésions de fibrose qui en résultent. Les CE apoptotiques seraient impliquées dans cette résistance à l'apoptose des fibroblastes environnants. En effet, le milieu conditionné des CE apoptotiques est capable d'inhiber l'apoptose des fibroblastes en culture et d'induire leur différenciation myofibroblastique par la voie de signalisation dépendant de PI3K (phosphoinositide 3-kinase) : ces myofibroblastes expriment alors plus d'α-sma, présentent des fibres de stress intra-cytoplasmiques et produisent plus de collagène 1 (108). Par ailleurs, les CE de patients ScS sont capables de moduler le switch phénotypique des CSMs saines ou sclérodermiques, retrouvées notamment au niveau péri-vasculaire, vers un phénotype de myofibroblastes. Ces mêmes CE semblent libérer, en coculture avec des CSMs, plus de TGFβ1 et de PDGF-BB (isoforme BB du facteur de croissance dérivé des plaquettes), qui sont des facteurs pro-fibrotiques impliqués largement dans la physiopathologie, comme nous le reverrons plus tard (109).

Le lien entre vasculopathie et fibrose ne se fait pas seulement par la communication croisée entre CE et fibroblastes-ScS mais aussi par l'hypoxie tissulaire occasionnée par la vasculopathie, qui contribue à la progression des phénomènes de fibrose chez les patients (110). En effet, l'hypoxie induit dans des fibroblastes dermiques, la sur-expression de gènes codant pour des protéines constitutives de la matrice extra-cellulaire (MEC) ou impliquées dans son turnover, tels que les gènes codant pour la thrombospondine 1 (THBS1), le facteur de croissance ressemblant à l'insuline (IGF) 3, le pro-collagène 1 alpha 2 ou encore la fibronectine (FN) 1.

Enfin, le système vasculaire contribue à la physiopathologie de la ScS du fait de sa participation à la formation du pool de myofibrobastes responsables de la fibrose, au travers d'un phénomène appelé transition endothélio-mésenchymateuse (endoMT). En effet, sous l'effet de cytokines (TGFβ1 et ET1 par exemple) et de facteurs environnementaux (stress oxydant par exemple (111)), les CE subissent,un changement phénotypique et acquièrent une morphologie de type fibroblastique. Les CE perdent alors leurs marqueurs spécifiques (CD31 par exemple) et acquièrent ceux des myofibroblastes. Cette endoMT a été observée au cours de différentes pathologies comme l'HTAP (112), notamment dans le cadre d'une ScS (113). Le facteur « Friend Leukemia Integration » 1 (Fli1) semble être largement impliqué dans la transition endoMT, comme l'a confirmé le développement d'une fibrose plus sévère chez des souris déficientes en Fli-1(114). Récemment, dans une étude menée sur un modèle murin de fibrose pulmonaire induite par la bléomycine, les auteurs ont observé cette endoMT des CE sous l'effet de la bléomycine (115). *In vitro*, l'exposition de CE à des sérums de patients atteints de ScS induit également cette endoMT, tout comme une stimulation au TGFβ1 (115,116).
Par ailleurs, les CE ne sont pas les seules cellules du système vasculaire à se trans-différencier et contribuer ainsi au pool de cellules constitutives du tissu fibreux. En effet, les progéniteurs endothéliaux seraient également impliqués (117). Leur trans-différenciation précoce en myofibroblastes chez les patients atteints de ScS contribue au phénomène de fibrose et aggrave la vasculopathie en préférant la différenciation du progéniteur endothélial vers le fibroblaste plutôt que vers la CE (118,119).

4) Fibrose

Le tissu fibreux caractéristique de la ScS est composé d'une part d'un tissu conjonctif épais résultant de dépôts de collagènes, de glycosaminoglycanes, d'acide hyaluronique ou encore de FN, et d'autre part de myofibroblastes. L'ensemble rigidifie le tissu et en modifie son architecture, aboutissant ainsi au dysfonctionnement de l'organe atteint. Cette fibrose est donc secondaire à une production excessive de MEC, notamment par les fibroblastes, qui n'est pas compensée par des mécanismes suffisants de remodelage. Les fibroblastes sont donc des acteurs majeurs dans la constitution de la fibrose. Au cours de la ScS, ils dysfonctionnent :

- d'une part, du fait d'anomalies intrinsèques qui les prédisposent à se différencier en myofibroblastes, à produire de la MEC et à s'auto-activer,
- et d'autre part, du fait de facteurs extrinsèques environnementaux, qui précipitent leur transdifférenciation en myofibroblastes et contribuent à leur activation.

a) *Anomalies intrinsèques aux fibroblastes-ScS*

Anomalies phénotypiques.

Les fibroblastes-ScS présentent un phénotype de cellules différenciées en myofibroblastes, exprimant fortement α-sma. Ils prolifèrent plus vite et leur capacité d'invasion et de motilité est augmentée. Les fibroblastes-ScS sont résistants à l'apoptose induite par la voie Fas (120,121), résistance favorisée notamment par le secrétome des CE apoptotiques (108). Cette résistance à l'apoptose promeut la persistance de leur phénotype pathologique et invasif dans les tissus atteints.

Par ailleurs, ces fibroblastes-ScS sont responsables en grande partie des phénomènes de fibrose puisqu'ils synthétisent en plus grande quantité des constituants de la MEC, tels que les collagènes, les protéoglycanes ou encore la FN (122). Ils n'améliorent pas pour autant le remodelage de cette MEC produite en excès, puisqu'ils sécrètent notamment de plus grandes quantités d'inhibiteurs tissulaires des métalloprotéinases (TIMPs) (123), tels que TIMP1 et TIMP3 (124). De même, ils produisent des lysyl oxydases qui sont responsables d'un assemblage de collagènes plus résistants à ces TIMPs déjà sous exprimés (125). Ces lysyl oxydases peuvent être des cibles thérapeutiques intéressantes. En effet, des travaux ont montré un effet anti-fibrotique de molécules inhibant la lysyl oxydase de type 4 (126), connue pour être sur-exprimée par les fibroblastes de patients atteints de ScS. Par ailleurs, il semble que la sous-population de fibroblastes-ScS résistants à l'apoptose, prélevés chez des patients en phase tardive de la maladie, sécrètent encore moins de métalloprotéinases (MMPs) (127), mettant en lumière la contribution de la dérégulation du remodelage dans l'instauration progressive d'une fibrose difficilement réversible.

Enfin, il est intéressant de souligner la présence, au niveau d'un tégument cliniquement sain de patients sclérodermiques, de cellules présentant des anomalies semblables à celles provenant de zones cliniquement atteintes par la sclérose cutanée (128). Cette constatation soutient fortement l'hypothèse que les fibroblastes des patients ont des anomalies intrinsèques, les prédisposant à évoluer vers un phénotype malade.

Auto-activation des fibroblastes-ScS.

Les fibroblastes-ScS sont responsables de la perte d'homéostasie tissulaire, s'expliquant par l'activation permanente de voies de signalisation notamment pro-fibrosantes, par des facteurs environnementaux, sur lesquels nous reviendrons plus tard, chapitre [V)4)c], ou par des mécanismes intrinsèques que nous allons discuter maintenant.

Tout d'abord, on observe une auto-activation de la voie TGFβ1, comme en témoigne l'accumulation de SMAD3 indépendamment d'une stimulation exogène par du TGFβ1 par exemple (129). De plus, les fibroblastes-ScS sur-expriment des récepteurs aux TGFβ (TGFβR), ce qui explique leur plus grande sensibilité au TGFβ1 environnemental (130,131), que ce soit dans les formes de d-ScS ou l-ScS (132,133).

Ensuite, les fibroblastes-ScS produisent de façon excessive du facteur de croissance du tissu conjonctif (CTGF) (134), cytokine capable d'induire la transdifférenciation des fibroblastes en myofibroblastes et stimulant leur prolifération (135). D'ailleurs, les malades présentent des taux sériques plus élevés de CTGF circulant, comparé à des sujets sains. Or, le CTGF est un facteur de croissance des fibroblastes mais aussi des CE qui produisent à leur tour du CTGF. De plus, l'hypoxie induit l'expression de CTGF sur les fibroblastes (136). Pourtant, le blocage de cette boucle de régulation autocrine par des anticorps anti-CTGF n' a pas montré d'effet thérapeutique convaincant, suggérant que cette voie n'est qu'une parmi bien d'autres (137,138). Toutefois, un analogue de la prostacycline (Iloprost), utilisé dans le traitement des atteintes vasculaires sévères, semble réduire les taux sériques de CTGF (139). Enfin, le CTGF ne semble pas agir seul mais aussi par l'intermédiaire d'interactions avec le facteur de croissance des fibroblastes (FGF) ou encore la protéine chimio-attractive des monocytes (MCP) de type 1 (140).

De même, les fibroblastes-ScS produisent différentes FRO qui activent à leur tour la prolifération des fibroblastes ainsi que leur synthèse de collagènes. De plus, les FRO participent à la phosphorylation de la kinase impliquée dans l'adhésion focale des fibroblastes, dont sa surexpression dans les fibroblastes-ScS a été démontrée (141). Ce phénomène d'auto-activation par les FRO contribuent notamment à la persistance des fibroblastes pathologiques adhérant dans les tissus.

Par ailleurs, plusieurs études rapportent des taux élevés de gènes régulateurs de la méthylation, tels que les gènes de la DNA méthyltransférase (DNMT) de type 1, les protéines de liaison au CpG méthylé de type 1 et de type 2 (MBD1 et MeCP2), suggérant une hyper-méthylation de l'ADN des fibroblastes de peau de patients sclérodermiques. Cette hyper-méthylation est responsable entre autre du blocage de l'expression de gènes régulateurs du collagène tels que Fli1 par exemple (142) ou d'antagonistes de la voie pro-fibrosante Wnt (143). Une étude récente a d'ailleurs démontré que le TGFβ1 induit une sur-expression de DNMT3a dans les fibroblastes-ScS de peau, responsable d'une hyper-méthylation de l'ADN à l'origine d'une sous-expression de SOCS3 (suppresseur de la signalisation des cytokines de type 3). La diminution de SOCS3 entraîne l'activation de STAT3 qui favorise la transdifférenciation du fibroblaste vers le myofibroblaste (144). D'autre part, une étude du génome à large échelle a montré une hypo-méthylation globale de l'ADN de fibroblastes-ScS (145), potentiellement à l'origine d'une sur-expression de gènes pro-fibrosants.

D'ailleurs, l'hypoxie induirait l'expression de TET1 (Tet méthylcytosine dioxygénase de type 1), protéine elle-même impliquée dans la déméthylation de l'ADN. Finalement, c'est une fine dérégulation de la balance entre méthylation et déméthylation de l'ADN qui permettrait la modulation de l'expression de certains gènes particulièrement impliqués dans le processus de fibrogenèse.

Enfin, les fibroblastes-ScS sont responsables de la stimulation des autres acteurs de la maladie, que sont les CE et le système immunitaire. En effet, les fibroblastes-ScS sécrètent en plus grande quantité différentes cytokines pro-inflammatoires et chimio-attractives, telles que le TNFα, l'IL6 ou MCP-1. En outre, les fibroblastes-ScS sécrètent plus d'ET1 que les fibroblastes sains (146). Finalement, l'activation des autres acteurs de la maladie va auto-entretenir leur propre activation.

b) *Origine des fibroblastes-ScS*

L'origine des fibroblastes évoluant vers un phénotype de myofibroblastes n'est pas totalement élucidée à ce jour. Outre les CE, les progéniteurs endothéliaux (113,117) et les fibroblastes résidents des tissus, d'autres cellules pourraient contribuer à enrichir la population de myofibroblastes (147).

Tout d'abord, différents travaux notamment sur la fibrose rénale, ont montré que des cellules épithéliales pouvaient perdre leur polarité et acquérir un phénotype fibroblastique, sous l'influence de différentes cytokines (TGFβ1, FGF2, le facteur de croissance épidermique (EGF), IGF2) et de certaines protéases qui dégradent la membrane basale (148). Ce phénomène est appelé transition épithélio-mésenchymateuse (TEM). Chez les patients sclérodermiques, cette TEM est observée au niveau des kératinocytes, notamment sous l'influence du TGFβ1 (149).
De plus, on retrouve un nombre plus important de péricytes au niveau du derme de malades que de donneurs sains et ces péricytes évolueraient vers un phénotype myofibroblastique (150), notamment sous l'influence du TGFβ1 qui induit l'expression de l'isoforme 12 de la métalloprotéinase comprenant un domaine désintegrine (ADAM 12)(151).

En dehors de ces cellules résidentes, des cellules circulantes pourraient également contribuer à ce pool de myofibroblastes. Premièrement, les monocytes sanguins sont les premières cellules circulantes qui ont fait débat. En effet, des monocytes sanguins de patients atteints d'ostéomyélosclérose et de la maladie d'Engelman, se transforment en culture en cellules fibroblastiques capables de sécréter du collagène de type 1 par exemple (152). Deuxièmement, les fibrocytes circulants, isolés du pool des cellules mononuclées du sang

périphérique (PBMCs) grâce à une culture pauvre en sérum sur une surface recouverte de FN sont capables de se différencier en myofibroblastes sous l'influence du TGFβ1 (153,154) alors que la protéine amyloïde du sérum P est capable d'inhiber cette différenciation (155). Troisièmement, les CSMs circulantes pourraient aussi intervenir, chez l'adulte, dans ce pool de cellules pro-fibrosantes. En effet, dans un modèle murin de fibrose pulmonaire induite à la bléomycine, des souris malades et greffées par des CSMs de moelle osseuse (MO) marquées à la GFP+ (protéine fluorescente verte) présentaient une plus forte proportion de CSMs GFP+ dans les zones fibreuses du parenchyme pulmonaire que des souris non malades. Ces CSMs GFP+ sur-exprimaient le collagène 1, contribuant au processus de fibrose (156).

Enfin, des cellules progénitrices du tissu adipeux (TA) qui perdent leurs marqueurs adipocytaires et expriment des marqueurs de myofibroblastes pourraient également contribuer à cette population de myofibroblastes. En effet, dans différents modèles animaux de ScS, on observe une perte du TA intradermique. Cette transformation de TA en tissu conjonctif est connu chez les patients depuis 1976 (157). De cette observation, Maragoni *et al* ont alors démontré que des progéniteurs intradermiques à adiponectine positive se différenciaient en myofibroblastes (158). Par ailleurs, la stimulation de CSMs adipocytaires par du TGFβ1 induit leur production de CTGF ainsi que leur switch phénotypique myofibroblastique, ce que le CTGF seul ne pourrait cependant pas faire (159). En outre, les adipocytes matures du TA blanc peuvent se trans-différencier en myofibroblastes contractiles par un processus appelé "transition adipocytes à myofibroblastes » (TAM), caractérisé par une diminution des marqueurs adipocytaires conjointement à l'augmentation de marqueurs mésenchymateux. La TAM est favorisée par le TGFβ1 mais aussi par la protéine FIZZ-1 (Found in inflammatory zone). Enfin, une hyper-activation de la voie Wnt/SMAD est observée dans les biopsies de peau de patients ScS (160) alors que Wnt3a semble responsable de la répression de PPARγ (récepteur activé par les prolifférateurs de peroxysomes γ) qui est le principal acteur de la différenciation vers la voie adipocytaire (161). Ainsi, Wnt3a favorise la différenciation des progéniteurs mésenchymateux adipocytaires vers le myofibroblaste. D'ailleurs, l'expression de PPARγ est diminuée dans les fibroblastes-ScS de peau et leur phénotype est amélioré par l'utilisation d'agonistes de PPARγ (162), tout comme celui de fibroblastes pulmonaires stimulés par le TGFβ1 (163). Il est intéressant d'observer que les niveaux sériques d'adiponectine, marqueur reflétant l'activité de PPARγ, sont inversement corrélés à l'atteinte clinique cutanée chez les patients (164). Enfin, un variant de PPARγ a été rapporté comme un gène de prédisposition à la ScS (78).

Au total, différents progéniteurs mésenchymateux semblent intervenir dans la constitution du pool de myofibroblastes activés responsables de la fibrose. Ainsi, sont-ils eux-mêmes porteurs d'anomalies prédisposant à donner naissance à des fibroblastes pathologiques ? Contribuent-ils seulement à la constitution de ce pool sous l'influence de l'environnement pathologique sans anomalies intrinsèques au départ ? En ce sens, se pose la question du rôle des CSMs dans la physiopathologie de la ScS et nous détaillerons ce point, dans la 2ème partie de cette introduction bibliographique consacrée aux CSMs.

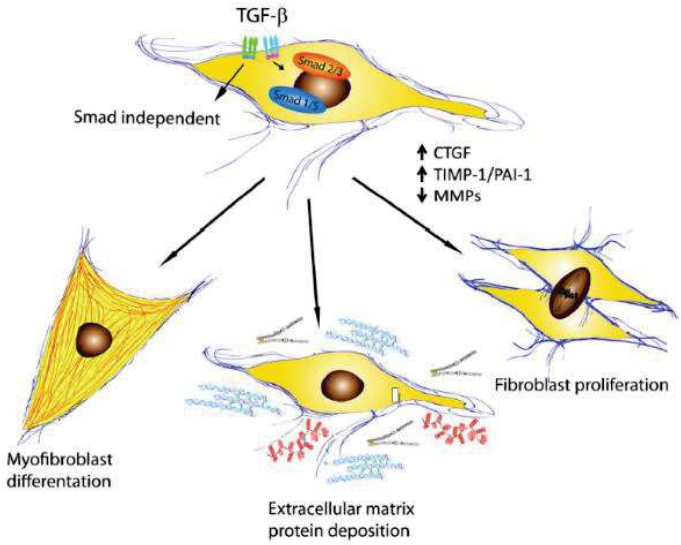
c) *Influence de facteurs extrinsèques*

De nombreux facteurs extrinsèques, moléculaires ou cellulaires, tendent à faciliter cette transition d'un fibroblaste à priori sain vers un phénotype pathologique mais aussi à exacerber et auto-entretenir ce processus majeur dans le développement de la fibrose.

Le TGFβ1

Parmi ces facteurs extrinsèques, on retrouve tout particulièrement le TGFβ1 qui joue un rôle majeur dans la fibrogenèse au cours de la ScS (165). Cette cytokine immunosuppressive est connue pour avoir également une activité pro-fibrosante (166), en stimulant la synthèse de collagènes, de FN, de TIMPs, de THBS1 ou encore de CTGF par l'activation sous-jacente des voies de signalisation canoniques et non canoniques du TGFβ1 dans les fibroblastes (167–170) (Figure 4).

Figure 4 – Effet du TGFβ1 sur le phénotype des fibroblastes.



CTGF: facteur de croissance du tissu conjonctif ; TIMP : inhibiteur de métalloprotéinase ; PAI: inhibiteur de l'activateur du plasminogène 1; TGFβ : Tranforming growth factor

D'après Biernacka A, Growth Factors (2011)

Le TGFβ1 serait effectivement présent en plus grande quantité au niveau de prélèvements de peau de patients atteints de l-ScS mais aussi au niveau du sérum de patients présentant une sclérodermie de type Morphée (171). Cependant, une autre équipe a montré que les taux sériques de forme active du TGFβ1 étaient inversement corrélés au score de Rodnan dans les formes de d-ScS (172). L'activation du TGFβ1 se produit sûrement au niveau local par des mécanismes paracrine et autocrine. Les fibroblastes-ScS contribuent à cette activation à l'aide notamment des intégrines α(v)β3 et α(v)β5 qu'ils sur-expriment (173,174) et de la THBS1 qu'ils sécrètent en grande quantité (175,176). Quoiqu'il en soit, une activité biologique cellulaire est induite par le TGFβ1 dans les tissus de ces patients, comme en témoignent l'expression forte des gènes cibles du TGFβ1 dans les fibroblastes-ScS (177) et la production importante de FN et de fibrilline (178,179). D'ailleurs, certains patients atteints de d-ScS ayant une signature de gènes induits par le TGFβ1 au niveau de leur tégument atteints ont des formes cliniques plus sévères que les malades n'ayant pas cette signature génétique (180,181).

De plus, comme nous l'avons vu précédemment, le TGFβ1 agit en promouvant la TEM, la TAM ainsi que la différenciation de CSMs et des péricytes en myofibroblastes. Le TGFβ1 associé à l'ET1 promeut également l'endoMT (182).

Les fibroblastes-ScS sur-expriment le TGFβR en comparaison à des fibroblastes sains mais ils produisent des quantités semblables de TGFβ1 en culture (183,184). L'excès de TGFβ1 dans les tissus des patients provient donc probablement d'autres partenaires impliqués dans la physiopathologie de la ScS, tels que les CE ou encore les cellules immunitaires comme les PBMCs et les mastocytes (185,186). Le blocage de la voie du TGFβ1 permet *in vitro* de diminuer la production de collagènes par les fibroblastes-ScS (183) et de nombreux travaux se sont intéressés à bloquer cette voie à visée thérapeutique (187). Malheureusement, un essai clinique évaluant l'efficacité thérapeutique d'un Ac spécifique du TGFβ1 s'est révélé décevant (188).

L'endothéline 1

L'ET1 (189) est présente en excès chez les patients comparés à des sujets sains, que ce soit au niveau sérique ou dans la peau des patients (190). Elle a un fort pouvoir vasoconstricteur connu mais elle est aussi responsable d'une activation des fibroblastes, de leur transformation en myofibroblaste, de leur synthèse de collagènes ainsi que de l'inhibition de leur production de MMP1 (191).

La sérotonine

L'activation plaquettaire présente au cours de la ScS est notamment responsable de la libération de sérotonine qui a des propriétés pro-coagulantes et vasoactives. La sérotonine va également induire la production de MEC en plus grande quantité par les fibroblastes-ScS que par des fibroblastes sains, notamment par qu'ils sur expriment l'un de ses récepteurs (5-HT2B) (192). Les inhibiteurs de la sérotonine peuvent ainsi bloquer l'activation de cette voie pro-fibrosante et présentent un intérêt thérapeutique potentiel (193).

Le CTGF

Le CTGF est une cytokine princeps de la physiopathologie de la ScS. Nous avons déjà abordé son importance dans la partie précédente puisqu'il participe à la prolifération des fibroblastes et leur transition vers un phénotype pathologique. Le CTGF est produit soit par les fibroblastes eux-mêmes (facteurs intrinsèques) soit par les cellules environnantes : CE et /ou immunitaires (facteurs extrinsèques). En tout cas, il est essentiel à l'activation de la voie de signalisation Smad2-ERK1/2 induite par le TGFβ (194). Le niveau sérique de CTGF est augmenté chez les malades ; les taux sont d'ailleurs corrélés à la présence d'une atteinte pulmonaire ainsi qu'à la sévérité de la maladie (195).

Le PDGF

Le PDGF joue un rôle important dans le processus de fibrose. Sur des fibroblastes en culture, après avoir activé son récepteur, le PDGF induit la voie ERK1/2 et la production de FRO. La voie de signalisation RAS sous-jacente est ainsi activée, ce qui entraîne la production de collagènes et la différenciation des cellules en myofibroblastes (196). On retrouve chez les patients des Ac dirgiés contre le récepteur au PDGF (PDGFR) qui sont capables de mimer exactement l'effet du PDGF en se liant sur son récepteur, activant ainsi cette voie pro-fibrosante (197). Le sumitinib, inhibiteur de tyrosine kinase, inhibe la phosphorylation du récepteur au PDGF et empêche ainsi l'induction de la ScS dans un modèle murin (198). Par ailleurs, miR-29a, connu pour ses propriétés anti-fibrosantes, a pour cible directe le PDGFR-BB (199,200).

Autres facteurs extrinsèques

D'autres cytokines sécrétées par les cellules du système immunitaire ou les CE vont également participer au processus de fibrogenèse, parmi lesquelles on peut citer l'IL4, l'IL6 ou encore l'IL13. D'ailleurs les fibroblastes-ScS sur-expriment l'IL4 (201) alors que l'expression de l'IL13 est polymorphe chez les patients (202). De ce fait, différents modèles *in vitro* ont été

développés en profitant des propriétés pro-fibrosantes de ces cytokines : nous y reviendrons dans le chapitre [VI)1)].

De plus, les kératinocytes de patients atteints de ScS seraient capables d'activer les fibroblastes-ScS par une voie indépendante du TGFβ, au travers de la libération de CCL5 (ligand de chimiokine 5 (motif C-C) ou RANTES) activant la voie NFκB et inhibant la voie PPARγ (203). Enfin, les aAc anti-fibrilline produits par les LBs et retrouvés dans le sérum de patients atteints de ScS sont également capables d'activer les fibroblastes en culture (204).

Finalement, les fibroblastes des patients ScS sont prédisposés à évoluer vers un phénotype pathologique mais leurs interactions avec les autres acteurs impliqués dans le développement de la maladie, comme les cellules immunitaires, jouent également un rôle majeur dans leur activation. Nous allons donc dans le prochain chapitre développer les différentes dérégulations du système immunitaire actuellement connues dans la ScS.

5) Dérégulation du système immunitaire

Au cours de la ScS, le système immunitaire intervient dans le développement de la maladie. Dès 1977, les travaux de Fleischmajer R *et al* ont mis en évidence la présence d'un infiltrat de cellules immunitaires sur les prélèvements tissulaires de patients (205). Par la suite, la caractérisation de cet infiltrat inflammatoire s'est précisée et des Ac sériques ont été détectés chez les malades. Ces observations témoignent de la présence d'anomalies du système immunitaire que nous allons détailler dans ce chapitre.

a) *Rôle des monocytes - macrophages*

La proportion de monocytes CD14+ est plus importante dans les PBMCs de patients que dans ceux des donneurs sains, témoignant de leur recrutement (206). En effet, les monocytes sont en première ligne dans la réponse au stress initial. D'une part, ils vont se différencier en macrophages au niveau des tissus atteints : on peut observer cet infiltrat de monocytes/macrophages (207) et notamment de macrophages activés (206) dans la peau des patients sclérodermiques. La polarisation des macrophages prédominerait vers le phénotype M2, responsable d'une importante sécrétion de cytokines, dont certaines sont connues pour être pro-fibrosantes (IL6, IL4, IL13, CTGF, IL10), ou encore d'enzymes telles que l'arginase-1 qui est capable de stimuler la synthèse de collagènes (208). D'autre part, les monocytes vont classiquement activer l'immunité cellulaire après s'être différenciés en cellules dendritiques ce qui leur confère un rôle de cellules présentatrices d'antigènes.

b) *Rôle des mastocytes*

Les mastocytes pourraient également jouer un rôle dans la physiopathologie de la ScS. Ils sont observés, chez des patients dont la maladie a débuté récemment, en plus grande proportion et en phase de dégranulation dans les zones de peaux atteintes cliniquement (209). Ces mastocytes contiennent du TGFβ1 actif, notamment au sein de vésicules dont certaines sont libérées dans le compartiment extra-cellulaire (186). De plus, dans le modèle murin Tight Skin 1 de ScS, on note une accumulation de mastocytes au niveau de lésions cutanées chez les souris (210).

c) *Rôle des TLR*

L'implication de l'immunité innée ne se limite pas à la seule stimulation des cellules du système immunitaire mais aussi à la stimulation directe de cellules stromales tissulaires.
En effet, parmi les différents TLR, le TLR4 peut être activé par des DAMPs (motifs moléculaires associés aux dégats) endogènes, tels que des composants de MEC résultant de lésions tissulaires. Dans la peau de patients sclérodermiques, le TLR4 est sur-exprimé au niveau des fibroblastes du derme mais aussi des kératinocytes de l'épiderme. De même, au niveau pulmonaire, il est sur-exprimé par les macrophages et les cellules épithéliales alvéolaires (211). La stimulation du TLR4 des fibroblastes-ScS en culture entraîne une augmentation de la synthèse de collagènes et de l'expression de gènes de remodelage de la MEC. Par ailleurs, ces fibroblastes sur-exprimant le TLR4 semblent plus sensibles à la stimulation par le TGFβ1, entraînant ainsi des réponses pro-fibrosantes dont l'activation de la voie SMAD et l'inhibition de l'expression de miR-29a. L'utilisation d'un inhibiteur du TLR4 permet de reverser le phénomène. D'ailleurs, le modèle de fibrose cutanée induite par la bléomycine est atténué chez des souris portant un TLR4 muté (212).
Par ailleurs, le TLR9 est sur-exprimé dans la peau de patients sclérodermiques et sa principale source serait les myofibroblastes (213), ce qui a été confirmé dans un modèle murin de fibrose cutanée induite par la bléomycine. L'activation du TLR9 entraîne la production de TGFβ1 dont on a déjà soulevé l'importance, capable d'activer sa propre production réalisant ainsi une boucle d'auto-amplification. Par ailleurs, l'activation des TLR explique en partie la signature interféron retrouvée notamment au niveau de poumons de patients ScS (208).

d) *Rôle des Lymphocytes T*

Les LT observés au niveau de la peau affectée des patients ScS sont principalement des LT CD4+ et la sous population Th2 semble prédominer (214). Les LT infiltrent la peau dans une phase précoce de la maladie et pourrait avoir un rôle dans l'activation des fibroblastes (215). D'ailleurs, les LT Th2 produisent entre autres des cytokines pro-fibrosantes

comme l'IL4 et l'IL13 (216). On retrouve les LT surtout dans les formes sévères précoces et rapidement évolutives (217) ce qui expliquerait l'efficacité des molécules immunosuppressives limitées à ce sous-groupe de malades. Dans le modèle murin de ScS induite par l'HOCl, on observe une évolution de la maladie en deux phases : une première phase dite « phase inflammatoire précoce » où l'afflux de macrophages et de LT prédomine ; puis une deuxième phase dite « phase de fibrose matricielle établie » où les dépôts de collagène sont majeurs alors que l'inflammation a diminué et qu'on n'observe plus de LT (Annexe 2) (96). Parmi les autres sous-populations de LT CD4+, les LT de type Th17 semblent également présents en proportion plus importante que chez des patients sains (218), ce qui corrèle avec les taux circulants d'IL23 et/ou d'IL17 plus élevés chez les malades, même si leur rôle reste encore à préciser (219).

D'autres études ont également mis en évidence la présence de LT CD8+ au niveau de la peau (220) et des poumons de patients (221). Ils participeraient au développement de la maladie par la production de cytokines pro-inflammatoires et pro-fibrosantes (220,221). Au niveau des LT circulants, il semble avoir une diminution de la proportion de CD8+ (222).

Enfin, le rôle de LT allo réactifs dans la physiopathologie de la ScS est également discuté. Durant la grossesse, on observe un passage de cellules fœtales vers le sang maternel qui peuvent y rester plusieurs années. Ce phénomène, appelé microchimérisme, est retrouvé chez 4% des individus sains et 46% de femmes atteintes de ScS (223). De plus, certaines cellules fœtales seraient retrouvées dans la peau de ces patientes. L'hypothèse principale serait que les cellules fœtales se différencieraient en LT alloréactifs chez la mère et s'activeraient secondairement dans les suites d'un stimulus externe entraînant ainsi une réaction de type « greffon contre l'hôte » (GVH). La maladie du GVH est décrite par ailleurs comme très proche de la ScS. Cette donnée est controversée dans un autre travail qui n'a pas retrouvé de différence entre une population de malades et de sujets sains, où le microchimérisme concernait 55% des sujets (224).

e) *Rôle des Lymphocytes B*

La plupart des patients sclérodermiques présentent des aAc sériques. La diversité des aAc retrouvés est vraiment importante et le nombre d'aAc nouvellement identifiés chez les malades s'accroît avec l'accumulation des travaux scientifiques. Différentes associations entre certains types d'aAc et certaines atteintes de la ScS ont été décrites, comme nous l'avons vu précédemment. Certaines cibles de ces aAc sont des molécules solubles ou exprimées sur des cellules impliquées directement dans la physiopathologie de la maladie ; citons par

exemple les fibroblastes, les CE, la fibrilline ou encore le PDGFR (225). Pour autant, il n'y a ce jour aucune preuve de causalité directe entre ces aAc et la survenue ou la progression de la ScS. Pourtant, leur implication dans l'activation des fibroblastes par exemple a pu être montrée *in vitro*, comme pour les aAc anti-Scl70 (226). Dans le cas des aAc anti-fibrilline 1, ils seraient à l'origine de la libération de TGFβ1 séquestré dans les microfibrilles de la MEC, qui irait ensuite activer les fibroblastes (204). Les aAc anti-fibroblastes semblent favoriser la production de MMP1 par les fibroblastes sans modifier pour autant leur phénotype (227). De leur côté, les aAc anti-CE favorisent la libération d'ET1 par les CE *in vitro*, ainsi que leur apoptose (199).

La perte de tolérance d'antigènes du soi à l'origine de la production de ces aAc n'est pas totalement comprise. Le clivage de certains autoantigènes ciblés dans la ScS, comme l'ADN topoisomérase 1 ou l'ARN polymérase III, est assuré par la granzyme B *in vitro* et lors de la mort induite par les granules des lymphocytes cytotoxiques. Ce clivage génère des fragments uniques de ces autoantigènes qui ne sont pas retrouvés lors de toute autre forme d'apoptose, comme après un clivage par la caspase 8. Ce phénomène est lié à la présence d'acides aminés en position P2 et P3 au niveau du site clivage. En conséquence, ces mêmes cibles antigéniques clivées par d'autres enzymes génèrent des fragments qui ne sont pas reconnus par les aAc (228). La fibrilline, elle, pourrait provenir de l'excès d'apoptose de CE (106). Par ailleurs, une étude a montré une hyper-méthylation de l'ADN des LT qui pourrait réguler négativement FoxP3 et expliquer en partie le déficit en LT régulateurs et la perte d'immunotolérance qui en résulte (229).

La grande diversité des aAc retrouvés chez les patients ainsi que l'environnement cytokinique délivré par les LT CD4+ Th2 amènent à penser que les LB sont hyper-activés au cours du développement de la ScS. De plus, la quantité sérique de facteur activateur des lymphocytes B (BAFF) est plus élevée chez les patients que chez des sujets sains et corrélée à la sévérité de la maladie (230). BAFF est une cytokine connue pour réguler la prolifération, la survie et l'activation des LB. Elle pourrait être en partie secrétée par les cellules inflammatoires situées au niveau de la peau, dans les formes débutantes de la maladie. De surcroît, les LB des patients sur-expriment la molécule de costimulation CD19 qui est responsable notamment de la synthèse d'immunoglobulines (231), ce qui pourrait expliquer en partie la production anormale d'aAc. L'homéostasie des LB circulants semble altérée chez les patients atteints de ScS : les LB mémoires sont moins nombreux et moins activés (232). Ces anomalies ont également été rapportés dans le modèle murin de ScS induite par l'HOCl (233). Enfin, les LB des patients sont responsables d'une hyper-production d'IL6 pro-inflammatoire et ont une capacité réduite de production d'IL10 après stimulation ; cette

réduction est corrélée à une altération de la phosphorylation de mTOR (mechanistic target of rapamycin) dans les LB (232,234).

Les LB sont donc impliqués dans la physiopathologie de la ScS par leur production d'Ac mais aussi par leur sécrétome, notamment par la libération d'IL6 qui participe à l'activation des myofibroblastes (235). D'ailleurs, différentes stratégies thérapeutiques ciblant les LB sont en cours d'évaluation (236) tels que les anti-CD20 (rituximab) et les anti-IL6 (tocilizumab). Les données pré-cliniques sont très encourageantes (237) mais il semble que le tocilizumab pourrait être efficace principalement en phase inflammatoire débutante de la maladie sur les atteintes pulmonaires parenchymateuses (238).

VI) Les modèles d'étude de la ScS

1) Modèles d'étude *in vitro*

Comme nous venons d'en discuter, l'identification de différentes cytokines impliquées dans le développement de la pathologie chez les patients sclérodermiques a permis de reproduire certaines caractéristiques de la maladie sur des fibroblastes et/ou des CE dans différents modèles *in vitro*.

Parmi les différents modèles d'étude des fibroblastes développés *in vitro*, l'utilisation d'une stimulation par l'IL4, l'IL13 ou encore le TGFβ1 semble induire un phénotype pathologique à des fibroblastes sains. Tout d'abord, l'IL4 utilisée à des concentrations comprises entre 2.5 et 10 ng/mL induit un changement phénotypique des fibroblastes vers le myofibroblaste et stimule leur production maximale de collagènes et de FN (239,240). Ensuite, la stimulation de fibroblastes de peau saine en culture par 10 ng/mL d'IL13 durant 24 heures induit une augmentation significative de leur production de collagènes qui est supérieure à celle induite par 10 ng/mL d'IL4 mais qui reste inférieure à une stimulation par 5 ng/mL de TGFβ1 (202). Finalement, le modèle d'étude *in vitro* le plus robuste reste celui des fibroblastes stimulés au TGFβ1. En effet, le TGFβ1, du fait de ses propriétés pro-fibrotiques, induit la production de MEC et de collagènes par les fibroblastes en culture. De plus, la stimulation au TGFβ1 majore l'expression des gènes codant pour l'α-sma ou encore le CTGF, le PDGFR ou la THBS1 qui vont contribuer à l'activation des fibroblastes (241–250). Différentes concentrations (généralement entre 2 et 10 ng/mL) de TGFβ1 sont utilisées et les stimulations durent 24 h à 72 heures, en fonction des études. Mais comme pour les fibroblastes de peau (202), une stimulation de fibroblastes de poumon par 5 ng/mL de TGFβ1 durant 24 heures est à priori suffisante pour augmenter significativement l'expression d'α-sma (251). L'augmentation de la concentration en TGFβ1 ne semble pas faire mieux (252) puisqu'avec

10ng/mL les auteurs relatent une multiplication par trois de l'expression d'α-sma à l'échelle transcriptionnelle ou protéique (253,254), ce qui est semblable aux données obtenues avec 5ng/mL. Concernant la durée de la stimulation, stimuler moins de 16heures semble insuffisant (252) et stimuler 48 heures n'apporte pas de bénéfices supplémentaires à 24 heures de stimulation (245). Cependant, cette condition a régulièrement été utilisée (244,246,247,255). Ces diverses conclusions peuvent s'expliquer par la grande variabilité entre les cellules utilisées (primaires ou lignée commerciale), du tissu source des fibroblastes, de la protéine recombinante utilisée, du milieu de culture employé (notamment s'il est dépourvu ou restreint en sérum), mais aussi des outils utilisés pour l'évaluation de l'effet du TGFβ1 sur les cellules. Chaque équipe doit donc s'assurer d'avoir mis en place un modèle robuste, dans les conditions du laboratoire, avant de l'utiliser pour modéliser l'effet d'une molécule thérapeutique ou pour étudier une voie de signalisation par exemple.

En résumé, du fait de l'importance du rôle du TGFβ1 dans la physiopathologie de la ScS (165) et des nombreuses études disponibles ayant montré l'effet du TGFβ1 sur différents fibroblastes en culture, avec les précautions précédemment citées, le modèle d'étude *in vitro* de fibroblastes activés par le TGFβ1 nous semble être un outil pertinent pour surseoir au manque de prélèvements de patients tout en reproduisant au moins en partie la pathologie sur des cellules au départ saines. L'avantage des expérimentations *in vitro* est de pouvoir étudier plus spécifiquement une voie de signalisation et/ou un acteur impliqué ou dérégulé dans le fonctionnement des cellules mais aussi de pouvoir réaliser plus aisément différents tests thérapeutiques afin de déterminer plus précisément une ou des molécules d'intérêt à évaluer chez l'animal. Ce principe répond à la règle des 3R et aux considérations éthiques visant à limiter l'utilisation d'animaux quand cela est possible. Cependant, la ScS étant une maladie multifactorielle impliquant de nombreux acteurs, on ne peut se limiter aux modèles d'études *in vitro* pour répondre à de nombreuses problématiques et c'est pourquoi différents modèles d'études *in vivo* sont utilisés.

2) Modèles d'étude *in vivo*

Différents modèles d'étude de la ScS chez l'animal sont rapportés dans la littérature mais la majorité de ces modèles ne reproduit qu'une partie des caractéristiques de la maladie (256). Ils sont souvent issus de modèles d'autres pathologies qui présentent certaines caractéristiques similaires à celles de la ScS, comme les modèles d'étude de la fibrose pulmonaire idiopathique par exemple. Toutefois ces modèles de fibrose souvent localisée ne constituent pas toujours un modèle de ScS en raison de la nature systémique de la maladie (257). Nous discuterons, dans cette partie, des modèles génétiques et non génétiques les plus fréquemment rapportés et reproduits dans la littérature.

a) *Modèles génétiques*

A ce jour deux modèles génétiques spontanés et six modèles génétiques induits sont les plus fréquemment rapportés dans la littérature.

Modèles génétiques spontanés.

Le modèle des poulets de l'université de Californie à Davis (UCD) lignée 200 a été décrit en 1981 (258). Cette lignée de poulet développe spontanément, après une 1ère phase inflammatoire durant 1 à 3 semaines, une phase de fibrose puis de nécrose cutanée des pattes et de la crête. A quelques semaines d'évolution, la maladie touche différents organes et notamment l'œsophage, les reins, le cœur et les poumons. La mortalité est importante du fait de phénomènes vaso-occlusifs. Dans ce modèle sont retrouvés des aAc anti-CE et des AAN sans spécificité Scl-70 ou centromères (259). L'apparition successive d'une phase inflammatoire puis vasculaire évoque une cascade d'évènements proche de ce que l'on observe dans la ScS humaine. Cependant, l'utilisation du poulet comme sujet d'étude est difficile en raison du nombre limité de laboratoires agréés pour leur élevage et du faible nombre de réactifs disponibles pour l'étude des poulets.

Le modèle murin Tight skin 1 (Tsk1/+) a été décrit en 1976 (260). Ces souris ont une mutation constitutionnelle de la fibrilline 1 et développent à quelques semaines de vie une fibrose cutanée, une hypertrophie du myocarde et un emphysème pulmonaire, parmi d'autres anomalies du tissu conjonctif. Cependant, même dans les zones riches en collagène 1 et 3, il n'est pas noté la présence de TGFβ1 pourtant essentiel dans la physiopathologie de la ScS humaine (261). De plus, l'absence de mutation de la fibrilline-1 chez l'homme soulève la question de la pertinence de ce modèle, même s'il a attiré une attention particulière lors de la découverte d'aAc anti-fibrilline chez certains patients.

Modèles génétiques induits.

Le modèle de souris transgénique sur-exprimant Fra2 (antigène 2 lié à Fos) est caractérisé par une vasculopathie précoce secondaire à une apoptose des CE dans les premières semaines de vie, suivie d'une fibrose cutanée et pulmonaire progressive à l'âge de 12 semaines (262). Les souris présentent également une HTAP, ce qui en fait un modèle d'étude tout particulièrement intéressant pour cette atteinte fréquemment retrouvée chez les patients sclérodermiques (263).

Les souris mutées pour Fli1 développent des anomalies fibrillaires des collagènes et une hyperperméabilité capillaire (264) alors que les souris sur-exprimant Wnt-10b présentent une raréfaction de leur TA ainsi qu'une fibrose de leur derme. Ce remodelage tissulaire secondaire est également observé chez les patients atteints de ScS ce qui rend ce modèle Wnt10b particulièrement intéressant (265).

Dans le modèle de souris transgéniques possédant un récepteur au TGFβ de type II sans kinase associée (TGFβRIIΔk), les fibroblastes sur-expriment ce récepteur et les souris développent une fibrose cutanée et pulmonaire (266). En revanche, il n'y a pas d'aAc ni de stigmates de phase inflammatoire chez ces animaux. Ce modèle permet d'étudier en particulier la fibrose myocardique qui est plus rarement rapportée dans les autres modèles animaux (267).

Les souris MRL/lpr et déficientes en récepteur de l'IFNγ développent une maladie proche du lupus, au cours de laquelle on observe également une fibrose cutanée mais aussi d'organes (poumon, rein, foie) associée à un infiltrat inflammatoire et à une atteinte vasculaire des organes évoquant les lésions des patients atteints de ScS (268).

Enfin, le modèle de souris déficientes en relaxine, peptide hormonal connu pour ses propriétés anti-fibrosantes et vasodilatatrices, se traduit par un développement progressif d'une fibrose du derme avec un excès de collagènes 1 et 3 ainsi qu'une disparition de l'hypoderme (269). Dans ce modèle, l'apport exogène de relaxine réverse le phénotype pathologique des souris, sauf au stade avancé de fibrose. Ce modèle mime une part de l'atteinte cutanée de la ScS mais le rôle physiopathologique de la relaxine chez l'homme n'est pas démontré.

La ScS n'est pas une maladie héréditaire : les mutations présentes dans ces modèles animaux sus-cités ne semblent donc pas pertinentes pour l'étude étiologique de la maladie humaine. Même si ces modèles ne prennent pas en considération une ou des spécificités de la ScS, leur utilisation reste intéressante puisqu'ils permettent l'étude des mécanismes impliqués dans la fibrose et/ou la vasculopathie ainsi que l'évaluation de l'effet thérapeutique de différentes molécules.

b) *Modèles non génétiques*

Modèle de GVH.

Les premiers modèles animaux de GVH ont été décrits en 1963 chez le rat (270) puis en 1985 chez la souris (271). Ils s'inspirent des patients souffrant d'une réaction de GVH, souvent dans les suites d'une greffe de moelle osseuse (MO) pour une hémopathie maligne. Ces malades développent alors une fibrose cutanée mais aussi viscérale et leur

symptomatologie clinique est très proche de celle de la ScS. Une optimisation de la réaction de GVH chez des souris Balb/c déficientes pour RAG2 a permis d'obtenir un modèle se rapprochant de la ScS humaine à l'aide d'une injection systémique de splénocytes de souris B10.D2 : les souris présentent alors une fibrose multiviscérale, une réaction inflammatoire, une vasoconstriction et des stigmates d'auto-immunité (272).

Ce modèle est bien caractérisé et reproduit une maladie proche de la ScS décrite chez l'homme. Cependant, il démontre la présence d'une forte réaction inflammatoire dans les tissus atteints ce qui ne correspond pas aux tendances observées dans la pathologie humaine où l'infiltrat inflammatoire est moins prononcé. Par ailleurs, ce modèle est difficile à mettre en place, chez ces souris immunodéprimées et fragiles, nécessitant un confinement adapté de l'animalerie.

Modèles induits par des agents externes.

Au vu de l'importance des facteurs environnementaux dans la physiopathologie de la ScS, l'utilisation de modèles induits par un agent chimique externe permet de mimer la pluralité des mécanismes qui entraînent secondairement la fibrose et la vasculopathie : en ce sens, ces modèles sont plus proches de la maladie humaine que les modèles génétiques basés sur la dérégulation d'un seul gène ou d'une seule voie de signalisation.

Un des modèles les plus fréquemment utilisés est l'utilisation de la bléomycine chez des rongeurs, soit par des injections sous-cutanées, soit par l'inhalation intra-trachéale. La bléomycine induit une inflammation majeure locale à l'origine d'une fibrose limitée au tissu directement exposé. Certains travaux avaient décrit l'induction d'une fibrose systémique et notamment pulmonaire dans les suites d'injections sous-cutanées répétitives de bléomycine, mais ce modèle de fibrose plus généralisée est finalement rarement reproduit dans la littérature (92).

Une autre approche consiste en l'injection sous-cutanée de chlorure de vinyle chez des souris femelles Balb/C gestantes après leur accouplement avec des males C57BL/6. Les souris, ayant des cellules fœtales circulantes, développent une fibrose cutanée localisée (93). Ce modèle a pour intérêt de reproduire un environnement oxydant ainsi qu'un microchimérisme. Cependant, comme la bléomycine, le chlorure de vinyle n'induit pas une fibrose systémique. Ces deux modèles sont donc plus éloignés de la ScS humaine au cours de laquelle la fibrose touche différents organes.

Enfin, un dernier modèle induit par des agents externes a été décrit pour la première fois en 2009. Il consiste en des injections intra-dermiques quotidiennes d'HOCl, chez des souris femelles Balb/C âgées de 6 semaines. Il induit une fibrose cutanée et pulmonaire, une atteinte vasculaire notamment rénale ainsi que la production d'aAc anti-Scl70 (94). Ce modèle présente comme avantage d'induire une maladie systémique très proche de la forme humaine tout en mettant en valeur l'importance physiopathologique de l'environnement oxydant dans le déclenchement de la maladie et c'est pourquoi nous avons choisi d'utiliser ce modèle murin. De plus, nous et d'autres équipes avons pu reproduire et caractériser ce modèle de ScS ce qui en fait un outil particulièrement robuste (96,233,273–279).

VII) Les traitements de la ScS

1) Traitements symptomatiques

En pratique clinique courante, les patients bénéficient principalement de traitements symptomatiques des différentes atteintes de la ScS. Ainsi, la rééducation et l'ergothérapie sont les principaux outils pour maintenir une fonctionnalité de la préhension la plus pérenne possible. L'oxygénothérapie et les programmes de réhabilitation respiratoire peuvent aider à suppléer la déficience respiratoire. Les thérapeutiques habituellement proposées dans l'HTAP (analogue de la prostacycline, antagonistes des récepteurs de l'ET, inhibiteur de la phosphodiestérase, par exemple) peuvent soulager certains patients. De plus, les traitements de l'HTAP, les inhibiteurs calciques et l'éducation thérapeutique sont les armes utilisées pour lutter contre les phénomènes vaso-occlusifs des extrémités et leurs lourdes conséquences. La prise en charge symptomatique de l'insuffisance cardiaque (le cas échéant), notamment à l'aide de diurétiques mais aussi d'inhibiteurs de l'enzyme de conversion, peut aussi apporter un bénéfice fonctionnel. Les thérapeutiques anti-reflux gastrique (inhibiteurs de la pompe à protons), les pro-kinétiques (erythromycine), les laxatifs et les analogues de la sandostatine viseront à soulager les patients présentant des atteintes digestives hautes et/ou basses.

L'ensemble de ces thérapeutiques ne permet en aucun cas de limiter l'évolution de la ScS en phase active. Ces traitements visent seulement à suppléer, lorsqu'ils le peuvent encore, la défaillance d'organe ou du tissu atteint afin de soulager la gêne fonctionnelle du patient. Dès lors qu'ils ne suffisent plus, seule une transplantation de l'organe défaillant (poumon et/ou cœur) permet d'apporter une solution au patient pour allonger sa survie, mais les complications, notamment péri-opératoires, sont sévères (280). De plus, la transplantation est rarement possible du fait de l'état général du patient et de nombreuses contre-indications.

2) Traitements curatifs

Dans une approche curative, à ce jour, aucun traitement visant l'ensemble des mécanismes physiopathologiques n'a fait ses preuves pour stopper ou reverser la maladie. Certaines molécules sont proposées, en fonction des atteintes viscérales du patient et de la présentation clinique, afin de limiter ou arrêter la progression de la ScS. De nombreuses thérapeutiques sont en cours d'études cliniques (281) et pour certaines encore en phase pré-clinique.

a) *Traitements immunomodulateurs*

Lors de la prise en charge précoce des patients, notamment en phase inflammatoire de la ScS, les traitements immunomodulateurs semblent apporter un bénéfice aux patients, même s'ils ne sont pas dénués d'effets secondaires potentiellement graves. Dans ce cadre, on soulève ici l'intérêt de différents traitements, comme la corticothérapie par voie générale, mais dont la posologie prescrite ne peut être élevée du fait du risque de déclenchement d'une crise rénale sclérodermique. Les immunomodulateurs dits conventionnels tels que le méthotrexate ou le mycophénolate mofétil (MMF) ont un bénéfice seulement modéré, sur les atteintes articulaires et cutanées. Cependant, le MMF ou encore le cyclophosphamide limitent l'extension de la fibrose dans les atteintes pulmonaires précoces et rapidement progressives (282). Il en est de même pour le rituximab, traitement qui induit d'ailleurs moins d'effets secondaires que les médicaments cités précédemment (283).

L'utilisation d'immunoglobulines polyvalentes ainsi que d'autres biothérapies, telles que les anti-IL1 et les anti-TNFα, ne semble pas apporter de bénéfice clinique. Concernant les anti-IL6, après avoir soulevé un grand espoir notamment en phase 2, un essai récent de phase 3 n'a pas montré de différence significative d'efficacité thérapeutique du tocilizumab entre le groupe placebo et le groupe de patients traités (238). Il semblerait qu'un sous-groupe de malades pourrait en bénéficier, mais de nouvelles études sont nécessaires pour le prouver.

b) *Traitements anti-fibrosants*

Un autre versant thérapeutique de la ScS est de cibler la fibrose. Ainsi de nombreuses études évaluent le potentiel anti-fibrosant de molécules ciblant certains médiateurs impliqués dans la physiopathologie tels que le TGFβ1, le PDGFR, le VEGF, PPARγ, c-abl, l'IL4, la voie Notch, la voie Wnt ou encore la voie endocannabinoïde. Certaines molécules ne sont encore qu'au stade d'étude pré-clinique alors que d'autres constituent l'objet de différents essais cliniques (pour revue,(281)). On soulignera ici l'intérêt de la pirfenidone et du nindetanib (284) qui suscitent un grand espoir thérapeutique notamment pour la composante pulmonaire de la maladie (285).

De même, les agonistes des récepteurs de type endocannabinoïdes (lenabasum), en limitant l'inflammation médiée par la réponse immune innée et de fait la fibrose induite, semblent sûrs et prometteurs en terme d'efficacité clinique, mais aussi à l'échelle moléculaire et histologique (286). L'essai clinique de phase 3 actuellement en cours devra bien sûr le confirmer avec un plus grand nombre de patients et une durée de suivi adéquate.

3) La thérapie cellulaire

L'intérêt pour la thérapie cellulaire, et notamment la transplantation de cellules souches hématopoiétiques (CSH), est grandissant. En effet, la transplantation de CSH a montré une amélioration significative de la survie globale des patients atteints de ScS et réfractaires aux autres traitements (287). Elle est plus efficace qu'un traitement par cyclophosphamide seul pour les patients inclus dans les essais (288). Cependant cette approche n'est pas sans risque (289). Les effets secondaires semblent importants en phase précoce de la transplantation du fait de la toxicité cardiaque mais aussi en phase tardive du fait de développement de tumeurs malignes. Ici, et comme souvent, la sélection des patients, pour un traitement aussi lourd, est essentielle afin de ne pas être plus délétère que l'évolution naturelle de la maladie (290). De plus, l'amélioration des protocoles d'immunosuppression, prérequis obligatoire à la transplantation de CSH, pourra également contribuer à diminuer sa toxicité.

Une autre alternative à l'utilisation de CSH est celle de la greffe de CSMs qui pourrait apporter un bénéfice thérapeutique semblable à la transplantation de CSH mais qui est dépourvu des effets secondaires à l'immunosuppression obligatoire de la transplantation de CSH. Nous allons donc développer ce sujet au cours de la 2ème partie de l'introduction bibliographique de ce manuscrit, dédiée aux CSMs.

2ème partie : Les cellules stromales mésenchymateuses

Afin de comprendre en quoi les cellules stromales mésenchymateuses (CSMs) sont une alternative thérapeutique intéressante au cours de la ScS, nous allons d'abord définir les CSMs et présenter leurs différentes sources tissulaires, puis décrire leurs différents mécanismes d'action et rôles biologiques, et enfin entrevoir leur intérêt thérapeutique.

I) Définition des CSMs

1) Histoire et nomenclature

Les CSMs, ont été décrites pour la première fois par Alexander Friedenstein en 1968, suite à l'isolement d'une population de cellules de MO qui adhèrent au plastique en culture formant des CFU-F (unité formant des colonies fibroblastiques) et qui présentent une forme fibroblastique (291). Ces deux caractéristiques les distinguent des CSH et sous-tendent leur appartenance au compartiment stromal de la MO. Le terme de « cellules souches mésenchymateuses » n'apparaîtra qu'en 1991 suite à la nomenclature d'Arnold Caplan. Même si ce terme est couramment utilisé dans la communauté scientifique, il fait débat et n'a pas été retenu par le groupe de travail sur les CSMs de la société internationale de thérapie cellulaire (ICST). Pour le comprendre, revenons sur la définition d'une cellule souche (CS).

Une CS est une cellule capable d'auto-renouvellement illimité, qui peut se différencier en d'autres types de cellules matures sous certaines conditions. On distingue les CS d'origine embryonnaire et les CS de l'adulte. On peut également classer les CS selon leur potentiel de différenciation.

Tout d'abord, les CS peuvent être totipotentes, c'est-à-dire capable de se différencier dans tous les types cellulaires des 3 feuillets embryonnaires (ectoderme, endoderme et mésoderme), y compris en cellules germinales. Elles peuvent également se différencier en cellules du trophoblaste et donc générer des tissus extra-embryonnaires (placenta). Finalement, seules les CS du zygote ou du blastomère précoce (avant le 4ème jour de division) sont totipotentes.

Ensuite, les CS peuvent être pluripotentes : elles ont le même potentiel de différentiation que les CS totipotentes à la différence qu'elles ne peuvent pas générer les cellules du trophoblaste. Ceux sont les CS embryonnaires dérivés du blastocyste (292). Depuis 2006, une autre source de CS pluripotentes a été générée : ce sont les iPS (cellules souches pluripotentes induites).

Ce sont des cellules matures adultes qui sont reprogrammées en CS pluripotentes grâce à l'introduction de 4 gènes de pluripotence (293).
Ensuite, on distingue les CS multipotentes qui sont capables de se différencier en différents types de cellules matures provenant d'un même feuillet embryonnaire (exemple : CSH).
Enfin, les CS peuvent être unipotentes, c'est-à-dire qu'elles ne peuvent se différencier qu'en un unique type cellulaire (exemple : peau, foie, …).

Ainsi, les CSMs se situent parmi les CS adultes multipotentes. Pour autant, leur multipotence est débattue, car cette notion sous-entend qu'il y ait un progéniteur commun à tous les tissus dont l'origine est le feuillet mésodermique. De plus, leur capacité de différenciation en plusieurs tissus a été prouvée dans des conditions de culture *in vitro* parfois extrêmes, soulevant aussi les limites de leur caractère « souche » au sens fondamental du terme. La capacité d'auto-renouvellement illimité comprise dans le terme « souche » a été démontré *in vivo* dans un travail ayant décrit la capacité de progéniteurs mésenchymateux de MO CD146+ à générer des tissus osseux hétérotopiques après deux cycles d'isolement/réimplantation successifs (294). Finalement, ces désaccords terminologiques soulignent les difficultés observées pour comparer des travaux de recherche sur les CSMs, dont l'isolement et la caractérisation n'étaient pas toujours univoques et consensuelles (295). C'est notamment la raison pour laquelle l'ICST a essayé d'homogénéiser les méthodes d'isolement et de culture des CSMs ainsi que leur caractérisation phénotypique et fonctionnelle, en proposant une définition des CSMs d'origine humaine en 2006 (296) et a publié une note complémentaire clarifiant la nomenclature des CSMs en 2019 (297).

2) Définition des CSMs selon l'ICST

Depuis 2006, selon l'ICST, on peut considérer comme étant une CSM, toute cellule isolée à partir d'un tissu ou d'un organe répondant aux caractéristiques suivantes (296,297) :

- Les cellules adhèrent au plastique dans des conditions de culture classique
- Au moins 95% des cellules étudiées doivent présenter les marqueurs membranaires CD73, CD90, CD105, et ne pas porter les marqueurs de surface CD34, CD11 ou CD14, CD45, CD19 ou CD79a, HLA-DR
- Les cellules doivent être capables de se différencier, *in vitro*, en adipocytes, en chondrocytes et en ostéoblastes.

Concernant le second point de cette définition, il semble important de souligner, que la liste des marqueurs de surface des CSMs proposée ici n'est pas exhaustive. En effet, de nombreux travaux essaient de trouver un marqueur spécifique de la CSM mais aucun ne semble

répondre à cette spécificité (298). Parmi toutes ces études, certains marqueurs sont fréquemment recherchés, tels que des molécules d'adhésion comme CD29 ou des récepteurs comme CD44. Finalement, c'est la combinaison de la présence et de l'absence de certains marqueurs qui permet de retenir un « phénotype » dit de CSMs. De plus, l'expression de certaines molécules peut varier en fonction des conditions de culture cellulaire, mais aussi en fonction de la source de tissu d'où proviennent les CSMs. Par exemple, le CD34 est présent sur des CSMs humaines d'origine adipocytaire puis disparait au cours des passages en culture (299). Par ailleurs, concernant la caractérisation des CSMs provenant d'autres espèces animales non humaines, certains de ces marqueurs peuvent ne pas être exprimés : ce sont alors les deux autres points de définition de l'ICST qui permettent de considérer les cellules isolées comme des CSMs (300). Par exemple, les CSMs murines présentent à leur surface membranaire Sca-1 (Stem cells antigen-1) et CD44, et sont négatives pour le CD45 et le CD11b. Au cours de l'isolement, la sélection positive par le CD34 permettrait d'enrichir la population de CSMs murines en culture, alors que son expression disparaitrait également au cours des passages en culture (301).

Concernant le troisième point de cette définition, les CSMs sont des cellules qui doivent pouvoir se différencier *in vitro* en adipocytes, en chondrocytes et en ostéoblastes, dans des conditions d'induction propres à chaque lignage de cellule mature (302). En 1999, le premier travail ayant démontré cette propriété des CSMs avait également démontré que des fibroblastes de peau de nouveau-né mais aussi de glande mammaire adulte n'en étaient pas capables (303). Ce point est important à souligner car, comme leur dénomination l'indique, les CSMs seraient à l'origine des fibroblastes, mais leur phénotype « fibroblastique » et l'absence de marqueur de surface spécifique aux CSMs peuvent laisser penser qu'elles seraient en fait elles-mêmes des fibroblastes. D'ailleurs, certaines études ont montré que des fibroblastes humains de peau ou de poumon pourraient avoir une différenciation semblable en culture à celle des CSMs (304,305) tout comme l'ont prouvé des fibroblastes de peau de souris (306). Ce point est toujours sujet à controverse bien que des signatures génétiques et épigénétiques pourraient distinguer ces deux types cellulaires. Certaines revues font la synthèse et discutent ces interrogations (307,308).

Enfin, les CSMs peuvent se différencier vers d'autres types cellulaires tels que les ténocytes (309), les myocytes (310), les cardiomyocytes (311), les hépatocytes (312), les CE (313) et même vers des cellules neuronales fonctionnelles (314). Pour cela, les CSMs sont placées dans des conditions de cultures souvent éloignées du contexte de leur niche physiologique chez l'homme. Cette plus large capacité de différenciation des CSMs suggérée par ces différentes études *in vitro* n'est peut-être pas une caractéristique naturelle des CSMs et cette transdifférenciation des CSMs est, une fois de plus, débattue dans la communauté scientifique.

3) Importance de la niche environnementale des CSMs

Le potentiel de différenciation des CSMs est donc dépendant du micro-environnement naturel dans lequel évolue la cellule. Cet environnement est appelé « niche ». Il régule le devenir de la CSM mais aussi sa survie dans le tissu. Cette niche est composée des cellules souches mais aussi des cellules stromales et de la MEC, qui varient en fonction des tissus. Ainsi, le destin de CSMs provenant d'une niche articulaire ou d'une niche médullaire ne sera pas le même par exemple. De plus, en pathologie, des modifications de la niche environnementale des CSMs peuvent déréguler leur processus de différenciation vers des cellules matures potentiellement impliquées dans le développement de maladies.

II) Sources tissulaires des CSMs

Les CSMs ont été initialement isolées à partir de MO. Pour les obtenir, il faut réaliser un prélèvement invasif et douloureux pour les patients, ou bien récupérer des prélèvements osseux issus d'interventions chirurgicales. Les cellules sont rares et le rendement est faible à l'isolement, ce qui implique des étapes d'amplification en culture avant leur utilisation (303). Ainsi, d'autres sources de CSMs ont rapidement été recherchées.

Par la suite, les CSMs ont donc été isolées à partir de TA. Ces CSMs sont appelés ASCs (pour cellules stromales mésenchymateuses du tissu adipeux). Elles sont comprises dans la fraction stroma-vasculaire (FSV) du TA dont le prélèvement par liposuccion est moins invasif qu'un prélèvement de MO. Elles intéressent particulièrement la communauté scientifique et médicale pour leur facilité d'accès et leur haut rendement (1 000 à 10 000 fois supérieur à celui de la MO) (315).

Les CSMs ont également été isolées à partir de la pulpe dentaire (316), de liquide synovial et du synovium (317,318), de la limbe et du trabéculum dans l'œil (319), de liquide amniotique et de la gelée de wharton (320), de cordon ombilical (321) ou encore de placenta (322). Finalement, les CSMs pourraient être présentes dans tous les tissus et les organes, comme le suggère une étude menée chez la souris (323). En effet, même si cette information est encore une fois un sujet à controverse, un sous-groupe de CSMs est localisé au niveau périvasculaire, ce qui pourrait expliquer que finalement tous les tissus et les organes en contiennent (324).

III) Propriétés des CSMs

Le rôle biologique des CSMs ne s'arrête pas à la seule régénération du tissu dans lequel elles se trouvent. Elles sont dotées de propriétés pléiotropiques, qu'elles exercent à l'échelle locale mais aussi systémique. En effet, les CSMs peuvent interagir directement avec leurs cellules cibles mais communiquent également à distance grâce à la libération de différents médiateurs, solubles dans l'environnement ou contenus au sein de vésicules extracellulaires (EVs) (325). Nous intégrerons globalement le rôle de ces médiateurs dans chaque fonction biologique des CSMs dans la prochaine partie de ce manuscrit et nous nous intéresserons tout particulièrement au rôle des microARNs dans la fonction des CSMs.

1) Les différentes fonctions biologiques des CSMs

a) *Renouvellement des cellules matures*

Par définition, les CSMs ont une fonction de renouvellement tissulaire et permettent par exemple, au niveau de la MO, de renouveler les ostéoblastes et les adipocytes, maintenant ainsi l'homéostasie du tissu squelettique.

b) *Fonction trophique*

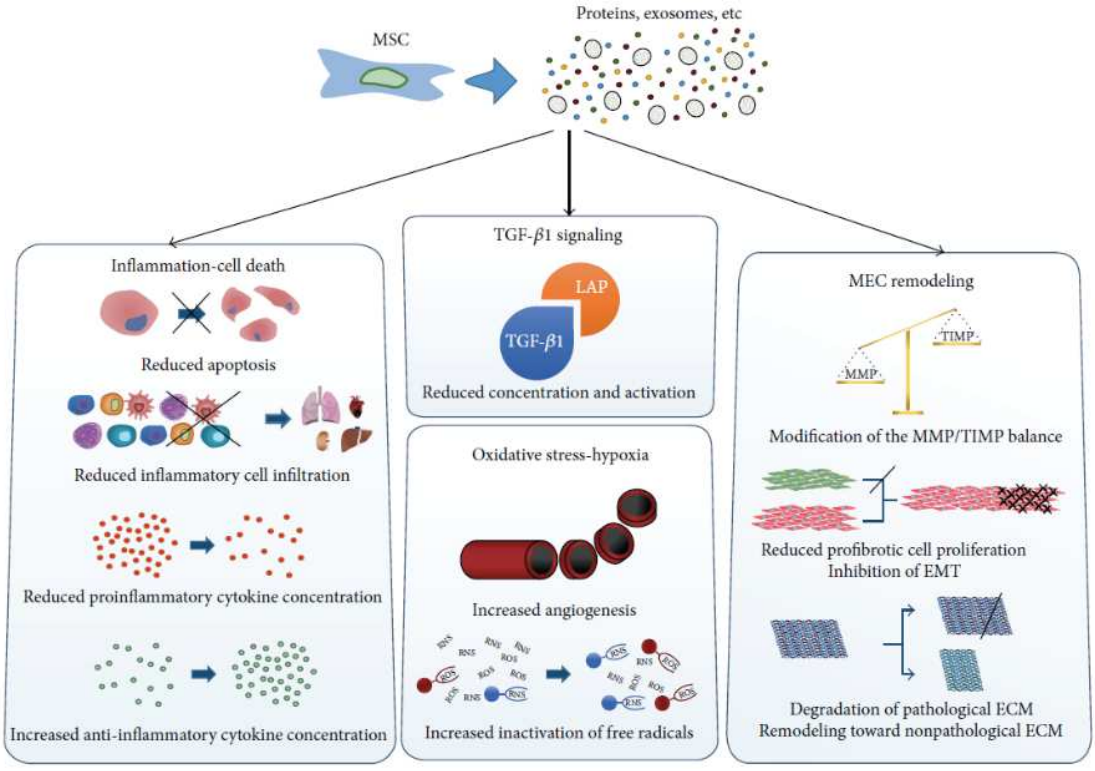
Les CSMs contribuent également à l'homéostasie tissulaire en raison de leur fonction trophique essentielle à d'autres cellules. Par exemple, dans la MO, les CSMs sont essentielles à la survie des CSHs et participent à la régulation de leur prolifération et de leur différenciation. De plus, elles sécrètent une grande partie des molécules de la MEC dans laquelle les CSHs sont contenues.
De façon plus générale, les CSMs assurent cette fonction « trophique » grâce aux nombreuses molécules secrétées et nous nous intéresserons plus particulièrement à leurs effets anti-fibrotiques, pro-angiogéniques, anti-apoptotiques et immunosuppressifs qui ciblent les trois axes de dysfonctionnement de la ScS (Figure 5).

Propriétés anti-fibrotiques.

Les CSMs exercent leur pouvoir anti-fibrotique par des mécanismes d'action ciblant directement les cellules pro-fibrotiques, la voie du TGFβ1 ainsi que le remodelage de la MEC. En effet, les CSMs diminuent l'expression du TGFβ1 dans un modèle murin de fibrose hépatique mais aussi de fibrose péritonéale (326,327), notamment par la réduction de la phosphorylation de SMAD 2 (327). Elles diminuent également l'expression du CTGF dans un

modèle de rectite chez le cochon (328). Par ailleurs, elles augmentent l'expression de certaines MMPs dans différents modèles de fibrose (MMP13 par exemple (329,330)) et réduisent l'expression de certains TIMPs, tel que TIMP1 dans un modèle animal de fibrose rénale (330) ou TIMP2 dans un modèle de coculture avec des cardiomyocytes (331). Ainsi, dans de nombreuses études, l'expression et la concentration des collagènes sont diminuées après un traitement par CSMs (332). Pour se faire, les CSMs sécrètent différents médiateurs anti-fibrotiques (297), parmi lesquels on note le facteur de croissance hépatocytaire (HGF)(333,334,335) ou encore le gène stimulé par le TNF de type 6 (TSG6) (336). L'HGF est un puissant médiateur anti-fibrotique capable d'agir sur différentes cibles. Il diminue l'expression du TGFβ1 et augmente la libération de MMP1 favorisant ainsi la dégradation du collagène (334). Il inhibe la synthèse des collagènes I et IV par les cellules hépatiques stellaires en induisant l'expression de miR-29a (333). Le TSG6 agit en inhibant l'activation des macrophages ce qui entraîne une réduction des taux de TNFα et permet également d'obtenir un rapport TGFβ1/TGFβ3 plus faible, témoignant d'une fibrose moins importante (336).

Figure 5 – Les différentes propriétés des CSMs d'intérêt pour la ScS



ECM: extracellular matrix, EMT: epithelial to mesenchymal transition, LAP: latency associated protein, MMP: matrix metalloproteinase, MSC: mesenchymal stromal cell, TGF-β1: transforming growth factor β1, and TIMP: tissue inhibitor of metalloproteinase.

D'après Usunier et al, Stem Cells International (2014).

De plus, les CSMs luttent contre la fibrose en ciblant le stress oxydant. En effet, elles sont capables d'augmenter la production de superoxide dismutase (SOD) par l'activation du facteur nucléaire 2 similaire au dérivé d'érythroïde 2 (Nrf2 ou NFE2L2) dans un modèle de fibrose hépatique (337). De même, elles augmentent l'expression et/ou la concentration de certaines enzymes anti-oxydantes telles que la NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) quinone oxydoréductase, la glutathione réductase ou encore l'hème oxygenase 1 (338,338). L'inhibition du stress oxydant permet notamment de contrecarrer indirectement l'activation du TGFβ1, en empêchant le clivage de son peptide de latence associé.

Enfin, les CSMs ont des propriétés pro-angiogénique et immunomodulatrice qui contribuent à lutter contre la fibrose en réduisant notamment l'hypoxie et l'inflammation. Nous les détaillerons dans les deux prochaines sous-parties.

Même si tous les mécanismes moléculaires anti-fibrotiques ne sont pas encore élucidés, de nombreux travaux ont démontré l'effet thérapeutique des CSMs dans différents modèles de fibrose secondaire à des pathologies ischémiques rénales (339), cardiaques (331) ou encore neurologiques (340).

Propriétés pro-angiogéniques

Les CSMs ont démontré leur rôle pro-angiogénique en promouvant la vasculogenèse et l'angiogenèse dans différents modèles d'étude *in vitro* et *in vivo* (341), qu'elles soient d'origine murine ou humaine (341,342), isolées à partir de MO (343,344), de cordon ombilical (345) ou encore de TA (346,347). Les CSMs exercent ce pouvoir pro-angiogénique au travers de la sécrétion de différents facteurs tels que le VEGF, le FGF2, l'IL6, la MCP1, le Placental Growth Factor, l'IL6, l'angiopoietin 1, l'IGF1, l''érythropoïétine (EPO), l'HGF, le facteur dérivé des cellules stromales 1 (SDF1), l'ET1 et le CXCL16 (ligand de chimiokines (motif C-X-C) 16) (348–351). Le surnageant de culture des CSMs possède un effet pro-angiogénique semblable à l'utilisation des CSMs : il favorise la prolifération des CE et réduit leur apoptose (352). De plus, la trans-différenciation des CSMs vers les péricytes pourraient contribuer à la formation de nouveau vaisseaux *in vitro* et *in vivo* (353,354).

Les propriétés pro-angiogéniques des CSMs sont potentialisées par l'environnement dans lequel elles évoluent. En effet, la culture des CSMs en condition d'hypoxie augmente leur potentiel angiogénique en augmentant les taux d'EPO et de VEGF (355). De même, une stimulation au PDGF majore la quantité d'EVs produites par les ASCs, ainsi que leur potentiel angiogénique en augmentant leur contenu en MMP9, en oncostatine M et en thrombopoïétine notamment (356). Il est intéressant de noter qu'*in vitro*, le milieu de culture de CSMs provenant de MO de patients atteints de ScS semble avoir un pouvoir pro-angiogénique plus fort que

celui de CSMs de donneurs sains mais équivalent à celui de CSMs saines stimulées par le SDF1 (357). Ces CSMs pourraient être finalement « pré-conditionnées » chez le patient. Concernant les CSMs provenant de TA de patients atteints de ScS, une étude récente a montré qu'elles conservent leur pouvoir pro-angiogénique sur des CE lorsqu'elles sont en coculture mais il serait plus faible que celui de CSMs saines (358). En revanche, la FSV du TA des malades ne présenterait pas d'altérations de ses propriétés pro-angiogéniques (359).

Ainsi, les CSMs ont prouvé leur effet thérapeutique dans des modèles précliniques d'ischémie cardiaque et rénale (348,355) ou encore d'ischémie de membres (343,345), tout comme leur surnageant de culture l'a démontré dans le traitement de l'infarctus du myocarde (360). Au niveau clinique, les CSMs ont montré un bénéfice thérapeutique dans le traitement d'ulcères de patients diabétiques ou de patients atteints de la maladie de Buerger (361). Finalement, les CSMs sont capables de réparer des tissus lésés, notamment en promouvant l'angiogenèse (pour revue, (362)). Dans le traitement de la ScS, une injection locale de CSMs ou de la FSV semble également améliorer les lésions ulcéreuses (363,364), même si nous sommes en attente de résultats d'un essai clinique de plus grande ampleur (SCLERADEC II).

Propriétés anti-apoptotiques.

Les CSMs et/ou leur surnageant de culture limitent l'apoptose de cardiomyocytes (365), de fibroblastes de poumon (366), de cellules musculaires (367), des cellules épithéliales du tube proximal du rein (368) ou encore des CE (352), *in vitro* et/ou *in vivo*. Elles limitent également les lésions d'ischémie-reperfusion de moelle épinière en diminuant les phénomènes apoptotiques (340). Parmi ces nombreuses études, différents facteurs semblent médier le rôle anti-apoptotique des CSMs ; citons le VEGF, le TGFβ1, le facteur stimulant les colonies de granulocytes et de macrophages (GM-CSF), l'HGF, le FGF, l'IL6, XCL1 (le ligand de la chimiokine (C motif) 1) ou encore l'IGF1 (367,368). Ces propriétés anti-apoptotiques des CSMs nous intéressent tout particulièrement dans la ScS, puisque la vasculopathie est en partie secondaire à une apoptose excessive des CE.

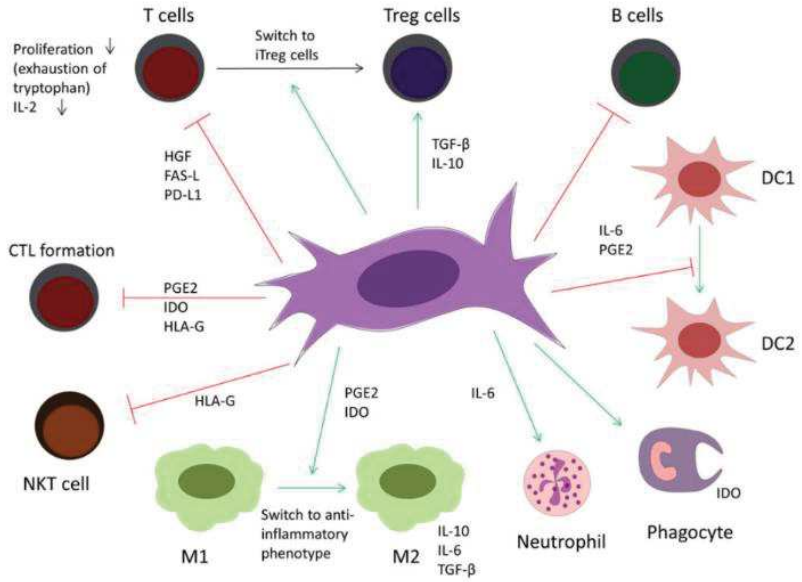
Propriétés immunomodulatrices.

Les CSMs possèdent différentes propriétés immunomodulatrices et peuvent ainsi interagir avec les nombreux acteurs de l'immunité innée et adaptative, par contact direct avec les cellules cibles mais également par la libération de médiateurs (Figure 6). Leur sécrétome va dépendre de l'environnement physiologique ou pathologique dans lequel elles se trouvent. De ce fait, la plupart des études *in vitro,* visant à étudier les propriétés immunomodulatrices des CSMs, sont conduites sur des CSMs stimulées par l'IFNγ, le TNFα et/ou l'IL1β afin de

reproduire l'environnement pro-inflammatoire dans lequel elles seraient dans l'organisme, suite à une lésion tissulaire et/ou une infection (369).

Ainsi, les CSMs sont capables d'inhiber la prolifération des LT, notamment par la sécrétion d'indoleamine 2,3-dioxygénase (IDO) (370). Elles peuvent induire la différentiation de certains LT vers une population de LT régulateurs, à l'aide de la prostaglandine E2 (PGE2) (371,372). Concernant l'interaction des CSMs sur les LB, les données de la littérature sont assez controversées mais il semblerait que la coculture de CSMs avec des LB permet de réduire leur prolifération, leur différenciation et leur production d'Ac (373). Les CSMs vont également influencer la différenciation de macrophages vers un phénotype anti-inflammatoire notamment par la sécrétion de PGE2 (374) mais aussi influencer la maturation, la différenciation et la fonction des cellules dendritiques (375). Enfin, en interaction avec les cellules natural killer (NK), les CSMs sont capables d'inhiber leur prolifération et diminuer leur cytotoxicité, notamment par la libération de PGE2, IDO (Indoléamine 2,3-dioxygenase) et TGFβ1 (376). Parmi les médiateurs immunomodulateurs libérés par les CSMs murines, on peut souligner l'important de PGE2 et d'iNOS (377) sans qui les CSMs perdent leur effet anti-inflammatoire dans un modèle murin d'arthrite. Outre ces molécules, le rôle de l'IL1RA, HLA-G, IL6 ou encore GILZ (glucocorticoid-induced leucine zipper) (378,379) ainsi que de certains microARNs ont également été démontré (380).

Figure 6 – Effet immunomodulateur des CSMs



CTL: cytotoxic T lymphocytes ; DC: dendritic cell ; FAS-L: FAS ligand ; HGF: hepatocyte growth factor ; HLA-G: human leucocyte antigen-G; IDO: indoleamine 2,3-dioxygenase; IL: interleukin; PD-L1: programmed death-ligand 1; PGE2: prostaglandin E2; NK: natural killer T; TGF-β: transforming growth factor-β; Treg: regulatory T.

D'après Mehler V et al, Stem Cell Translational & Clinical research (2018)

Enfin, les CSMs sont faiblement immunogènes, exprimant peu de CMH de classe I et de molécules de co-stimulation (381). Elles sont tout de même reconnues par le système immunitaire et peuvent être lysées par les cellules NK. Cependant, les CSMs limitent par elle-même la réaction immunitaire grâce à leurs propriétés immunosuppressives (382,383).

***Autres propriétés**.*

Les CSMs présentent d'autres propriétés biologiques qui sont moins étudiées dans le cadre de la ScS. Elles possèdent des propriétés chémoattractantes en sécrétant un large panel de chimiokines capables de recruter différents types cellulaires (CSH, cellules du systèmes immunitaires, progéniteurs endothéliaux) en fonction du contexte physiologique ou même pathologique dans lequel elles se trouvent (pour revue, voir (384)). Les niveaux d'expression de ces différentes chimiokines varient en fonction de la source de CSMs ainsi que de leur condition de culture et/ou coculture. Elles possèdent également des propriétés antibactériennes en sécrétant des facteurs anti- ou pro-inflammatoires selon le contexte, mais aussi en stimulant la phagocytose (385). De plus, elles sécrètent des peptides anti-microbiens, tels que la cathelicidine LL-37 (386), la défensine β (387), l'hepcidine (388) ou encore la lipocaline 2 (389), qui luttent directement contre certains germes. Différentes études *in vivo* ont montré que les CSMs améliorent la survie de souris présentant une septicémie (390,391) et plusieurs essais cliniques sont en cours.

2) Le rôle des microARNs dans la fonction des CSMs

Les microARNs sont des petits ARNs non codants (19 à 25 nucléotides), ayant un rôle dans la régulation post-transcriptionnelle des ARNm. Ils inhibent l'expression de leur cible en favorisant leur dégradation ou en empêchant leur traduction en protéines (278). Ils seraient responsables de la régulation post-transcriptionnelle d'au moins 30% des gènes humains (392). Une étude a même démontré que plus de 60% des ARNm sont des cibles prédictives de microARNs (393). L'expression des microARNs est modulée par différents facteurs (stress, environnement, …). Un même microARN peut réguler des centaines de gènes différents et un gène peut être réguler par plusieurs microARNs (394). Les fonctions des microARNs sont donc multiples et variables selon le contexte environnemental mais aussi selon la cellule sur laquelle ils agissent.

a) *Fonctions des microARNs*

La fonction des microARNs est donc essentielle dans de nombreux processus physiologiques. D'ailleurs, l'inhibition de l'enzyme nécessaire à la maturation de microARNs au cours de leur biogenèse (Dicer) entraîne le décès des embryons de souris dès les stades précoces de l'embryogenèse (395). La dérégulation des microARNs semble également impliquée dans la physiopathologie de cancers (396), de pathologies hépatiques (397), de pathologies neuro-dégénératives (398) ou encore de l'obésité et du diabète (399,400). Les connaissances scientifiques et le nombre de publications sur les microARNs augmentent de manière exponentielle depuis ces vingt dernières années. Ainsi, l'étude des microARNs est devenue un outil pour la compréhension de mécanismes physiopathologiques mais aussi un outil diagnostic et thérapeutique prometteur dans certains domaines comme dans le traitement de l'hépatite C par exemple (401).

***Fonction des microARNs dans la ScS*.**

Au cours des pathologies fibrosantes, plusieurs microARNs se sont révélés être des acteurs importants, soit par leur fonction anti-fibrotique tels que miR-29a (402–407), miR-122 (408), miR-129-5p (409), miR-133 (410) et miR-214 (411), soit par leur rôle pro-fibrotique tels que miR-17-5p (412), miR-21 (413–415), miR-33a (416), miR-132 (417,418), miR-192 (419), miR-199a-5p (420), miR-199b (421). Leurs cibles sont nombreuses et varient en fonction du microARN en question et de la cellule cible. Par exemple, miR-132 régule la transition du fibroblaste vers un phénotype de myofibroblaste au travers de la modulation de MeCP2, un facteur de transcription inhibiteur d'α-sma. Dans un modèle murin d'infarctus du myocarde, la suppression de miR-132 par une approche d'antagomiR réduit *in vitro* et *in vivo* son action ciblée sur MeCP2, diminuant ainsi l'expression d'α-sma et inhibant la prolifération des fibroblastes et leur différenciation en myofibroblastes (418).

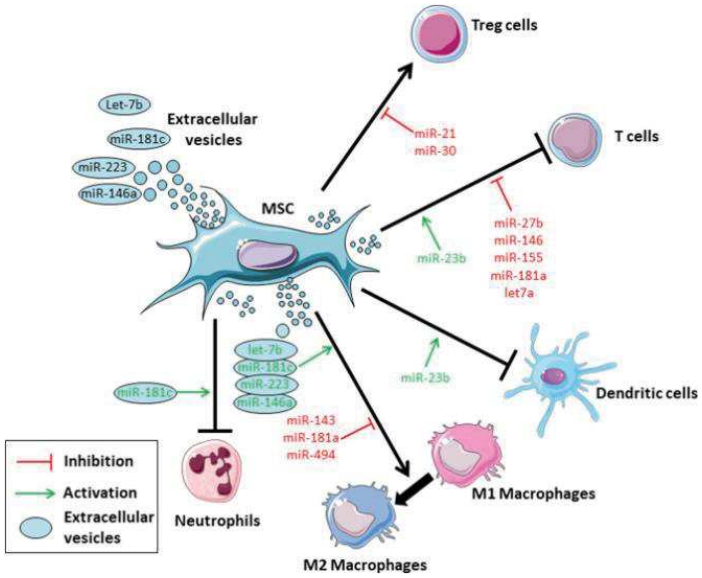
Au cours de la ScS, différents microARNs ont été identifiés comme étant dérégulés au niveau sérique mais également au niveau des tissus atteints par la fibrose (422).
Tout d'abord, plusieurs études ont montré la sous-expression de miR-29a dans les fibroblastes-ScS comparée à des sujets sains (423), mais nous reviendrons en détails sur miR-29a dans le paragraphe suivant [III-2)b)].
Ensuite, miR-21 est sur-exprimé dans les fibroblastes-ScS (424) et son expression est directement corrélée à celle des collagènes (425). MiR-21 a des propriétés pro-fibrosantes en inhibant SMAD7, lui-même inhibiteur de la voie du TGFβ1 (426). In fine, miR-21 induit donc la production d'α-sma, de collagène 1 ou encore de FN1 en activant indirectement la voie du TGFβ1. D'ailleurs, le TGFβ1 peut lui-même induire la sur-expression de miR-21 dans la peau des patients. La sous-expression de miR-21 pourrait augmenter l'apoptose des fibroblastes-

ScS en augmentant le ratio Bax/Bcl2 (protéine Bcl-2–associated X/B-cell lymphoma 2) (427). Dans la fibrose rénale, l'inhibition de miR-21 par une approche d'antagomiR réverse la maladie (413) et un essai clinique de phase 1 évalue la sécurité et l'effet d'un antagomiR-21 (RG-012) chez des patients atteints de syndrome d'Alport. Nous sommes en attente des résultats.
Enfin, d'autres microARNs sont dérégulés dans les fibroblastes-ScS. Ainsi, miR-92a et miR-202-3p sont augmentés dans les fibroblastes-ScS et contribuent aux anomalies de remodelage de la MEC, en ciblant directement MMP1 (428,429). La dérégulation de miR-150 et miR-196a augmente l'expression du collagène 1 et de phospho-SMAD3 au sein des fibroblastes-ScS (430,431). MiR-135b est sous-exprimé dans les fibroblastes-ScS et responsable d'une sur-production d'IL13, cytokine pro-fibrosante (432). Par ailleurs, let-7a est sous-exprimé au niveau de fibroblastes-ScS et let-7d/b est négativement corrélé à la présence d'HTAP chez les patients (433). Au niveau des fibroblastes-ScS provenant de parenchyme pulmonaire, miR-155 est sur-exprimé et serait responsable de la production de collagène médiée par l'inflammasome, dépendant de NLRP3 (434). Enfin, la sur-expression dans les fibroblastes-ScS de miR-130b est inversement corrélée à celle de PPARγ qu'il cible directement. Ainsi, la diminution d'expression de PPARγ entraîne la levée d'inhibition d'une cascade d'expression de gènes pro-fibrotiques (435).

Fonction des microARNs dans les CSMs.

Parmi les différentes molécules qui assurent la fonction des CSMs, les microARNs sont largement impliqués (436). Les premiers travaux ont démontré leurs rôles dans la différenciation des cellules souches (pour revue, (437)). Ensuite, leur implication dans la fonction immunosuppressive des CSMs a été soulevée (Figure 7) (pour revue, (380)), les microARNs étant eux-mêmes dérégulés dans un environnement inflammatoire et pathologique (438). Enfin, le rôle des microARNs dans la fonction pro-angiogénique et anti-fibrotique des CSMs, notamment au cours de pathologies cardio-vasculaires, a également été exploré (pour revue, (439)).

Figure 7 – Rôle des microARNs dans l'effet immunomodulateur des CSMs



D'après Pers YM et al, Biochimie (2018)

D'ailleurs, plusieurs études ont montré que l'effet thérapeutique de CSMs *in vivo* est médié par des microARNs libérés au sein d'EVs : le tableau 3 de ce manuscrit vous présente une synthèse de ces travaux, réalisée en 2018 par Qiu G *et al* (440), mettant en évidence le rôle des microARNs dans l'effet thérapeutique des CSMs et contenus dans leurs EVs.

Parmi l'ensemble des microARNs dérégulés au cours de la ScS, nous nous sommes tout particulièrement intéressés à miR-29a pour deux raisons que nous allons développer dans le paragraphe suivant : 1- miR-29a est impliqué dans les phénomènes de fibrose au cours de la sclérodermie, 2- notre équipe était parmi les premiers à démontrer sa forte expression par les CSMs et son rôle anti-apoptotique au cours de la chondrogenèse (441). De plus, le rôle de miR-29a dans l'effet immunosuppresseur des CSMs était en cours de validation, ce qui suggérait qu'il puisse jouer un rôle prépondérant dans l'effet thérapeutique des CSMs dans la ScS.

Tableau 3 – Rôle des microARNs dans l'effet thérapeutique de CSMs

Table 3 Studies demonstrating the MSC-EV-mediated transfer of miRNAs in animal models

References	Disease model	Treatment with MSC-EVs or MSCs, dose, duration, and route	Major findings	miRNA transferred	Target proteins
Collino et al. 2015 [48]	Mouse, rhabdomyolysis-induced acute kidney injury	MSC-EVs with Drosha knockdown or control, 2.2×10^8 EV particles, on day 3 of injury, tail veil	Drosha knockdown blocked morphologic and functional recovery in acute kidney injury	Reduction in all miRNAs in EVs from Drosha knockdown	ND
Wang et al. 2016 [50]	Mouse, renal fibrosis from ureteral obstruction	MSCs with miR-let7c overexpression, 1×10^6 cells, 0 h after obstruction, intravenous	Attenuated kidney injury and fibrosis	miR-let7c	TGF-β receptor 1
Gu et al. 2016 [51]	Rat, renal ischemia-reperfusion injury	MSC-EVs, 100 μg, 0 h after reperfusion, intravenous	Protect the kidney from the injury by inhibition of mitochondrial fission	miR-30	DRP1
Feng et al. 2014 [52]	Mouse, MI	MSC-EVs from ischemic precondition, 1 μg, 0 h after ischemia, intramyocardium	Reduced infarct size	miR-22	Methyl-CpG-binding protein 2
Yu et al. 2015 [53]	Rat, MI	EVs from MSCs with GATA-4 overexpression, harvested from 4×10^6 MSCs, 0 h after ischemia, intramyocardium	Reduced infarct size, promoted cardiac function recovery	miR-19a	PTEN
Wang et al. 2015 [54]	Mouse, sepsis-induced cardiac dysfunction	MSC-EVs, 2 μg/g body weight, 1 h after cecal ligation and puncture model, intravenous	Protection against cardiac dysfunction, apoptosis, and inflammatory response	miR-223	Sema3A and Stat3
Lou et al. 2015 [56]	Nude mouse, hepatocellular carcinoma	MSC-EVs, 50 μg, intra-tumor injection	Increased the antitumor efficacy of sorafenib on hepatocellular carcinoma	miR-122	Cyclin G1 and IGF1R
Lou et al. 2017 [57]	Mouse, CCl4-induced liver fibrosis	MSCs with miR-122 overexpression, 1×10^5 cells, 1 day after the fourth injection of CCl4, tail vein	Alleviated liver fibrosis and collagen deposition	miR-122	Cyclin G1, IGF1R, and P4HA1
Chen et al. 2018 [58]	Mouse, experimental autoimmune hepatitis	EVs from MSCs with miR-223 overexpression; dose not shown; on days 21, 28, and 35 of 42-day experiment; intraperitoneally	Reduced inflammation and reversed liver injury	miR-223	NLRP3
Qu et al. 2017 [59]	Mouse, CCl4-induced liver fibrosis	EVs from MSCs with miR-181 overexpression, 40 μg, twice weekly for 8 weeks, intrasplenic injection	Inhibited liver fibrosis and activated autophagy	miR-181	Bcl-2 and Stat3
Xin et al. 2012 [60]	Rat, stroke from middle cerebral artery occlusion	MSCs, 3×10^6 cells, 24 h postischemia, tail vein	MSCs increased miR-133b level in the brain, MSC-EVs elevated neurite outgrowth in vitro	miR-133	RhoA (candidate)
Xin et al. 2013 [61]	Rat, stroke from middle cerebral artery occlusion	MSCs with miR-133 overexpression or knockdown, 3×10^6 cells, 24 h postischemia, tail vein	miR-133 enhanced axonal plasticity and neurite remodeling	miR-133	Connective tissue growth factor and RhoA
Xin et al. 2017 [62]	Rat, stroke from middle cerebral artery occlusion	EVs from MSCs with miR-17-92 cluster expression, 100 μg, 24 h postischemia, intravenous	Increased neural plasticity and functional recovery	miR-17-92 cluster	PTEN

MSCs mesenchymal stem cells, *EVs* extracellular vesicles, *MI* myocardial infarction, *IGF1R* insulin-like growth factor receptor 1, *P4HA1* prolyl 4-hydroxylase alpha 1, *ND* not determined

D'après Qiu G et al, Stem Cell Research & Therapy (2018)

b) *Particularités de miR-29a*

MiR-29a et ScS.

En 2008, miR-29a-3p, la forme mature prépondérante de miR-29a, est découvert pour la première fois dans le domaine de la fibrose, tout particulièrement cardiaque, humaine et murine. Il était sous-exprimé et son expression était inversement corrélée à l'expression des collagènes (442). Par la suite, l'implication de miR-29a dans d'autres pathologies fibrosantes a été montrée, notamment au cours de la fibrose rénale (443), pulmonaire (404) et hépatique (444). Concernant la ScS, plusieurs études ont montré sa sous-expression dans les fibroblastes-ScS comparée à des sujets sains (423), mais aussi au niveau de cheveux (445) ou encore du sérum des patients (422,446). La même observation fut notée sur des prélèvements cutanés de souris sclérodermiques (modèle bléomycine). D'ailleurs, le TGFβ1, le PDGF et l'IL4 entraînent une diminution d'expression de miR-29a sur les fibroblastes sains et sclérodermiques *in vitro*.

L'effet anti-fibrotique de miR-29a est secondaire à plusieurs mécanismes. Tout d'abord, il diminue la production de collagènes 1 et 3, en ciblant directement leurs ARNm dans des fibroblastes-ScS (423). Ensuite, miR-29a module le remodelage de la MEC grâce à la répression d'une autre cible directe : la protéine de liaison à la kinase de type 1 (MAP3K7) activée par le TGFβ (TAB1) (402). En effet, en inhibant l'expression de TAB1, miR-29a bloque la voie de signalisation non canonique du TGFβ1 et réprime ainsi l'expression de TIMP1 (447). De plus, miR-29a agit sur le remodelage tissulaire au travers de son effet inhibiteur sur d'autres MMPs, telles que MMP2, MMP9 (448,449) ou encore les ADAMs (405), mais aussi sur l'IL33 (407). En outre, miR-29a peut améliorer la fibrose par son effet pro-apoptotique sur les fibroblastes-ScS en modulant le ratio Bax/Bcl2 (450). Enfin miR-29a cible directement plusieurs gènes impliqués dans la méthylation de l'ADN, tels que la méthylase DNTM3A (403,451) ou la déméthylase TET1 (145,452) et nous avons déjà évoqué l'importance de la dérégulation de la méthylation de l'ADN des fibroblastes-ScS dans l'expression de gènes codant pour des molécules impliquées dans la fibrogenèse.

Par ailleurs, il a été montré que miR-29a cible directement le PDGFR (199) dans des cellules musculaire lisses mais aussi dans cellules hépatiques stellaires (453). Ainsi, on peut imaginer que l'apport de miR-29a bloque cette voie de signalisation importante dans la fibrogenèse (454).

Finalement, devant l'ensemble des mécanismes anti-fibrotiques attribués à miR-29a et sa sous-expression chez les patients atteints de ScS, il semble important de restaurer des niveaux de miR-29a chez les malades. D'ailleurs, l'apport de miR-29a, à l'aide d'un vecteur non viral, dans un modèle murin de fibrose pulmonaire a montré son effet thérapeutique (406).

Cependant, au début de ma thèse, aucune étude n'avait étudié le rôle de miR-29a dans le mécanisme d'action des CSMs dans la ScS.

MiR-29a et CSMs.

Concernant plus précisément le rôle de miR-29a dans la fonction des CSMs, notre équipe avait montré précédemment la forte expression de miR-29a par les CSMs humaines et son rôle important au cours de la différenciation chondrocytaire des CSMs (441). Aucune autre donnée concernant le rôle de miR-29a dans les CSMs n'était disponible au démarrage de ma thèse en 2017.

En 2018, une équipe a démontré que les CSMs isolées à partir de placenta, ainsi que leurs EVs, étaient capables de diminuer l'expression de gènes de fibrose dans des myoblastes de patients atteints de la maladie de Duchenne et que cet effet anti-fibrotique était en partie médié par miR-29c (455). Plus récemment, la sur-expression de miR-29a ou miR-29b dans des CSMs de MO, permettait de générer des EVs contenant des taux élevés de ces molécules. Ces EVs étaient efficaces pour améliorer la symptomatologie clinique des rats dans un modèle de maladie d'Alzheimer (456). Ces deux derniers mois, trois études sur le rôle de miR-29a dans les CSMs ont été publiées. L'une a démontré son implication dans leur effet anti-fibrotique sur les processus d'adhésion intra-utérine au niveau de l'endomètre (457), une seconde dans leur effet pro-angiogénique *in vitro* en ciblant VASH1 (Tubulinyl-Tyr carboxypeptidase 1) dans les CE (458) et une troisième dans leur effet pro-apoptotique sur des myocytes en réprimant la follistatin-like 1 dans les CSMs (459). Enfin, notre équipe a démontré que miR-29a joue un rôle dans l'effet immunosuppresseur de CSMs, notamment en inhibant PSAT1 (Phosphoserine Aminotransferase 1) (Bony et al, manuscrit soumis).

En résumé, les CSMs possèdent de nombreux rôles biologiques qui sont médiés par différents facteurs, notamment les microARNs. L'ensemble de ces propriétés biologiques leur confère une fonction régénératrice majeure. Ainsi, les CSMs sont, depuis plusieurs années, l'objet de nombreux travaux évaluant leur potentiel thérapeutique dans différents domaines. L'utilisation de CSMs en thérapie cellulaire parait d'autant plus séduisante qu'elles semblent dépourvues des contraintes liées au rejet de greffe de cellules dans les approches allogéniques grâce à leurs propriétés immunosuppressives et leur faible immunogénicité.

IV) Intérêt thérapeutique des CSMs

1) Généralités

Parmi les données scientifiques publiées, un traitement par CSMs semble efficace pour freiner l'évolution de maladies ostéoarticulaires telles que l'arthrose ou encore la polyarthrite rhumatoïde (pour revue, voir (460)), de pathologies neurologiques telles que la sclérose en plaque, la sclérose latérale amyotrophique ou les accidents vasculaires cérébraux, ou encore de maladies auto-immunes telles que le lupus (461). Mais leur efficacité thérapeutique dans des essais cliniques de phase 3 reste à évaluer. Concernant les maladies cardiovasculaires, les CSMs ont démontré leur potentiel thérapeutique dans différents modèles pré-cliniques de pathologies ischémiques. Les premières évaluations cliniques étaient encourageantes mais les CSMs ont montré leurs limites dans des essais de plus grande ampleur (462). De même, l'utilisation de CSMs en prévention ou en traitement de réactions de GVH dans le cadre de transplantation de CSH avait suscité beaucoup d'espoir après plusieurs succès chez des patients, alors qu'une méta-analyse réalisée par la Cochrane en 2019 n'a pas mis en évidence de bénéfice (463). Ces données montrent que l'utilisation de CSMs en clinique nécessite encore de nombreuses investigations afin d'optimiser leur efficacité thérapeutique dans des essais de grande cohorte.

En effet, l'injection de CSMs chez l'homme soumet les cellules à un environnement pathologique qui n'évolue pas strictement à l'identique des modèles pré-cliniques, ce qui peut moduler la fonction biologique des CSMs. De plus, différents paramètres peuvent largement influencer l'effet thérapeutique des CSMs et pourraient expliquer une partie de ces échecs thérapeutiques dans les essais cliniques de grande ampleur. Parmi ces paramètres, on note : la sélection de la « sous-population » de malades à traiter, la quantité de cellules injectées, la source tissulaire de CSMs utilisées, le temps d'injection en fonction de l'histoire naturelle de la maladie, ou encore l'utilisation de CSMs dans une approche allogénique ou autologue. Ce dernier point est important à souligner. Comme nous l'avons vu précédemment, la niche environnementale dans laquelle les CSMs évoluent module leurs propriétés biologiques. Ainsi, un environnement tissulaire pathologique peut altérer les CSMs et moduler leur comportement biologique. Partant de ce postulat, il est licite de se demander si les CSMs de patients auront la même efficacité clinique que des CSMs de donneurs sains.

2) Effet thérapeutique des CSMs dans la ScS

Les propriétés anti-fibrotiques, pro-angiogéniques et immunomodulatrices des CSMs peuvent cibler les différents axes de dysfonctionnement impliqués dans la physiopathologie de la ScS. C'est pourquoi plusieurs équipes ont évalué leur potentiel thérapeutique dans différents modèles pré-cliniques de ScS. Les résultats encourageants obtenus à l'issu de ces travaux ont conduit à la réalisation de plusieurs essais cliniques, actuellement en cours. Comme nous l'avons vu dans les parties précédentes, nous savons d'une part que le fibroblaste joue un rôle central dans la physiopathologie de la ScS et d'autre part que la CSM a plusieurs liens directs et indirects avec le fibroblaste. Ainsi, il semble possible que la CSM pourrait jouer un rôle, tout comme le fibroblaste, dans le développement de la ScS : sont-elles porteuses d'anomalies intrinsèques responsables de leur différenciation en cellules matures pathologiques ? sécrètent-elles différents facteurs pro-fibrotiques à l'origine de l'évolution du fibroblaste vers le myofibroblaste ? dans une niche environnementale altérée, se trans-différencient-t-elles vers un phénotype myofibroblastique contribuant au pool de cellules constitutives des tissus fibreux ? Quoiqu'il en soit, ces questions soulignent l'importance, dans une approche thérapeutique, de s'assurer que les CSMs provenant de malades conservent des propriétés biologiques identiques à celles de donneurs sains.

a) *Article 1 : revue de la littérature*

Nous avons donc passé en revue l'ensemble des données de la littérature ayant évalué d'une part, l'effet thérapeutique des CSMs dans la ScS et d'autre part, les caractéristiques et les fonctions des CSMs de patients atteints de ScS. Ce travail a fait l'objet d'une publication en 2018 dans la revue Frontiers in Immunology, intitulée : « Mesenchymal Stem Cells in Systemic Sclerosis: Allogenic or Autologous Approaches for Therapeutic Use? ».

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REVIEW
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Mesenchymal Stem Cells in Systemic Sclerosis: Allogenic or Autologous Approaches for Therapeutic Use?

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Systemic sclerosis (SSc) is a rare autoimmune disease, which is potentially lethal. The physiopathology of the disease is still incompletely elucidated although the role of fibroblasts, endothelial cells (ECs), immune cells. and the environment (i.e., oxidative stress) has been demonstrated. This is an intractable disease with an urgent need to provide better therapeutic options to patients. Mesenchymal stem cells (MSCs) represent a promising therapeutic approach thanks to the number of trophic and pleiotropic properties they exert. Among these, MSCs display anti-fibrotic, angiogenic, and immunomodulatory capacities that might be of interest in the treatment of SSc by acting on different processes that are dysregulated in the disease. In the recent years, the therapeutic effectiveness of MSCs has been demonstrated in different preclinical animal models and is being investigated in phase I clinical trials. Both allogenic and autologous transplantation of MSCs isolated from bone marrow or adipose tissue is being evaluated. The rationale for using allogenic MSCs in SSc, as well as in other autoimmune diseases, is based on the possibility that autologous MSCs might be altered in these diseases. In SSc, reports from the literature are controversial. Nevertheless, the role of the oxidative environment and of the crosstalk with neighboring cells (fibroblasts and ECs) on the functional properties of MSCs has been reported. Here, we review the preclinical and clinical data reporting the interest of MSC-based treatment in SSc and question the use of autologous or allogeneic MSCs in perspective of clinical applications.

Keywords: mesenchymal stem cell, systemic sclerosis, allogenic, cell therapy, bleomycin, HOCl

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INTRODUCTION

Systemic sclerosis (SSc) is a rare autoimmune disease, which affects most frequently middle-age patients with a prevalence ranging from 100 to 300 per million depending on the country. The pathophysiology of SSc is still not completely understood even though three main axes of dysfunction are reported: fibrosis, vascular activation and immune abnormalities. The disease is characterized by vascular damage and diffuse fibrosis, which mainly affects skin and lung tissues but heart and digestive tract could also be involved (1). SSc is typically classified as limited or diffuse according to the extent and distribution of skin fibrosis (2). One of the earliest and most frequent symptom is the Raynaud's Phenomenon but vasculopathy is also responsible of other

clinical signs including digital ulcers, pulmonary arterial hypertension, and telangiectasia (3). All of these symptoms are responsible for increased morbidity and lead to functional disability (reduced mouth opening and loss of hand function, for example), pain, and psychological consequences. This impacts not only the patient's quality of life but also reduces his life expectancy. In at least half of the cases, patients will die from SSc-related disorders and the other half from higher incidence of malignancies and cardiovascular diseases compared to the general population (4–6).

There is no curative treatment to date. Only symptomatic treatments are commonly proposed to patients to alleviate pain and improve function. Novel therapeutic strategies are being envisaged among which mesenchymal stem cells (MSCs)-based therapy, which is currently under evaluation in the clinics. The first clinical trial has evaluated the safety of MSCs-containing autologous stromal vascular fraction (SVF) (7) but the option of using allogeneic MSCs is also under evaluation since publications have reported potential alterations of MSCs from SSc patients. Here, we review the available literature on MSC-based cell therapies in SSc from pre-clinical models to clinical applications and discuss the interest of using allogeneic or autologous MSCs for future clinical trials.

GENERALITIES ON MESENCHYMAL STEM CELLS

MSCs are adult multipotent progenitor cells, which have been first identified in the bone marrow (BM) (8). In addition to bone marrow-derived MSCs (BM-MSCs), they have been described in different niches and isolated from several tissues, including adipose tissue (ASCs), umbilical cord (UC-MSCs), placenta, or dental pulp (9). Actually, MSCs have been proposed to be present in virtually all organs as pericytes, which show identical differentiation capacities *in vitro*. However, recent lineage-tracing experiments indicate that *in vivo*, pericytes do not contribute to other cell lineages (10). These results therefore challenge the concept that MSCs, or at least part of them, are pericytes behaving as multipotent tissue progenitors.

MSCs are defined by 3 criteria, as proposed by the International Society for Cell Therapy: (1) plastic adherence, (2) expression of the cell surface markers CD73, CD90, CD105, and lack of expression of the hematopoietic markers CD11b or CD14, CD19 or CD79α, CD34, CD45, HLA-DR, and (3) capacity of differentiation into adipocytes, chondrocytes, and osteoblasts (11). Along with their potential of multilineage differentiation, MSCs exert a number of paracrine functions: they support survival and differentiation of hematopoietic stem cells, induce cell proliferation and have anti-fibrotic, anti-apoptotic, pro-angiogenic, anti-bacterial, and anti-inflammatory effects (12). These effects are mediated primarily through the secretion of soluble mediators but can be enhanced upon contact of MSCs with the target cells (13). The soluble factors are released in the extracellular milieu or within extracellular vesicles that protect them from degradation and allow their transfer throughout the organism [for review, see (12, 14)]. Although, MSCs from different tissue sources share similar properties, they may display some differences in their differentiation potential or immunomodulatory capacity (15, 16).

Thanks to this pleiotropic activity, the therapeutic efficacy of MSCs has been investigated in different pathological conditions, from inflammatory diseases to acute or degenerative diseases. So many different applications as rheumatic diseases (17), stroke (18), lupus and scleroderma (19), heart diseases (20), or bone defects (21) have been evaluated. To date, thousands of patients have been enrolled in hundreds of clinical trials and safety of MSC injection has been proved. In 2016, a meta-analysis has reported no acute toxicity, death, infection, organ systemic failure or risk malignancy after MSC implantation (22). Only transient fever was frequently observed. Efficacy of MSC-based treatments as compared to standards of care is still however under evaluation but promising results have been achieved mostly in phase II trials. For SSc, very few trials have been initiated and results from a couple of registered phase I/II clinical trials are still pending.

THERAPEUTIC EFFECT OF MSCs IN PRECLINICAL MODELS OF SSc

Before the initiation of clinical trials, the interest of using MSCs in the treatment of SSc has been evaluated in different preclinical models. Three models have been used: the model of bleomycin-induced pulmonary fibrosis, the model of hypochlorous acid (HOCl)-induced SSc and the tight skin (Tsk1/+) mouse model of SSc.

The Model of Bleomycin-Induced Fibrosis

The model of bleomycin-induced fibrosis is the most widely used model to replicate scleroderma, or dermal or pulmonary fibrosis in mice or rats. According to the mode of administration, fibrosis can be induced in skin when bleomycin is injected daily sub-dermally or in lung after a single intra-tracheal instillation. Fibrosis in lung, skin, and internal organs may be induced via the use of osmotic pumps that deliver a constant amount of bleomycin over a number of days (23, 24). It is believed that bleomycin causes breaks in DNA, resulting in overproduction of reactive oxygen species (ROS) and inflammatory response that activate fibroblasts and subsequently fibrosis formation. Importantly, the bleomycin models replicate some of the earliest patterns of SSc but do not present with the typical clinical signs and autoantibody patterns of SSc.

The therapeutic potential of MSCs has been first demonstrated in the mouse model of lung fibrosis. One intravenous injection of 500,000 allogenic BM-MSCs on the day of bleomycin instillation was shown to reduce collagen content and inflammation in the lungs (25). At day 14 after injection, the authors could detect engraftment of BM-MSCs in areas of bleomycin-induced injury where they adopted an epithelium-like phenotype. Another study confirmed that systemic injection of allogenic BM-MSCs after intra-tracheal instillation of bleomycin protected mice from injury and fibrosis through the suppression of inflammation (26). They also reported that BM-MSCs differentiated into distinct lung cell phenotypes and secreted chemokines that

might have attracted endogenous cells. Due to the relatively low numbers of transplanted MSCs and persistence of some injury in BM-suppressed animals, the authors suggest that both endogenous and exogenous MSCs likely contribute to the repair process. Another study comparing minimally cultured (2 h) to conventionally cultured (9 days) BM-MSCs indicated that both types of cell preparations ameliorated as efficiently inflammatory and progressive fibrotic lung injury (27). Minimally cultured BM-MSCs had a higher proliferative capacity, expressed higher levels of stem cell markers, and chemokine receptors but lower levels of type I procollagen, α-smooth muscle actin (α-sma) and transforming growth factor-β (TGFβ) that might be more advantageous for cell-based therapy.

The therapeutic effect of BM-MSCs has been also confirmed in rat models of bleomycin-induced lung fibrosis. One intravenous injection of five millions of BM-MSCs resulted in decreased levels of TGFβ1, platelet-derived growth factor-A (PDGF-A), PDGF-B, insulin growth factor-1 (IGF1), and in lower collagen content in lungs (28). Another report has shown that systemic injection of syngeneic BM-MSCs ameliorated fibrosis and decreased inflammatory and angiogenic markers as well as nitric oxide metabolites (29).

In a dermal fibrosis model induced by daily subcutaneous injection of bleomycin for 4 weeks, syngeneic BM-MSCs were injected subcutaneously into the lesion skin every day (30). Treatment with BM-MSCs resulted in a basket-weave organization of collagen arrangement similar to normal skin, with few inflammatory cells and α-sma-positive myofibroblasts as well as down-regulation of TGFβ, type I collagen, and heat-shock protein 47 (HSP47) expression in skin.

The impact of MSCs from different species or tissues on treatment efficacy has been evaluated in the mouse model of bleomycin-induced lung fibrosis. Human UC-MSCs injected systemically 24 h after bleomycin instillation in SCID mice were reported to reduce inflammation markers, collagen content, and inhibit expression of TGFβ, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) (31). In this study, improvement of tissue remodeling was shown with increased levels of matrix metalloproteinase-2 (MMP-2) and decreased levels of their endogenous inhibitors, tissue inhibitor of MMP (TIMP). Of interest, another report demonstrated that both human amnion- and chorion-derived fetal MSCs displayed similar reduction in the severity of bleomycin-induced lung fibrosis as allogeneic murine amnion- and chorion-derived fetal MSCs (32). Amniotic fluid MSCs were shown to inhibit collagen deposition and to preserve pulmonary function, which could be related to transient increase of MMP-2 (33). Cells were observed to localize within fibrotic lesions with a preferential targeting to the area of fibrosis. Similarly, compared to BM-MSCs, human amnion-derived MSCs reduced collagen deposition and increased MMP-9 activity (34). Finally, ASCs attenuated lung fibrosis induced by repetitive intra-tracheal administrations of bleomycin, namely hyperplasia of Club cells (Clara cells) and cuboidal alveolar epithelial cells, infiltration of the perialveolar ducts by inflammatory cells, septal thickening, enlarged alveoli, and extensive fibrosis (35). It also led to suppression of epithelial cell apoptosis and expression of TGFβ suggesting that irrespective of the tissue or species origin, MSCs are potent inhibitors of lung fibrosis.

The time of cell injection after disease induction may be one important factor to control for better efficacy of cell therapy. Most frequently, MSCs have been implanted at the time or within the few hours after bleomycin instillation. Interestingly, early treatment (day 0) with murine or rat BM-MSCs on the day of bleomycin instillation resulted in a significant reduction of fibrotic changes that was not seen when BM-MSCs-based treatment was delayed (day 7) (25, 36). Similarly, two injections of low (2.8×10^6cells/kg) or high (5.6×10^6cells/kg) doses of human BM-MSCs at day 7 and 15 after fibrosis induction did not lead to improvement of lung function or rescue of damage tissue (37). Injection of autologous ASCs via the trachea at day 15 of the disease did not improve the severity of lung injury but prevented further aggravation of lung damage (38). In this report, the majority of ASCs did not penetrate inside the lung region at week 3 but some cells had sprouted deep into the distorted architecture of the lung at week 6 after disease induction. By contrast, injections of murine amniotic fluid-derived MSCs at either day 0 or day 14 were both efficient to inhibit fibrosis indicating improvement of the disease in both acute and chronic remodeling events (33). Injections of amniotic MSCs or BM-MSCs at day 3 or 6 were even more efficient to reduce lung inflammation than the treatment at day 1 (39). In addition, in a model of repeated injections of bleomycin at day 0 and 7, BM-MSCs injected at day 10 were beneficial in terms of collagen deposition reduction and MMP-9 down-regulation (34).

The age of the donors may be another parameter that might change the therapeutic potential of MSCs. One study compared ASCs from aged (>22 months) and young (4 months) mice (40). At day 21 after bleomycin instillation, mice receiving young ASCs exhibited decreased fibrosis, MMP-2 activity, oxidative stress, and markers of apoptosis vs. untreated controls. Improved treatment with young-donor ASCs was associated with decreased mRNA expression of MMP-2, IGF receptor, and protein kinase B (AKT) activation.

For more a decade since 2003, the skin or lung bleomycin-induced model of local fibrosis was the single model used to evaluate the therapeutic effect of MSCs. However, it does not reproduce the main characteristics of the diffuse human SSc. With respect to the systemic nature of the disease, relevant models of systemic fibrosis have to be used to better evaluate the efficacy of MSCs in models closer to human SSc.

The Model of Bleomycin-Induced Lung and Skin Fibrosis in Aged Mice

A study relied on the use of an established aged (18–22-month-old) mouse model of bleomycin-induced lung fibrosis to test the hypothesis that fibrosis may develop simultaneously in multiple organs and notably affects lung, skin and wound healing. Mice developed irreversible lung and skin fibrosis as well as delayed wound closure at day 21 (41). In this model, intravenous single injection of allogeneic ASCs attenuated lung fibrosis as evaluated by semi-quantitative Ashcroft score on Masson's-trichrome stained histological sections. ASCs also accelerated

wound healing as shown by increased total wound size and wound gaps. This effect was associated with higher levels of caveolin-1 and lower level of $\alpha_v$integrin, TNFα, and miR-199-3p in lung and skin wounds. These results therefore support the hypothesis that ASCs may prevent systemic fibrosis and enhance wound healing but rely on the use of aged mice that are expensive and difficult to get.

The Murine Model of HOCl-Induced SSc

In order to evaluate the therapeutic effect of MSCs in a systemic model of scleroderma, the murine model of HOCl-induced SSc is relevant, cheap and reproducible. After daily intradermal injections of HOCl for 6 weeks, mice develop a systemic disease with the main features of the human form, including skin and lung fibrosis, vascular abnormalities and the production of Anti-Scl-70 autoantibodies, which are anti-nuclear topoisomerase antibodies mainly detected in diffuse systemic scleroderma (42). Our group demonstrated the therapeutic effect of a single intravenous injection of syngeneic BM-MSCs in preventive (day 0) but also curative (day 21) approaches (43). Interestingly, the dose escalation study revealed that the best improvement of both skin and lung fibrosis was obtained with the lowest dose of 2.5×10^5 BM-MSCs. We also compared different sources of cells, i.e., allogenic, xenogeneic, and syngeneic BM-MSCs as well as xenogeneic ASCs, which seemed to be the most potent as compared to BM-MSCs (44). The therapeutic effect was observed on all features of SSc, including reduction of fibrotic, inflammatory. and oxidative markers, but also reduction of autoantibodies Scl-70 while matrix remodeling markers were increased. Finally, infused GFP-expressing allogeneic BM-MSCs were entrapped in the lungs where they were detected at days 1 and 2 but not at day 7. Interestingly, either murine allogenic or human xenogeneic BM-MSCs did not migrate to the skin although skin fibrosis was reduced, indicating a systemic effect of BM-MSCs (44, 45).

The Model of Tight Skin (Tsk1/+) Mouse

The tight skin 1 (Tsk1/+) mouse occurred as a spontaneous mutation in fibrillin-1, which controls the biologically active levels of TGFβ through binding to the latent TGFβ binding proteins (LTBP) 1 and 4 (46). It has therefore been suggested that activation of the TGFβ signaling axis is involved in the development of the phenotype. This genetic model of SSc is characterized by hyperplasia of the sub-cutaneous loose connective tissue with abnormal dermis, osteopenia and deregulation of the interleukin-4 (IL4)/IL4 receptor (IL4R) signaling pathway. Interestingly in this model, MSCs have been shown to ameliorate osteopenia by rescuing impaired lineage differentiation of the recipient BM-MSCs (47). In this interesting study, the authors further demonstrated that MSC-derived extracellular vesicles containing miR-151-5p rescued the disease phenotypes via regulation of the IL4R pathway in recipient BM-MSCs.

Altogether, the beneficial effect of MSCs-based treatment has been proved in the different models of local or systemic scleroderma (**Table 1**). Some clues of their mechanism of action have also been investigated as discussed below.

MECHANISMS OF ACTION OF MSC-BASED THERAPY IN SSc

The capacity of MSCs to differentiate into several cell types of musculoskeletal tissues was the first property that has attracted the attention of researchers and clinicians (58). Since then, several mechanisms of action have been proposed to play important roles in various disorders and diseases via the secretion of many soluble mediators as summarized in (12). Besides anti-apoptotic and anti-bacterial functions, the support of hematopoietic stem cells, the chemoattracting effect, the proliferative, and protective role of MSCs has been largely exemplified. In SSc, the main functions of MSCs are proposed to be anti-inflammatory to counteract the dysregulation of the immune system, anti-fibrotic to down-regulate the excessive production of collagen associated to thickening of skin and internal organs but also pro-angiogenic to counteract the widespread vasculopathy. A number of reviews have discussed the interest of using MSCs for the treatment of SSc (19, 59, 60). In the next paragraph, we focused our attention on the mechanisms that have been deciphered specifically in preclinical models of SSc.

Systemic administration of MSCs results in their homing in lungs and cell engraftment is increased in lungs from bleomycin-induced mice, in particular in areas of injury where they adopt an epithelial cell morphology and display anti-oxidative role (25, 48). Of interest, in the HOCl-induced systemic model of SSc, both human or green fluorescent protein (GFP)-positive murine BM-MSCs were shown to be retained in the lungs of mice after intravenous administration while no cells were detected in the skin suggesting a systemic effect of infused BM-MSCs (43, 45). In addition, tracheal instillation of BM-MSC supernatants decreased the number of apoptotic cells, collagen content, inflammation and fibrotic scores in lungs in the model of bleomycin-induced lung fibrosis (49). These results suggest that BM-MSCs preferentially act through the secretion of soluble mediators that are distributed throughout the body but they can also act directly in contact with damaged lungs where they preferentially reside.

Some secreted mediators involved in their therapeutic effect including IL1-RA, hepatocyte growth factor (HGF) have been identified in murine models of lung fibrosis (50, 51). However, novel strategies of MSC-based treatments have been tested in order to improve their efficacy by enhancing survival after *in vivo* administration or increasing their trophic action. Indeed, preconditioning of BM-MSCs before administration was shown to enhance their therapeutic efficacy. Namely, oncostatin M- or hypoxia-preconditioned BM-MSCs improved pulmonary respiratory function thanks to decreased inflammatory and fibrotic markers as compared to unconditioned BM-MSCs and this effect was mediated at least through the upregulation of HGF (52, 53). Pre-treatment of BM-MSCs with N-acetylcysteine was able to increase both treatment efficacy in a murine model of bleomycin-induced lung injury and BM-MSC survival through the production of anti-oxidant factors (54). In order to improve their therapeutic function, strategies to over-express factors have also been tested. Overexpression

TABLE 1 | Summary of studies on the role of mesenchymal stem cells in preclinical models of systemic sclerosis.

Model/species	Number and origin of MSCs	Route of injection	Injection time	Main results	Mechanisms	References
Bleomycin intratracheal/mouse	5×10^5 allogenic BM-MSCs	IV	d0 or d7	Reduction of collagen content and inflammation in lungs after treatment at d0	ND	(25)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	H6	Reduction of inflammation and fibrosis in lungs	Differentiation into distinct lung cell phenotypes	(26)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	d3	Reduction of collagen content and inflammation in lungs	ND	(27)
Bleomycin intratracheal/rat	5×10^6 syngeneic BM-MSCs	IV	H12	Reduction of TGFβ1, PDGF-A, PDGF-B, IGF1 and collagen content in lungs	MSC differentiation into alveolar epithelial cells	(28)
Bleomycin intratracheal/rat	1×10^6 syngeneic BM-MSCs	IV	d4	Diminution of inflammation, collagen content, angiogenic markers and nitric oxide metabolites in lungs	ND	(29)
Bleomycin subcutaneous/mouse	1×10^6 syngeneic BM-MSCs	SC	Daily injection during 4 weeks	Improvement remodeling matrix responsible for normal collagen arrangement in skin. Reduction of inflammation and α-sma-positive myofibroblasts	Down-regulation of TGFβ, type I collagen and HSP47 expression	(30)
Bleomycin intranasal/mice	1×10^6 human UC-MSCs	IV	d1	Reduction of inflammation, collagen content and TGFβ expression and improvement remodeling of matrix	ND	(31)
Bleomycin intratracheal/mouse	4×10^6 IP or 1×10^6 IV ou IT xenogeneic & allogeneic amnion- and chorion-derived fetal MSCs	IP or IV or IT	d0	Reduction of lung fibrosis	ND	(32)
Bleomycin intratracheal/mouse	1×10^6 murine amniotic fluid MSCs	IV	d0 or d14	Inhibition of collagen deposition and preservation of pulmonary function	ND	(33)
Bleomycin intranasal/mouse	Human BM-MSCs or amnion-derived MSCs	IV	d0 and d7	Reduction of inflammation and collagen content in lungs	ND	(34)
Bleomycin intratracheal/mouse (every 2 weeks, 8 doses in total)	5×10^5 human ASCs	IP	4 doses at time of bleomycin injection	Reduction of lung fibrosis and inflammation	ND	(35)
Bleomycin intravenous/rat	5×10^5 syngeneic BM-MSCs	IV	d1 or d7	Reduction of lung fibrosis only after treatment at d1	ND	(36)
Bleomycin intratracheal/rat	2.8×10^6 or 5.6×10^6 human BM-MSCs /kg	IV	d8 or d15	Safety of MSC injection No amelioration of disease	ND	(37)
Bleomycin intratracheal/rat	Autologous ASCs	IT	d15	No improvement but prevention of lung damage aggravation	ND	(38)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	d1 or d3 or d6	Reduction of lung inflammation & fibrosis after d3 or d6 treatment	ND	(39)
Bleomycin intratracheal/mouse	5×10^5 syngeneic ASCs from old or young mice	IV	d1	Only young ASCs induced lower lung fibrosis, oxidative stress and apoptosis	Lower levels of MMP-2, IGFR and AKT activation	(40)
Bleomycin intratracheal/mouse	5×10^5 syngeneic ASCs	IV	d1	Reduction of lung & skin fibrosis Acceleration of wound healing	Decreased miR-199-3p and increased caveolin-1 in lungs and skin	(41)

(Continued)

TABLE 1 | Continued

Model/species	Number and origin of MSCs	Route of injection	Injection time	Main results	Mechanisms	References
HOCl intradermic injection/mouse (daily, 42 days)	2.5×10^5 syngeneic or allogeneic BM-MSCs, or human ASCs & BM-MSCs	IV	d0 or d21	Reduction of fibrotic, inflammatory and oxidative markers in skin & lungs. Improvement of matrix remodeling.	ND	(43) (44) (45)
Tsk1/+ mouse	1×10^5 allogenic BM-MSC /kg bodyweight	IV	8 weeks	Improvement of osteopenia	Downregulation of the IL4R pathway by miR-151-5p in MSC-EV	(47)
Bleomycin intratracheal/rat	2.5×10^6syngeneic BM-MSCs	IV	d0 or d7	Reduction of alveolitis, pulmonary fibrosis and oxidative stress	Conversion of BM-MSC into type II alveolar epithelial cells	(48)
Bleomycin intratracheal/rat	0.2mL allogenic BM-MSC supernatants	IT	H6 and d3	Reduction of collagen content, inflammation and fibrosis in lungs	ND	(49)
Bleomycin intratracheal/mouse	5×10^5 allogenic BM-MSCs	IV	d0	Reduction of IL1α lung level	IL1RN expressing MSCs antagonizing IL1α	(50)
Bleomycin intranasal/mice	5×10^4 allogenic or HGF KO BM-MSCs/g bodyweight	IV	H6 or d9	Reduction of lung fibrosis and inflammation, and increase of HGF	HGF release	(51)
Bleomycin intratracheal/mouse	2.5×10^5 syngeneic OSM-preconditioned BM-MSCs	IT	d3	Diminution of inflammation and fibrosis in lungs and improvement of respiratory function	Production of high level of HGF	(52)
Bleomycin intratracheal/mouse	2.5×10^5 syngeneic hypoxia-preconditioned BM-MSCs	IT	d3	Improvement of lung function and matrix remodeling. Decreased pro-inflammatory and fibrotic factors in lungs	Anti-apoptotic	(53)
Bleomycin intratracheal/mouse	2×10^5 NAC-pretreated human embryonic MSCs	IV	d1	Decrease of inflammation and lung fibrosis.	Increased antioxydative capacity of MSCs	(54)
Bleomycin intratracheal/mouse	5×10^5 human BM-MSCs overexpressing let7d	IV	d7	Reduction of collagen content and inflammation in lungs	Let7d over-expression	(55)
Bleomycin intratracheal/mouse	1×10^6 xenogenic UC-MSCs over-expressing ACE2/kg bodyweight	IV	d3	Decrease of collagen content, fibrotic and pro-inflammatory factors and increase of anti-oxidative mediators	ACE2 over-expression	(56)
Bleomycin subcutaneous/mice (daily, 21 days)	1×10^6 Trx-1-overexpressing BM-MSCs	SC	Daily	Reduction of skin fibrosis and apoptosis, promotion of BM-MSC survival and differentiation into endothelial cells	TRX1-mediated inhibition of oxidative stress	(57)

ACE2, angiotensin converting enzyme 2; AKT, protein kinase B; ASCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow mesenchymal stem cells; HGF, hepatocyte growth factor; HOCl, hypochlorite; IGF, insulin growth factor; IGFR, IGF receptor; IL, interleukin; IL1RN, interleukin 1 receptor antagonist; IP, intraperitoneal; IT, intratracheal; IV, intraveinous; KO, knock-down; MMP, metalloproteinase; MSC-EV, mesenchymal stem cell-derived extracellular vesicles; NAC, N-acetylcystein SC, subcutaneous; OSM, onconstatin M; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor; Trx-1, thioredoxin 1; Tsk1, tight skin; UC-MSCs, umbilical cord-derived mesenchymal stem cells.

of let7d, which is a microRNA known to have anti-fibrotic effects, in BM-MSCs resulted in slight decrease of collagen content in lungs and improved mouse survival when injected at day 7 in bleomycin-induced mice while naïve BM-MSCs did not have any beneficial effect (55). Similarly, human UC-MSCs over-expressing angiotensin-converting enzyme 2 (ACE2) were more efficient than naïve UC-MSCs to decrease collagen content, fibrotic, and pro-inflammatory factors while increasing anti-oxidative and anti-inflammatory mediators (56). Thioredoxin 1 (Trx-1)-overexpressing BM-MSCs were also shown to inhibit apoptosis and fibrosis under hypoxic conditions and to promote the formation of tubular-like structures by endothelial cells in bleomycin-induced lung injury (57). Indeed, strategies for enhancing MSCs survival and/or efficiency have been demonstrated to be of interest in preclinical models of SSc. A summary of the potential (or proven) mechanisms of action of MSCs in the treatment of SSc is provided in **Figure 1**.

CHARACTERIZATION OF MSCS FROM SSc PATIENTS

Early studies evaluating the phenotypic and functional characteristics of MSCs from SSc patients reported no major difference as compared with MSCs from healthy donors. Indeed, BM-MSCs from two patients with SSc have been compared to those of healthy donors 10 years ago (61). No major differences in their proliferative rate, adipogenic or osteogenic differentiation potential, expression of surface markers and immunosuppressive properties were reported between the two sources of BM-MSCs. Some years later, similar data were obtained using BM-MSCs from 12 scleroderma patients in comparison with 13 healthy controls (62). SSc and healthy BM-MSCs displayed the same phenotype, identical capacities to proliferate and form CFU-F, similar capacities to differentiate into adipogenic and osteogenic lineages, equal capacities to support long-term hematopoiesis, and similar anti-proliferative properties. SSc ASCs were also shown to have comparable phenotypic markers and functional characteristics (proliferative and differentiation potential) as healthy donor ASCs (63). However, this cohort of six SSc patients was characterized by cutaneous local forms of scleroderma (generalized morphea, linear and/or plaque scleroderma) and not diffuse systemic sclerosis. More recently, no difference in proliferative capacities and potential to support endothelial cell tube formation was noticed between ASCs from ten patients with diffuse SSc or eight healthy donors (64).

By contrast, other studies have reported some differences. As an example, MSCs isolated from the skin of scleroderma patients expressed more PDGF-R than those from healthy donors, resulting in the production of higher levels of ROS (65). Nevertheless, SSc-MSCs were still able to improve the antioxidant defenses by counterbalancing ROS accumulation. More recently, the impact of the pro-oxidant environment exerted by SSc sera on BM-MSCs has been investigated. Exposure of BM-MSCs to SSc sera enhanced their antioxidant capacities as well as their osteogenic and adipogenic potentials while their immunosuppressive functions were reduced (66). With the objective to evaluate the consequence of TGFβ activation in SSc, analysis of several members of the TGFβ family has been undertaken in BM-MSCs from nine patients with diffuse SSc (67). A highly significant increase in mRNA and protein levels of TGFβ receptor II (TβRII) was detected in SSc BM-MSCs as compared to healthy controls. Moreover, in response to TGFβ activation, the production of type I collagen and Smad-3 phosphorylation was up-regulated in SSc BM-MSCs and also in SSc fibroblasts. An earlier study reported that nerve growth factor receptor (NGFR)-positive BM-MSCs, a subpopulation of MSCs with high proliferative and clonogenic potentials, were less numerous in BM from SSc patients than from healthy controls (68). They displayed lower clonogenic potential (10-fold less), had a reduced fold expansion rate (105-fold less), showed signs of rapid aging and stress and, never differentiated into adipocytes or osteoblasts. In line with these data, Cipriani and coauthors have described that BM-MSCs from SSc subjects expressed up-regulated α-sma and transgelin (TAGLN or SM22α) genes and displayed reduced proliferative activity and migration potential (69). An increase in senescence markers (senescence-associated β-galactosidase (SA-βGal), p21, IL6, TGFβ) was also observed while their immunosuppressive activity on lymphocyte proliferation and regulatory phenotype induction was retained (70). Interestingly, the same team showed an impaired crosstalk between endothelial cells (ECs) and BM-MSCs (71). In coculture conditions, SSc ECs induced increased levels of VEGF-A in SSc MSCs and of TGFβ, PDGF-R, α-sma, type I collagen in both SSc and healthy MSCs. Despite the production of high levels of VEFG-A by SSc MSCs, they had lower angiogenic performance. The authors also detected increased expression of TGFβ and PDGF-BB in SSc ECs when co-cultured either with MSCs from healthy or SSc individuals. This impaired crosstalk between ECs and MSCs suggested that ECs may be involved in the early steps leading to fibrosis by producing factors that induce a phenotype switch of MSCs toward myofibroblast cells. In another study, SSc BM-MSCs exhibited altered differentiation into contractile and synthetic vascular smooth muscle cells when stimulated by connective tissue growth factor (CTGF) or b-FGF. Moreover, stimulation with TGFβ1 induced a myofibroblast-like phenotype with high production of α-sma and collagens, higher migration capacity but less proliferative capacity (72). Accordingly, SSc BM-MSCs expressed up-regulated levels of proangiogenic factors including VEGF, stromal derived factor-1 (SDF-1) and C-X-C chemokine receptor (CXCR4) (73). However, they noticed that SSc BM-MSCs promoted angiogenesis and improved capillary morphogenesis as compared to healthy MSCs.

A unique study has identified altered functions in SSc ASCs (74). No alteration in their phenotype or differentiation potential was noticed but their proliferative rate, metabolic activity and migration capacity were reduced as compared to healthy matched control ASCs. However, no data on the expression of TβRII or other fibrotic markers as well as on their immunosuppressive and angiogenic functions were available. Further functional analysis is therefore required to fully decipher the therapeutic potential of ASCs isolated from SSc patients.

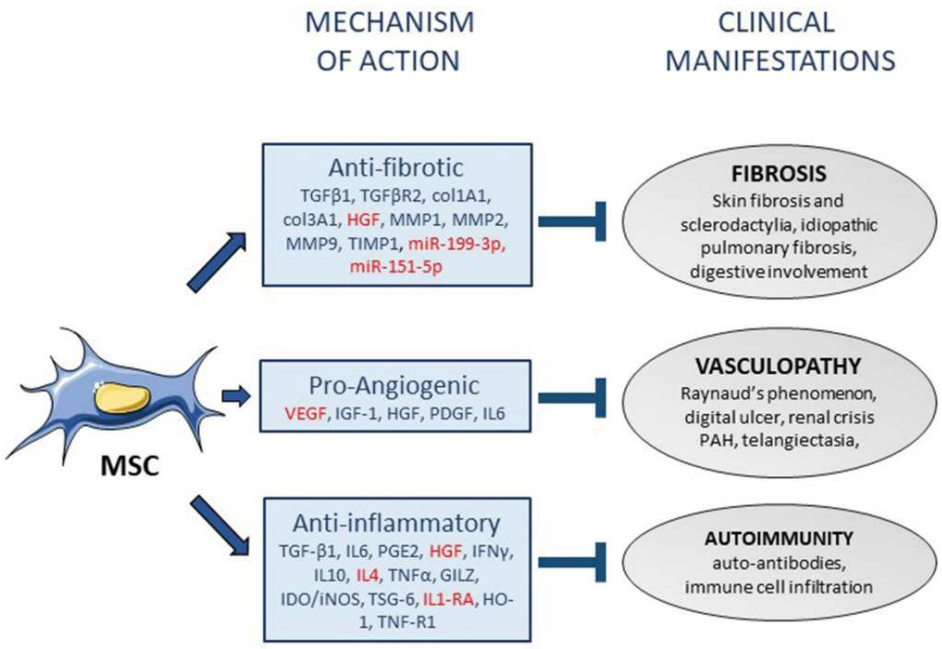

FIGURE 1 | Mechanisms of action of mesenchymal stem cells in systemic sclerosis. Mesenchymal stem cells (MSC) display various functions that might participate to their mechanisms of action in the treatment of systemic sclerosis (SSc), through the release of soluble mediators (arrows). The mediators that have been associated with anti-fibrotic, pro-angiogenic or anti-inflammatory functions of MSCs are listed in the squares (in red: mediators specifically identified in SSc). Three mechanisms of action of MSCs can be associated with the improvement of the three main clinical manifestations of SSc (blind-ended arrows). PAH, pulmonary arterial hypertension.

CLINICAL DATA ON MSC-BASED TREATMENT IN SSc

Case Reports Using Fat Injection

In recent decades, fat tissue grafting has been used to treat skin atrophy or fibrosis thanks to its biocompatibility and property of filling but also to its high content in multipotent stem or progenitor cells with regenerative potential. The first report on the implantation of fat in hands of patients who presented Raynaud's phenomenon was published in 2014 (75). Among the thirteen treated patients, nine patients suffered from scleroderma. There were no complications and the treatment showed some evidence of perfusion. In 2015, another study reported a significant improvement of digital ulcerations, hand grasping, and pain after injection of adipose tissue-derived cells into fingers of 15 SSc patients suffering from digital ulcers (76). More recently, the effect of one local injection of autologous SVF associated with platelet-rich plasma into malar and perioral areas in six patients showed improvement of skin elasticity and vascularization (77). Some indication of efficacy was suggested by these case reports but the role of ASCs contained within the SVF could not be drawn.

Case Reports Using MSCs

The first patient who received BM-MSCs in the treatment of progressive diffuse SSc has been reported in 2008 (78). A young female patient had severe disease, refractory to all immunosuppressive drugs. At time of implantation, she presented with six painful ulcerations and received 6×10^7 intravenous administration of allogeneic haploidentical-related donor BM-MSCs. No adverse events were reported and 3 months after treatment, a significant decrease in the patient's painful ulcerations was measured. Vascular improvement in the blood circulation of hands and fingers was noticed but level of Scl-70 autoantibodies was not decreased. In 2011, the same team reported four supplementary cases of allogeneic BM-MSC systemic injection (0.22 to 1.8×10^6 BM-MSCs/kg bodyweight) (79). Here again, improvement of vasculopathy and skin fibrosis was observed but the study underscored the need to set phase II clinical trials for efficacy assessment.

Local injection of autologous ASCs in combination with hyaluronic acid (HA) solution has been first tested in 2013 (63). Infiltration of 8×10^5 ASCs/mL HA was done in a single area for each patient, either the face or the arm chosen with the patient consent. The procedure improved considerably skin fibrosis for four out of six patients and moderately for one patient. All patients showed arrest of local disease progression (100%), four of them presented regression of dyschromia (67%), five patients increased skin softening (83%), four patients showed better sensitivity (67%), and one patient reported erythema reduction (17%). The study demonstrated that ASCs could be successfully implanted locally in patients with severe disease, thus representing a good, feasible, and efficient cell-based soft-tissue augmentation strategy. Of interest, the same team thereafter compared autologous fat and ASC transplantation to evaluate clinical improvement of mouth opening in two groups of five

TABLE 2 | Summary of clinical trials using mesenchymal stem cells for the treatment of systemic sclerosis.

Type of treatment	Route	Patient number	Clinical target	Clinical outcome	Adverse events	References
Autologous fat grafting (mean of 30 ml/hand)	Subcutaneous in hand	9	Raynaud's phenomenon	Improvement of perfusion and decrease of ulcer numbers	None	(75)
Autologous fat grafting (0.5–1 ml/finger)	Subcutaneous in hand	15	Digital ulcers	Improvement of digital ulcerations, hand grasping and pain	None	(76)
Combined platelet-rich plasma and lipofilling treatment	Subcutaneous in peri-oral location	6	Face skin fibrosis	Improvement of skin elasticity, labial rhyme opening and vascularization	None	(77)
Allogeneic BM-MSCs (10^6/kg bodyweight)	Intravenous	1	Systemic sclerosis	Reduction of ulceration and pain, improvement of hand vasculopathy	None	(78)
Allogeneic BM-MSCs ($0.22–1.8 \times 10^6$/kg)	Intravenous	5	Systemic sclerosis	Improvement of skin fibrosis and vasculopathy	Minor respiratory tract infection	(79)
Autologous ASCs (4 to 8×10^6)	Subcutaneous peri-oral location	6	Localized skin scleroderma	Disease stabilization for all patients and improvement of skin elasticity in 4/6 patients	None	(63)
Autologous fat (16 ml) or ASCs (3.2×10^6)	Subcutaneous peri-oral location	5	Face skin fibrosis	Improvement of skin fibrosis & mouth opening	None	(80)
Autologous SVF (5 ml/hand)	Subcutaneous in hand	12 (phase I trial)	Severe hand functional handicap	Improvement of pain, grasping capacity, finger edema, Raynaud's phenomenon, quality of life	None	(7) (81) (82)

patients (80). Both procedures obtained significant results but neither one emerged as a first-choice technique.

Clinical Trials With MSCs

The results of the first open phase I clinical trial evaluating the interest of MSCs, and more precisely of MSC-containing SVF, on 12 SSc patients with severe functional hand handicap were published in 2015 (7). Injection of autologous SVF into fingers was safe and a significant improvement in pain, grasping capacity, finger edema, Raynaud's phenomenon, and quality of life was recorded at 6 months. At 12 months follow-up, a significant improvement in skin sclerosis, motion, edema and strength of the hand was observed. SVF was therefore claimed as a promising therapy whose effect persisted at least 1 year after injection (81). Finally, at 22 and 30 months after treatment, safety, tolerability and efficacy were very encouraging (82). The same team is now including 40 patients in a randomized double blind phase II trial to evaluate efficacy of the approach.

There are only 4 clinical trials recorded in the United State National library of Medicine (www.clinicaltrials.gov). One trial has been completed but no result is available. Only one of those is recruiting in France since 2014. This is a phase I/II trial which assesses the effect of one intravenous injection of intrafamilial allogenic MSCs on 20 patients. The trial is still enrolling patients and data are not yet available. Another phase I/II clinical trial has started in November 2017. It evaluates the therapeutic effect of allogeneic BM-MSCs after injection in intramuscular areas of affected limbs in 20 scleroderma patients with digital ulcers and is recruiting (NCT03211793) (83).

In summary, data from case reports and clinical trials are scare and need to be taken with cautious in terms of efficacy (**Table 2**). One question still pending is whether autologous or allogeneic ASCs or BM-MSCs have to be used in the clinics since some studies have reported phenotypic or functional alterations of MSCs from SSc patients.

CONCLUSION

Many studies have now established the beneficial effect of the administration of BM-MSCs, ASCs or MSCs from other tissue sources in different preclinical models characterized by local or systemic fibrosis. Some evidence of safety and efficacy of MSC-containing SVF or culture expanded MSCs has been described from the clinics but efficacy needs to be further proved in phase II clinical trials that are ongoing. Both autologous and allogeneic MSCs from BM or adipose tissue are being assessed but the risk that the functional properties of MSCs isolated from SSc patients are altered is under debate. Contradictory results are reported in the literature but a number of reports discuss the reduction of the number of clonogenic cells, proliferative rate, differentiation, and angiogenic potentials. MSCs from SSc patients display a more mature and myofibroblast-like phenotype, probably related to microenvironmental signals dysregulated during the disease. They express higher levels of TβRII and TGFβ, which is released into the extracellular medium where it can act in an autocrine or paracrine manner. Moreover, the crosstalk between MSCs and ECs contribute to the altered expression of different molecules involved in angiogenesis, inducing a switch of perivascular MSCs toward a myofibroblast population, further supporting the fibrotic process. The finding that MSCs from SSc patients constitutively overexpress mediators involved in the fibrotic and

angiogenic processes might indicate that MSCs are altered by the environment secondary to the onset of the disease or, that they might participate to the physiopathology of the disease. With respect to the use of autologous MSCs for clinical applications, further investigation on their functional properties is likely needed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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b) *Mise à jour des connaissances depuis la publication*

***Données pré-cliniques**.*

Depuis notre publication, l'efficacité thérapeutique de CSMs d'origine adipocytaire ou de cordon ombilical a été prouvée dans un modèle murin de ScS induit par la bléomycine (464,465) ainsi que dans un modèle de rat (466). Par ailleurs, quelques travaux s'intéressant aux CSMs de patients sclérodermiques ont été publiés. Un travail récent montre que les ASCs de patients ont des propriétés anti-fibrotiques et pro-angiogéniques conservées *in vitro* (358). D'ailleurs, la FSV avait démontré une capacité pro-angiogénique semblable, malgré un profil moléculaire en transcriptomique différent entre la FSV de donneurs sains ou celle de malades. En effet, des molécules d'intérêt thérapeutique telle que l'IDO serait moins représentée dans la FSV des patients (359). En outre, nous avons démontré que l'iNOS (inductible oxyde nitrique synthase) dans les CSMs murines était essentielle à leur efficacité thérapeutique dans un modèle murin de ScS, soulignant l'importance de l'effet immunomodulateur et anti-oxydant des CSMs (467). Je vous joins ce travail en Annexe 3 de ce manuscrit.

Une équipe a récemment observé que l'expression de microARNs était dérégulée dans des CSMs de patients sclérodermiques, qu'elles proviennent de la MO ou du TA, comparés à des CSMs de sujets sains. Les microARNs différentiellement exprimés entre cellules saines ou ScS n'étaient pas les mêmes en fonction de la source des CSMs, soulignant l'effet de la niche environnementale sur les CSMs (468). Cette idée est également sous-tendue par d'autres études qui ont montré que l'exposition d'ASCs saines à du liquide interstitiel, provenant du derme prélevé chez les patients sclérodermiques à l'aide d'un système d'aspiration cutanée, induisait leur transdifférenciation vers un phénotype de myofibroblastes (469). Des résultats similaires ont été obtenus avec des sérums de patients ScS ou après stimulation par 10 ng/mL de TGFβ1 (470). Dans cette dernière étude, les auteurs montrent également que l'exposition au sérum de patients ScS altère la différenciation adipocytaire des ASCs saines, ce qui n'était pas observé avec du sérum de donneurs sains. Deux ans plus tôt, nous avions montrés que l'exposition de CSMs saines de MO au sérum de patients n'altérait pas leur capacité de différenciation adipocytaire alors que leur propriétés immunosuppressives l'étaient (471). La niche environnementale des CSMs de MO ou de TA n'est effectivement pas la même, ce qui pourrait expliquer les différences entre ces deux études.

Données cliniques.

Au niveau clinique, une équipe a récemment observé l'efficacité thérapeutique de l'injection locale de FSV autologue au niveau des doigts de patients atteints de ScS, sur la fibrose cutanée des mains, l'œdème et le handicap fonctionnel relaté par les patients. Ces données ont été observées au cours d'un essai clinique ouvert sur 18 patients et aucun évènement indésirable notable a été rapporté (472). Par ailleurs, un essai français ADUSE de phase II qui vise à évaluer l'injection locale d'ASCs au niveau des doigts de patients présentant des ulcères digitaux réfractaires aux traitements conventionnels va bientôt débuter (NCT04356755).

Concernant un traitement systémique par CSMs, deux nouveaux essais cliniques ont été publiés sur clinicaltrial.gouv. Le premier est mené en Colombie (NCT04432545). Il vise à évaluer l'injection de $2x10^6$ CSMs par kilogramme de poids corporel, chez des patients présentant des formes sévères de ScS avec atteinte pulmonaire (au moins de type HTAP), réfractaire aux traitements conventionnels. Les CSMs proviennent de gelée de Wharton. Le deuxième est mené à Montréal (NCT04356287). Cet essai de phase I/II vise à évaluer la sécurité et l'effet d'une ou plusieurs injections systémiques de CSMs provenant de cordon ombilical au cours de ScS sévère, diagnostiquée depuis plus de 2ans et réfractaire aux traitements conventionnels.

Au total, on peut donc conclure que les CSMs sont un espoir thérapeutique pour les patients sclérodermiques au vu des nombreuses données précliniques robustes et des différents essais cliniques en cours. Cependant, la thérapie cellulaire avec des CSMs de grade clinique nécessite des contraintes techniques importantes et un coût financier élevé (délai d'amplification, timing d'injection chez les patients, contrôles qualité …). De plus, l'optimisation du protocole de thérapie cellulaire n'est également pas simple et de nombreux paramètres importants restent à définir. Par exemple, il faut déterminer la source des CSMs injectées, la dose de cellules injectées, le nombre et la fréquence d'injection, la voie d'administration des CSMs, ou encore l'origine auto- ou allo-génique des cellules. Enfin, une thérapie cellulaire suscite toujours des questions d'effets secondaires, même si, à ce jour les données sont tout à fait rassurantes.

Différents travaux pré-cliniques ont démontré que l'injection de milieu de culture de CSMs reproduit les effets thérapeutiques observés après l'injection des cellules elles-mêmes. Ces études ont permis d'apporter la preuve de concept que les CSMs agissent en partie par des interactions directes avec leurs cellules cibles mais surtout au travers de la libération de médiateurs. Ces médiateurs sont libérés solubles dans l'environnement ou contenus au sein de vésicules extracellulaires (EVs). Ces EVs pourraient donc être une alternative acellulaire à l'utilisation des CSMs chez les patients. En effet, les facteurs contenus dans ces EVs sont protégés des enzymes de dégradation, par la double membrane lipidique des EVs et peuvent ainsi transiter jusqu'à leur cible thérapeutique. Différents travaux ont désormais apporté la preuve de la contribution des EVs dans l'effet des CSMs (473), effet notamment médié par certains microARNs contenus dans les EVs de CSMs (474). Ces informations ne sont cependant pas disponibles pour la ScS. Alors que notre équipe a démontré l'effet thérapeutique des CSMs dans un modèle murin de ScS, une partie de mon travail de thèse consistait donc à évaluer le rôle des EVs dans l'effet systémique des CSMs au cours de la ScS. Ainsi, nous allons approfondir l'ensemble des informations disponibles à ce jour sur les EVs dans la prochaine et dernière partie de cette introduction bibliographique.

3ème partie : Les vésicules extracellulaires

Nous allons parcourir successivement dans ce chapitre : la définition et la description des différents sous-types d'EVs, les différentes méthodes d'isolement puis de caractérisation des EVs, les mécanismes d'actions des EVs, leurs propriétés et leurs rôles biologiques, puis nous discuterons plus précisément des données concernant les EVs provenant de CSMs et leur intérêt thérapeutique potentiel.

I) Définition et différents sous-types d'EVs

1) Histoire et nomenclature

La première démonstration de l'existence des EVs remonte à 1946 (475). Ces fragments de membranes ou de cellules sont décrits pour la première fois en 1967 à l'aide de la microscopie électronique (476). Elles proviennent de plaquettes sanguines supposées mortes mais dont la preuve de leur viabilité a été apportée quelques années plus tard (477). Ce sont finalement les premières « microparticules » ou « microvésicules » décrites. Par la suite, différentes études démontreront que les microparticules peuvent provenir de nombreux types cellulaires différents.

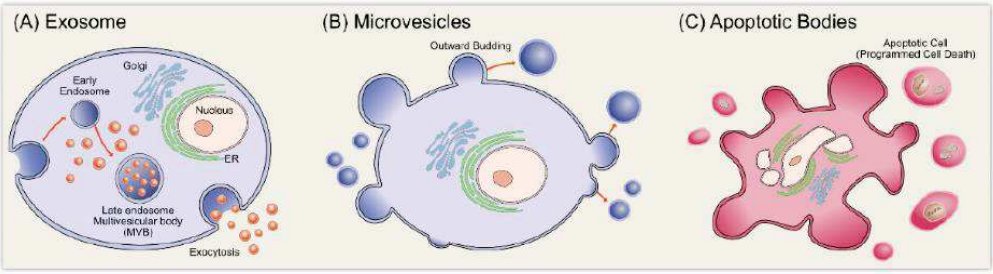
En 1981, les travaux de Trams *et al* décrivent deux populations distinctes en taille parmi les EVs : l'une dont le diamètre est compris entre 500 et 1000 nm, et l'autre dont le diamètre avoisine les 40nm. Ces dernières semblent provenir d'un mécanisme plus complexe qu'un simple bourgeonnement de la membrane plasmique notamment du fait de leur composition lipidique (478). C'est la première fois que le terme « exosomes » apparaît dans la littérature. En 1983, est observée l'exocytose de vésicules provenant de la fusion de corps multivésiculaires d'origine endosomale avec la membrane plasmique chez des réticulocytes (479). Quinze ans plus tard, il est démontré que les lymphocytes B (480) et les cellules dendritiques (481) produisent également des exosomes, contenant différentes molécules présentant un intérêt fonctionnel notamment dans la communication intercellulaire. Durant les années qui ont suivi, de nombreux travaux ont démontré que des EVs pouvaient être produites par différents types cellulaires. D'ailleurs, on sait aujourd'hui, que toutes les cellules d'organismes eucaryotes et procaryotes produisent des EVs ; on peut d'ailleurs les retrouver dans tous les fluides biologiques des organismes pluricellulaires.

Parmi les centaines de publications concernant les EVs au sens large, de nombreuses dénominations sont utilisées : microparticules, microvésicules, exosomes, corps apoptotiques, oncosomes, ectosomes, ... Ces termes portent souvent à confusion et il est parfois difficile de comprendre quelle était la sous-population d'EVs étudiée. Actuellement, trois sous-types d'EVs définis selon leur biogenèse sont communément admis dans la littérature scientifique, même si ce sujet fait toujours débat : les exosomes, les microvésicules et les corps apoptotiques. Les techniques disponibles actuellement de séparation et de concentration des EVs permettent principalement de les scinder en populations d'EVs de tailles différentes et non selon leur biogenèse : nous reviendrons sur ces techniques plus loin. Dans la littérature, il est fréquemment mentionné le terme d'« exosomes » pour des EVs de petite taille et celui de « microvésicules » (MV) pour des EVs de plus grosse taille. On voit donc que ce langage n'est pas exact puis que dans la population de petites EVs appelées « exosomes », il y aura probablement des MV de petite taille. Cette confusion de langage met en exergue la difficulté à caractériser précisément les préparations de vésicules utilisées dans les études scientifiques, dont le nombre a augmenté exponentiellement ces dernières années. Pour ces raisons-là, la société internationale des vésicules extra-cellulaires (ISEV) a mis à jour en 2018, des recommandations visant à préciser la nomenclature et la caractérisation de la population de vésicules obtenues. Ce consortium a ainsi proposé d'abandonner les termes « exosomes » et « microvésicules » qui portaient à confusion sur la pureté des échantillons obtenus, au profit des termes « petites » EVs et « grosses » EVs (482).

2) Description des sous-types d'EVs

Les EVs sont des particules délimitées par une bicouche lipidique (482) qui ne peuvent pas se répliquer, puisqu'elles n'ont pas de noyau cellulaire. Elles renferment des médiateurs de différentes natures : protéines, lipides, acides nucléiques et même des métabolites (483). Nous allons décrire ici les trois sous-types d'EVs distincts par leur biogenèse (Figure 8) (484).

Figure 8 – Les trois sous types d'EVs selon leur biogenèse



D'après Gurunathan S et al, Cells (2019)

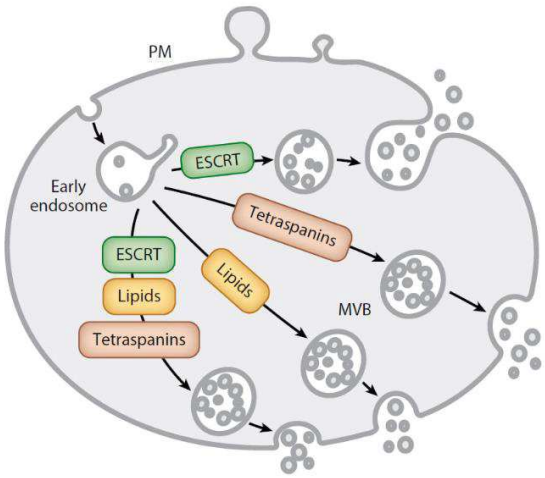
a) *Exosomes*

Les exosomes sont des EVs de petite taille : celle-ci peut varier entre 50 et 150nm. Pour un échantillon donné, la population d'exosomes présente une taille généralement homogène. La libération des exosomes par les cellules est constitutive. Ils sont issus du compartiment endosomal de la cellule et nous allons décrire ici les principales étapes de leur biogenèse.

L'endocytose est un processus physiologique permettant d'internaliser des éléments de la membrane plasmique et/ou du compartiment extracellulaire. Initialement, la membrane plasmique s'invagine pour former des vésicules d'endocytose qui vont alors fusionner avec un compartiment intracellulaire appelé endosome précoce. La membrane délimitant l'endosome précoce va, à son tour, s'invaginer, pour former des vésicules intra-luminales : cet endosome est alors appelé « corps multi-vésiculaire ». Puis, il va fusionner avec l'endosome tardif dont le contenu aura deux destins possibles : soit l'endosome tardif fusionne avec un lysosome afin de dégrader les molécules initialement endocytées ; soit l'endosome tardif retourne vers la membrane plasmique afin de recycler le contenu initialement endocyté. Ainsi, ces vésicules intra-luminales, adressées à la membrane plasmique pour être libérées dans le compartiment extracellulaire, sont en fait les futures EVs appelées exosomes.

Pour former les vésicules intra-luminales, plusieurs processus doivent intervenir (Figure 9). Tout d'abord, la formation des vésicules requière une réorganisation des protéines au niveau de la membrane de l'endosome par la formation de micro-domaines qui sont enrichis en tétraspanines (CD9, CD63, CD81, CD82) (485). Ensuite, le bourgeonnement des vésicules au sein de la lumière de l'endosome nécessite l'intervention du complexe ESCRT (Endosomal Sorting Complex Required for Transport). Cette machinerie est composée de plusieurs protéines assemblées dans un des 4 complexes – ESCRT0, ESCRT1 (comprenant Tsg-101 par exemple), ESCRT2, ESCRT3, ainsi que d'autres protéines associées à ces complexes (Alix par exemple). La formation des exosomes reste possible après blocage des 4 complexes ESCRT (486), ce qui laisse penser que d'autres mécanismes permettent la naissance des exosomes, comme la voie dépendante du facteur 6 de ribosylation de l'ADP (ARF-6), par exemple (487). Enfin, la formation des vésicules peut également se dérouler au niveau de micro-domaines de la membrane, enrichis en lipides appelés rafts lipidiques. Au niveau de ces rafts, les céramides issues notamment de l'hydrolyse de la sphingomyéline, permettent l'invagination de la membrane endosomale pour former la vésicule (488), qui bourgeonnera par la suite dans la lumière endosomale (489).

Figure 9 – Mécanismes moléculaires impliquées dans la biogenèse des exosomes



PM: plasmatic membrane ; ESCRT: Endosomal Sorting Complex Required for Transport ; MVB: multi-vesicular body.

D'après Colombo et al, Annual Review of Cell and Developmental Biology (2014)

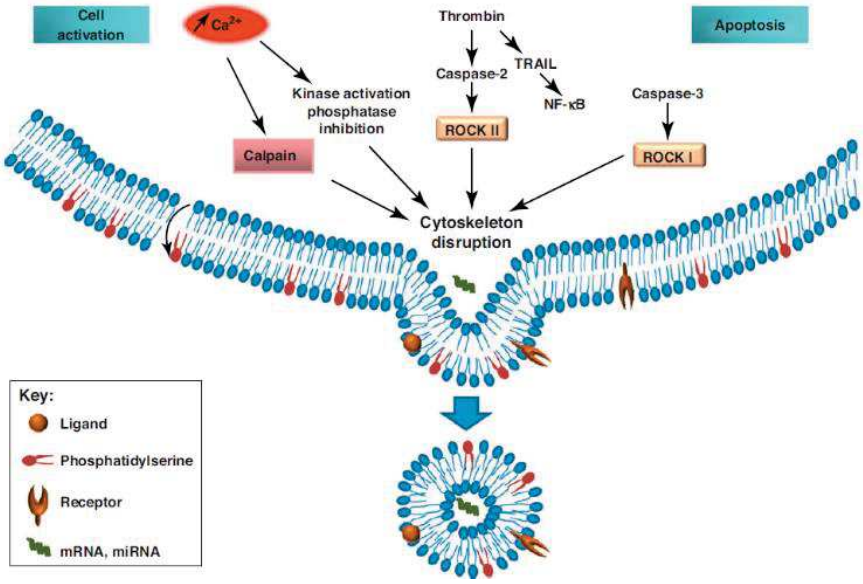
Une fois les vésicules intra-luminales formées dans le corps multi-vésiculaire, il est nécessaire que cet endosome soit réadressé à la membrane, se déplace jusqu'à la membrane puis qu'il libère les vésicules dans l'espace extracellulaire. Pour différencier les corps multi-vésiculaires destinés à fusionner avec le lysosome ou ceux réadressés à la membrane plasmique, la cellule doit pouvoir les distinguer. Il semblerait que la membrane des endosomes destinés à être recyclés soit riche en lipides et en tétraspanines, ce qui permettrait en partie de les distinguer de ceux destinés à être lysés. Ensuite, le déplacement du corps multi-vésiculaire recyclé jusqu'à la membrane fait principalement intervenir les protéines du cytosquelette, les kinésines et les protéines Rab (490). Enfin, la fusion des corps multi-vésiculaires avec la membrane plasmique nécessite l'intervention des protéines SNARE (Récepteur des protéines d'attachement du facteur soluble sensible au N-ethylmaleimide) qui permettent l'union des deux bicouches lipidiques qui se sont rapprochées (484).

b) *Microvésicules*

Les microvésicules (MV), appelées dans certaines études « microparticules », sont des EVs de taille moyenne, comprise entre 100nm et 500nm. Pour un échantillon donné, la taille des MV d'une même population est assez hétérogène. Les MV naissent du bourgeonnement de la membrane plasmique, c'est pourquoi les marqueurs de surface de la cellule d'origine peuvent aider pour la caractérisation des MV. La formation puis la libération des MV dans le compartiment extracellulaire ne sont pas constitutives comme pour les exosomes, mais induites par un signal environnemental.

En effet, l'activation de la cellule par le stimulus environnemental entraîne l'élévation des taux de calcium intracellulaire, lui-même responsable d'un réarrangement des phospholipides constitutifs de la membrane plasmique (491). Cette réorganisation fait intervenir différentes enzymes (floppases, flippases, scrambases) et aboutit à une courbure de la membrane plasmique (492). L'élévation des taux de calcium intracellulaire modifie, en parallèle, l'expression des enzymes régulatrices du cytosquelette (gelsoline, calpaïne) qui devient alors instable : la membrane peut bourgeonner et la vésicule ainsi formée (MV) peut être libérée dans le compartiment extracellulaire. Les étapes de la biogenèse des MV sont résumées dans la Figure 10.

Figure 10 – Mécanismes impliquées dans la biogenèse des MV



Ca2+: calcium ; NF-κB: nuclear factor-kappa B ; ROCK: Rho-associated, Coiled-coil Containig Protein Kinase

D'après Martinez et al, Trends in Pharmacological Sciences (2011)

c) *Corps apoptotiques*

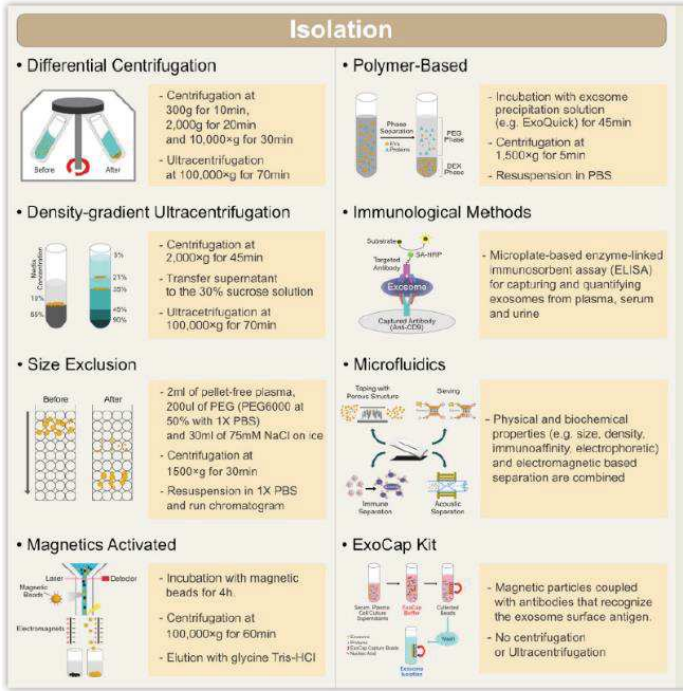
Les corps apoptotiques sont les plus grosses EVs, dont la taille est le plus souvent supérieure à 500nm. Les corps apoptotiques sont issus de la fragmentation de la cellule secondairement aux processus d'apoptose. Leur contenu est fait de résidus cytoplasmiques ou nucléaires, de nature protéique, lipidique ou d'acides nucléiques, en proportion variable selon la partie de la cellule fragmentée qui s'est vacuolisée pour être à l'origine du corps apoptotique. La suite de ce manuscrit se concentrera sur les exosomes et les MV.

II) Séparation et concentration des EVs

Le terme « isolement » des EVs est souvent employé mais ne correspond pas vraiment à la réalité car il sous-entend que nous isolons les EVs de tous les autres éléments non particulaires, ce qui n'est pas toujours le cas (contaminations par des agrégats protéiques ou lipidiques par exemple). L'usage de ce terme « isolement » est donc inexact mais il est très répandu dans la littérature et d'usage plus aisé que « séparation et concentration », et nous parlerons d'enrichissement en sous-types d'EVs.

Différentes techniques d'enrichissement des EVs ont été mises au point. Le choix dépend d'une part du sous-type d'EVs collectées, mais également du fluide (et notamment de son volume) à partir duquel on souhaite isoler les EVs. En effet, les EVs peuvent être isolées à partir de surnageants de culture cellulaire ou de fluides biologiques (493). Lorsque les volumes sont importants, certains procédés d'enrichissement ne pourront être utilisés. De même, des EVs peuvent être enrichies à partir de tissus, tels que des tumeurs (pour revue, (494)), du tissu hépatique (pour revue, (495)) ou encore des plaques d'athérome (496,497), compliquant le processus de séparation des EVs des autres composants tissulaires. Différentes techniques ont été évalués dans la littérature (Figure 11) et nous allons en détailler les modalités pour celles qui sont les plus couramment utilisés.

Figure 11 – Les différentes techniques d'isolement des EVs



D'après Gurunathan S et al, Cells (2019)

1) Méthodes par ultra centrifugation différentielle

La plus commune des techniques d'enrichissement des EVs est l'ultracentrifugation différentielle. Elle permet en effet de séparer les EVs par leur taille et leur densité. Après avoir soumis le fluide contenant les EVs à une centrifugation à faible vitesse (10 minutes à 300g puis 10 minutes à 2000g) afin d'éliminer les cellules et leurs débris, deux ultracentrifugations à vitesse différentielle permettront d'abord de séparer les grosses vésicules après 30 minutes d'ultracentrifugation à 10 000g puis les petites vésicules après 70 minutes d'ultracentrifugation à 100 000g, selon le protocole décrit initialement par C Théry (498). Les deux populations d'EVs collectées à chaque étape, doivent être soumises à une étape de lavage dans du PBS, par une nouvelle ultracentrifugation à 100 000g durant 70 min au moins. Cette méthode a été adaptée au fil des études : les vitesses et les temps d'ultracentrifugation peuvent donc varier en fonction des travaux mais l'ordre de grandeur reste le même : entre 10 000 et 20 000g pendant 30 min à 1 heure pour les grosses vésicules ; entre 80 000 et 120 000 g pendant 1h30 à 2h00 pour les petites vésicules. Les ultracentrifugations différentielles ne permettent pas de séparer les EVs des agrégats protéiques même si l'étape de lavage au PBS en élimine une grande partie. De plus, il n'est pas exclu que le stress mécanique induit par la vitesse de rotation n'engendre pas artificiellement la formation de petites vésicules (499). Certains protocoles d'ultracentrifugation à très haute vitesse utilisent des étapes à 120 000 voire 140 000g pour concentrer des exosomes.

Parfois, l'étape d'ultracentrifugation à 100 000g est réalisée sur un gradient de sucrose (ou un gradient d'iodixanol) afin de rassembler les exosomes, dont la densité est comprise entre 1,13 et 1,21g/mL, dans une même zone du tube (498). En effet, le gradient de densité, obtenu par des concentrations différentes de sucrose, permet de séparer les EVs par leur taille, leur masse et leur densité. Ainsi, la population d'EVs collectées sera composée principalement d'exosomes et non de MV de petite taille qui n'auraient pas la même densité (484). Cependant l'utilisation de ce protocole a mis en évidence différents sous-types d'exosomes qui sédimenteraient différemment sur le gradient (500), limitant ainsi l'exhaustivité de cette méthode de collecte en exosomes. Un des avantages de l'utilisation du gradient de sucrose est qu'il permet de séparer les EVs des agrégats protéiques.

2) Méthodes par filtration

Plusieurs outils basés sur la séparation des EVs par leur taille et leur poids moléculaires, à l'aide de membrane de filtration, sont également disponibles. Tout d'abord, l'ultrafiltration sur une membrane semi-perméable, permet de séparer les petites vésicules, dont le diamètre est inférieur à celui du pore de la membrane utilisée (généralement 200 nm),

des plus grosses. Ensuite, on peut réaliser l'ultrafiltration sur des colonnes composées de membranes capturant des EVs d'un poids moléculaire donné. Cela permet de séparer d'un côté les petites EVs et de l'autres les grosses EVs (ainsi que les corps apoptotiques). L'ultrafiltration sur colonne est disponible sous forme de kits commerciaux qui ne sont pas adaptés aux fluides de grands volumes, tels que les surnageants de culture par exemple. Enfin, il existe l'ultrafiltration séquentielle, qui consiste à filtrer le fluide en différentes étapes. Tout d'abord, le fluide passe au travers d'une membrane semi-perméable pour éliminer les gros éléments (cellules, débris, corps apoptotiques). Puis une filtration tangentielle du liquide recueilli sur une membrane dite « cut-off » à 500kD ne laisse passer que les protéines solubles. Enfin, les éléments retenus par la précédente membrane sont ultra filtrés au travers de pores dont le diamètre est de 100 nm afin de conserver seulement les petites EVs. Les effets secondaires au passage des EVs au travers des membranes de filtration ne sont pas tous connus. Il semblerait que cela puisse déformer les EVs et peut être, modifier leurs propriétés biologiques.

3) Méthodes par exclusion

Un autre outil permettant d'enrichir les EVs à partir d'un fluide, basé sur la taille des EVs et plus précisément sur leur diamètre hydrodynamique, est la chromatographie par exclusion de taille. Elle permet d'éliminer les protéines solubles qui passent au travers des pores et de retenir seulement les particules de plus gros diamètre hydrodynamique, dont les exosomes (501,502). Ici, les cellules mortes et les débris auront été éliminés au préalable par des centrifugations à faible vitesse. Les rendements sont importants par cet outil (503).

4) Méthodes par précipitation

La concentration d'EVs à partir d'un fluide peut également être réalisée à l'aide de polymères hydrophiles qui vont entraîner la précipitation des EVs, en utilisant leur propriété biochimique d'hydrophobie. Par la suite, ces EVs précipitées vont être culotées au fond du tube à l'aide de centrifugation à basse vitesse. Cette méthode est réalisée à l'aide de kit prêt à l'usage et de centrifugeuses de routine : elle est donc très facile à réaliser. L'inconvénient est que les EVs obtenues sont un pool d'EVs hétérogènes et que les contaminations par des agrégats protéiques et par les polymères utilisés sont fréquentes.

5) Méthodes par immuno-affinité

Enfin, des techniques de séparation d'EVs par la détection de protéines de surface, notamment de marqueurs du compartiment endosomal, sont disponibles (504). Elles sont basées sur l'utilisation de billes de capture magnétiques conjuguées à des anticorps spécifiques de ces protéines, généralement anti-CD9, anti-CD63 et anti-CD81. Ces méthodes sont plus spécifiques des exosomes, mais elles peuvent aussi purifier des protéines solubles dans le fluide. Par ailleurs, certains exosomes peuvent ne pas porter à leur membrane la protéine d'intérêt, ce qui limite l'exhaustivité de cette technique. De plus, elle pourrait purifier des MV qui exprimeraient à leur membrane les tétraspanines. Enfin, ces techniques d'immuno-affinité ont un coût élevé et ne peuvent s'appliquer à des fluides de grand volume.

6) Choix de la méthode

Parmi toutes ces méthodes d'enrichissement des EVs, l'ISEV en 2018 a publié des recommandations pour l'usage de ces techniques en fonction du sous-type d'EVs à isoler ainsi que du fluide biologique (ou surnageant de culture) à partir duquel les EVs seront concentrées (482). Par exemple, si l'objectif de rendement prévaut sur la spécificité de la séparation des sous-types d'EVs, alors on préfèrera l'utilisation de kit de précipitation avec des polymères. A l'inverse, si la spécificité des EVs concentrées est importante pour le projet, alors les techniques basées sur l'immuno-affinité seront surement les plus adaptées mais pour des fluides de faible volume. Entre les deux, on trouvera un juste intermédiaire entre rendement et spécificité avec l'utilisation d'un protocole d'ultracentrifugations différentielles ou encore de la chromatographie par exclusion.

III) Caractérisation des vésicules extracellulaires

Comme nous venons de le voir, il est extrêmement complexe de séparer les exosomes d'un côté et les MV de l'autre. Il n'y a actuellement aucun marqueur qui permet de les distinguer formellement, c'est pourquoi on utilise une combinaison de critères pour d'une part, valider la présence d'EVs et d'autre part, définir le sous-type d'EVs présentes dans la préparation. Ce panel de critères comprend la caractérisation de la morphologie et de la taille des EVs ainsi que l'identification de certains marqueurs au sein ou à la surface des EVs.

1) Détermination de la morphologie des EVs

La morphologie des EVs est généralement observée en microscopie électronique. Ainsi la microscopie à transmission électronique ou la cryomicroscopie à transmission électronique (cryoTEM) peuvent être utilisées. La cryoTEM présentera l'avantage de ne pas modifier la taille et la morphologie des EVs observées à leur état basal, à la différence de la microscopie à transmission électronique qui nécessite des étapes de fixation, de coloration et/ou de coupes ultrafines des échantillons. De plus, la visualisation de la double membrane lipidique délimitant le contour des particules est nécessaire pour attester la présence d'EVs : celle-ci sera visible en cryoTEM (505). La microscopie électronique peut également détecter la présence de protéines grâce à l'utilisation d'Ac couplés à des billes d'or (506). D'autres outils ont également été testés pour préciser les structures morphologiques des EVs, telles que la microscopie à force atomique (507) ou la microscopie à balayage (508,509). Tous ces outils permettent dans un même temps de mesurer la taille de chaque vésicule mais ne contribuent pas à quantifier le nombre total d'EVs.

2) Détermination de la taille des EVs

En sus des outils caractérisant la morphologie et la structure des EVs, on dispose de plusieurs technologies pour déterminer la taille des EVs : analyse de suivi des nanoparticules (Nanoparticle Tracking analysis (NTA)), analyse par diffusion dynamique de la lumière (Dynamic light scattering (DLS)), analyse de l'impulsion accordable à la résistivité (Tunable Resistive Pulse Sensing (TRPS)) (509).

Avec la DLS, on évalue la taille des EVs à partir d'un faisceau lumineux qu'elles diffusent en fonction de leur mouvement brownien, lui-même dépendant de leur taille (510,511). Cet outil permet ainsi de connaître la taille moyenne d'un échantillon. Lorsque la population étudiée est très hétérogène en taille, la DLS sous-estime la présence des EVs de petite taille car le signal émis par les grosses EVs masque celui des petites EVs. Cet outil est donc délaissé, depuis quelques années, au profit du NTA.

Le NTA consiste à suivre individuellement les particules d'un échantillon et analyser la taille de chaque particule en suspension dans un liquide selon ses propriétés de diffusion de la lumière, qui dépendent ici aussi de leur mouvement brownien (512). Ainsi, grâce à cet outil, on peut déterminer la concentration en particules ainsi que la distribution des EVs selon leur taille dans un échantillon donné, pour des vésicules dont la taille est comprise entre 10nm et 2µm. Par ailleurs, il est aussi possible de visualiser de la fluorescence permettant d'analyser spécifiquement certaines sous-populations de particules précédemment marquées par un fluorochrome (513).

Enfin, le TRPS permet de mesurer la taille de particules dont le diamètre est compris entre 50 nm et 4 µm et de déterminer la concentration d'EVs dans un échantillon en suspension (514). Chaque particule passe dans un pore situé dans une membrane isolante, séparant ainsi deux électrodes immergées dans un électrolyte entre lesquelles diffuse un courant ionique. La résistance électrique d'une particule à ce courant ionique entraîne un pic à chaque passage de particules dans le pore : l'amplitude de ce pic dépend de la taille de la particule. Cette technologie présente des limites pour des échantillons très hétérogènes en taille puisque le choix du pore utilisé dépend de la taille des vésicules à analyser. Ainsi un échantillon présentant des petites et des grosses vésicules ne pourrait pas être analysé sur un même pore pour déterminer les quantités de chaque sous-type de vésicules. Le TRPS, la DLS et le NTA ne permettent pas de distinguer les EVs de complexes protéiques.

La cytométrie en flux classique pourrait être un outil pour quantifier des vésicules par leur taille également mais elle ne permet pas de détecter des éléments dont la taille est inférieure à 200nm. De fait, de nouveaux outils de cytométrie de flux à l'échelle nanométrique (Nanoscale Flow Cytometer) ayant une meilleure sensibilité pouvant détecter des EVs de taille comprise entre 40 et 1000nm sont en cours de développement. L'avantage de ces nano-cytomètres est qu'ils permettent dans un même temps de déterminer la distribution et la quantité d'EVs dans un échantillon ainsi que d'identifier la présence de marqueurs protéiques grâce à l'utilisation d'Ac couplés (515,516). Ces outils ne sont pas encore utilisés en routine (pour exemples : CytoFLEX S chez Beckman (517), NanoAnalyze chez NanoFCM (516)).

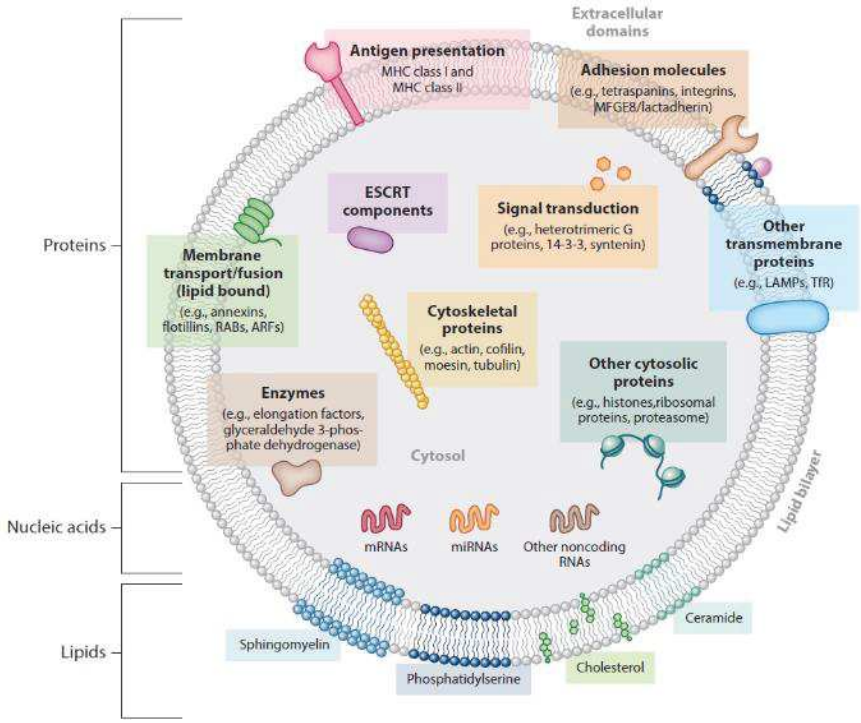
Toutes ces technologies permettent de quantifier un nombre de particules dans un échantillon, avec les limites mentionnées pour chacune d'entre elles. La mesure de la concentration en protéines des échantillons (Bradfort, microBCA) permet d'estimer également une quantité approximative d'EVs présentes par leur contenu en protéines, ce qui n'équivaut pas à une quantité de particules présentes. En effet, la quantité de protéines peut être modulée par la quantité de particules présentes mais aussi par le contenu des vésicules eux-mêmes mais peut surtout mésestimer la quantité d'EVs à cause de contaminants protéiques retenus lors des étapes de concentrations des EVs.

3) Détermination du contenu des EVs

Comme nous l'avons déjà soulevé, le contenu des EVs est de différentes natures (protéines, lipides, ADNc, ARNm, microARNs, ARNs longs non codant) (518–520) (Figure 12). Les informations disponibles à ce jour sur le contenu des exosomes sont recensées dans une base de données internationale appelée « exocarta ». Une ressource libre d'accès appelée « vesiclepedia » a également été développée (521).

En fonction des projets de recherche et des molécules étudiées, l'examen de ce contenu peut donc faire appel à de nombreux outils, que nous ne détaillerons pas ici. La caractérisation moléculaire des EVs est basée principalement sur l'identification des protéines témoignant de l'origine endosomale de certaines EVs (Alix, Tsg-101, Hsp70, CD9, CD63, CD81). Ainsi, pour les mettre en évidence, on peut utiliser des outils classiques de caractérisation protéique tels que le western blot ou les techniques immuno-enzymatiques (ELISA). Certains marqueurs peuvent également être validés en cytométrie en flux à l'aide d'Ac spécifiques. De plus, ces outils permettent aussi de s'assurer de l'absence de contamination dans l'échantillon, comme le requièrent les recommandations 2018 de l'ISEV (482). Par exemple, pour des EVs concentrées à partir de surnageant de culture cellulaire, il est nécessaire de s'assurer de l'absence de contamination par le sérum lorsqu'il est présent lors de l'étape de production du surnageant conditionné.

Figure 12 – Schématisation du contenu des EVs



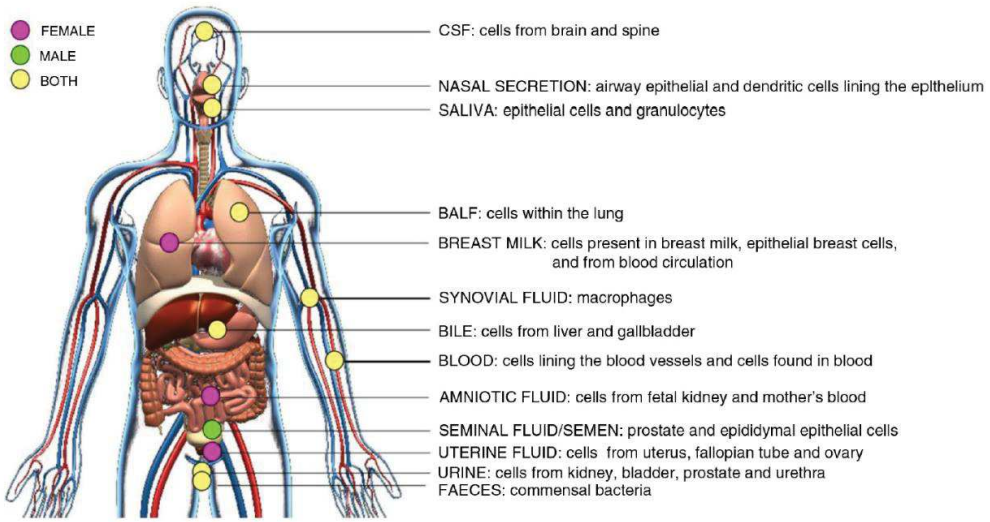
D'après Colombo M et al, Annual Review of Cell and Developmental Biology (2014)

Certaines études de protéomique (522) ont identifié des profils caractérisant certains sous-types d'EVs. De même, le profil lipidique des EVs semble varier selon les sous-types de vésicules et pourrait donc être exploité pour aider leur caractérisation (523). Mais, il semble important de souligner que ces études nécessitent des technologies non disponibles dans tous les laboratoires et ne peuvent être utilisées pour la caractérisation de routine des EVs (524).

IV) Mécanismes d'action des EVs

Les EVs permettent aux cellules de communiquer entre elles, localement (effet paracrine) mais aussi au niveau systémique (effet endocrine). En effet, on les retrouve dans tous les fluides biologiques (Figure 13) (493). Les EVs peuvent être également capturées par leur cellule d'origine (effet autocrine) (525).

Figure 13 – Représentation schématique de l'ensemble des fluides à partir desquels ont été isolés des EVs à ce jour



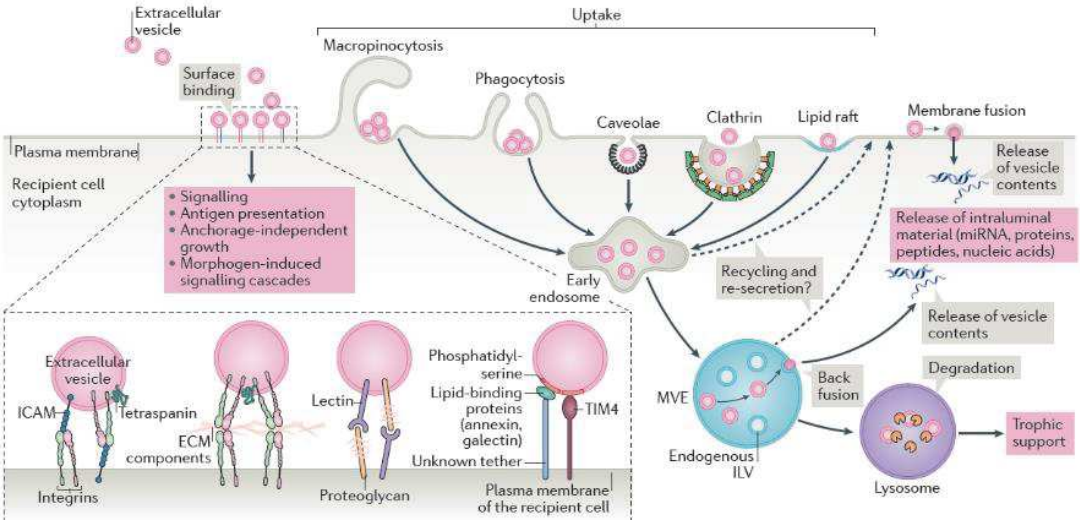
CSF: cerebrospinal fluid; BALF: bronchoalveolar lavage fluid.

D'après Yanez-Mo M et al (526)*, Journal of Extracellular Vesicles (2015)*

Elles contiennent de nombreux médiateurs dont la nature et la proportion sont variables, en fonction de la cellule qui a secrété les EVs ainsi que du contexte environnemental dans lequel elles ont été générées. Une fois libérées dans le compartiment extracellulaire, les EVs vont agir sur leur cible au travers de différents mécanismes (Figure 14) (527).

Tout d'abord, elles peuvent communiquer avec leurs cibles au travers d'interactions dites « ligand-récepteur ». Les EVs présentent à leur surface différentes molécules d'adhésion leur permettant notamment de se lier à un récepteur membranaire de la cellule cible et ainsi activer une ou plusieurs voies de signalisation après transduction du signal. Les interactions entre des protéines de surface des EVs et des récepteurs de membrane plasmique ont été démontrées pour différentes cellules cibles telles que les cellules dendritiques (528), les cellules dendritiques folliculaires (529), les neurones (530) ou encore les cellules épithéliales de l'intestin (531).

Figure 14 – Les différents mécanismes d'action des EVs sur la cellule cible.



ECM : extracellular matrix ; ILV : intraluminal vesicles ; MVE : multivesicular endosom ; ICAM : InterCellular Adhesion Molecule ; TIM4 : T-cell Immunoglobulin and mucin domain 4.

D'après Van Niel et al, Nature (2018)

Ensuite, les EVs peuvent également être internalisées dans les cellules cibles afin de leur transférer leur contenu. Il y a plusieurs voies d'internalisation des EVs décrites. D'une part, les EVs peuvent fusionner avec la membrane plasmique de la cellule cible et libérer directement leur contenu dans le cytoplasme, à l'image de ce qui est décrit dans les fusions de particules virales avec leurs cibles (532). Ici aussi, ce mécanisme a été démontré sur plusieurs types cellulaires, notamment sur les cellules cancéreuses et sur les cellules dendritiques (533). D'autre part, elles peuvent être internalisées par la cellule cible en suivant la voie classique d'endocytose. De ce fait, leur contenu pourra être soit dégradé, soit recyclé à la membrane, soit libéré dans le cytoplasme de la cellule cible. L'internalisation peut se faire selon la voie d'endocytose dépendante de la clathrine (534) ou selon les différentes voies d'endocytose indépendantes de la clathrine : macropinocytose, rafts lipidiques, phagocytose, cavéoles (535,536).

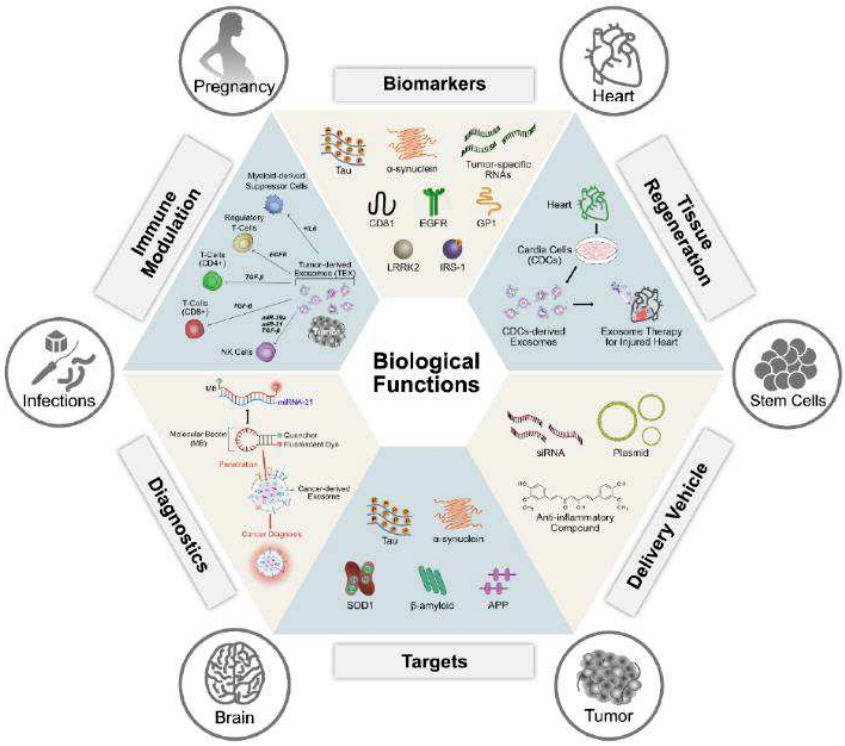
Enfin, l'ensemble de ces mécanismes d'action aboutit soit à l'activation/inhibition directe de voies de signalisation cellulaire au niveau de la membrane plasmique, soit à la libération au sein du cytoplasme de médiateurs qui vont à leur tour moduler leurs cibles (537). Finalement les EVs permettent aux cellules de s'échanger du matériel génétique (518) mais aussi des microARNs (538) ou des facteurs de transcription qui vont réguler certaines cibles et voies clés de signalisation dans les cellules receveuses (539). La capture des EVs par les cellules cibles dépend bien sûr de l'expression à leur membrane de différentes protéines et récepteurs, mais aussi des conditions environnementales (540).

V) Fonctions des EVs

1) Rôles des EVs

Ainsi, les EVs sont d'authentiques navettes permettant la communication entre différentes cellules : de fait, elles assurent de multiples rôles physiologiques (526) au cours du développement embryonnaire (541–543) mais aussi au cours de la vie d'adulte (544,545). Pour assurer cette fonction de communication, les EVs transfèrent leur contenu aux cellules cibles. La fonction d'une vésicule va donc varier selon son contenu, lui-même dépendant de sa cellule mère, de sa biogenèse et de l'environnement, physiologique ou pathologique, dans lequel elle a été produite (537,546). La plupart des travaux démontrent que les EVs assurent une (ou des) fonction(s) allouée(s) aux cellules dont elles proviennent. De plus, les EVs seraient également impliquées dans le développement de cancers (547), de maladies neuro-dégénératives (548), de maladies cardiovasculaires (549), de maladies auto-immunes (550–552) ou encore de maladies ostéoarticulaires (553). Ainsi, les EVs constituent aujourd'hui l'objet d'études visant à évaluer leur potentiel rôle de biomarqueurs de maladies ou d'outils pour le diagnostic. Enfin, les EVs sont également un outil thérapeutique en pleine expansion et nous allons tout particulièrement développer l'intérêt thérapeutique des EVs produites par les CSMs dans la prochaine partie. La multiplicité des fonctions allouées aux EVs est illustrée dans la figure 15 de ce manuscrit.

Figure 15 – Fonctions biologiques des EVs



D'après Gurunathan S et al, Cells (2019)

2) Fonctions des EVs de CSMs

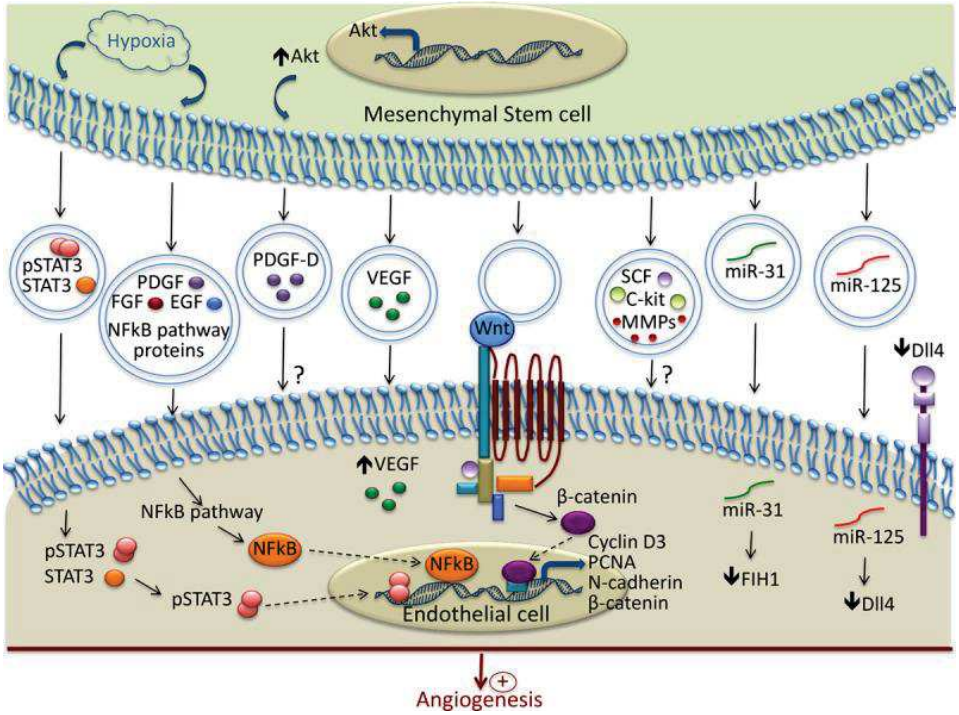
a) *Propriétés biologiques des EVs de CSMs*

Ces dernières années, différents travaux ont montré que les EVs produites par les CSMs sont capables d'assurer les propriétés biologiques de leur cellule mère (554).

Tout d'abord, nous avons vu que les CSMs avaient des propriétés anti-fibrotiques qui nous intéressent tout particulièrement dans le traitement de la ScS. Leurs EVs ont également fait leur preuve dans des modèles de fibrose de la cornée (555), de fibrose rénale secondaire à différentes étiologies (néphropathie diabétique, obstructive ou encore médicamenteuse) (556–558) ainsi que dans des modèles de fibrose hépatique (327,559–562). Plusieurs mécanismes d'action anti-fibrotiques des EVs sont possibles, notamment par le biais de médiateurs protéiques ou des microARNs. Par exemple, des exosomes de CSMs inactivent la voie de signalisation Hedgehog dans les cellules hépatiques stellaires d'un modèle de fibrose hépatique, grâce à miR-486-5p qu'elles contiennent (562). De même, des EVs adipocytaires délivrent miR-150-5p à des cellules hépatiques stellaires, inhibant ainsi CXCL1 et les voies pro-fibrotiques hépatiques sous-jacentes (563). Un autre exemple montre le rôle de certains médiateurs protéiques, tels que le facteur de croissance épidermique de globule de lait-facteur VIII (MFGE8) qui est capable d'inhiber la voie RhoA/ROCK impliquée dans la fibrose rénale (558). A noter que cette voie est également impliquée dans la fibrose pulmonaire développée par des souris sclérodermiques suites aux injections d'HOCl (278).

Ensuite, nous avons vu que les CSMs avaient un pouvoir pro-angiogénique. Les EVs produites par les CSMs favoriseraient également l'angiogenèse *in vitro* et *in vivo*. En effet, elles améliorent les lésions d'infarctus du myocarde chez le rat (564) mais aussi la neuroangiogenèse post-ischémique (565,566). De plus, dans un modèle d'ischémie de membres chez le rat, des EVs de CSMs provenant de cordon ombilical améliorent le flux sanguin et favorisent l'angiogenèse *in vitro* (567). L'effet pro-angiogénique des EVs est médié, une fois encore, par de nombreux facteurs protéiques, tels que le VEGF (568) ou le PDGF-DD (569), mais aussi des microARNs, tels que miR-125 (570) ou encore miR-31 (571). De plus, ce rôle pro-angiogénique au cours de pathologies ischémiques semblerait impliquer STAT3 (572) ainsi que la voie NF-κB (nuclear factor-kappa B) (573). L'implication de STAT3 a été démontrée dans un modèle murin d'HTAP où les exosomes inhibent le remodelage vasculaire en induisant notamment l'expression de miR-17 chez la souris. Dans cette même étude, des expériences *in vitro* complémentaires démontrent également l'effet anti-inflammatoire de ces EVs (574). Ce point est tout particulièrement intéressant pour nous, puisque l'HTAP est fréquente au cours de la ScS. Ces différents mécanismes d'action pro angiogéniques sont résumés dans la Figure 16 de ce manuscrit (575).

Figure 16 – Mécanismes impliqués dans l'effet pro-angiogénique des EVs de CSMs



Akt: Protein Kinase B ; STAT: Signal Transducer and Activators of Transcription ; PDGF: Platelet Derived Growth Factor ; FGF: Fibroblast Growth factor ; EGF: Epidermal growth factor ; NF-κB: Nuclear Factor-Kappa B ; VEGF: Vascular Endothelial Growth Factor ; SCF: Stem Cell Factor ; MMPs: metalloproteinases ; C-kit: tyrosine-protein kinase Kit (CD117) ; PCNA: proliferating cell nuclear antigen ; FIH1: factor inhibiting HIF-1 (hypoxia-inducible factor 1) ; Dll4: Delta-like ligand 4.

D'après Todorova D et al, Circ Research (2017)

Enfin, nous avions discuté des importantes fonctions immunosuppressives des CSMs. Ces propriétés passent également par leurs EVs. Différents travaux ont démontré le rôle des EVs dans la fonction immunosuppressive des CSMs sur les LT, les LB ou encore les macrophages ((576–579), pour revue (580)). Une récente étude a démontré l'effet immunosuppresseur des EVs de CSMs de cordon ombilical sur la prolifération des PBMCs en co-culture. Cet effet se perdrait au-delà du 3ème passage en culture des CSMs (581).

b) *Intérêt thérapeutique des EVs de CSMs*

Modèles pré-cliniques :

Il existe actuellement une centaine de publications qui ont évalué l'effet thérapeutique des EVs dans des études pré-cliniques et qui ont démontré qu'elles étaient, au moins, aussi efficaces que les CSMs. Ainsi, les EVs ont démontré leur efficacité dans le traitement de pathologies inflammatoires de l'œil (582), de l'épilepsie (583,584), de lésions cérébrales post-traumatiques (585), de lésions neurologiques chez les nouveau-nés prématurés (586–588), d'accidents vasculaires cérébraux (565), de maladies infectieuses (589,590), d'infarctus du

myocarde (591–593) ou encore de l'insuffisance rénale aiguë (568,594) et chronique (595). De plus, au vue du bénéfice apporté par les EVs dans des modèles pré-cliniques d'insuffisance respiratoire aiguë (596–598), elles sont actuellement à l'étude dans le traitement de la pneumopathie à SARS-CoV2 responsable de notre pandémie mondiale (599). Lors de maladies ostéo-articulaires, elles sont également une bonne alternative pour traiter l'arthrose ou l'arthrite (600,601). D'ailleurs, notre équipe a démontré que l'ARNm codant pour la protéine induite par le TGFβ (TGFβi) est contenu dans les EVs et a un effet chondroprotecteur dans des modèles *in vitro* et *in vivo* d'arthrose (602) (Annexe 4). Finalement, les EVs ont un grand potentiel de régénération tissulaire notamment pour la régénération du foie (327,603,604), de cellules neuronales (605) et de tissu osseux dans les suites de fracture (606), ainsi que pour la cicatrisation de plaies cutanées (607).

Utilisation clinique de EVs libérées par les CSMs :

Les EVs libérées par les CSMs ont fait leurs preuves dans plusieurs domaines en pré-clinique et suscitent ainsi un grand intérêt en thérapeutique clinique. Véritables outils de communication vers un tissu cible, les EVs peuvent être utilisées telles qu'elles ou après avoir modifié leur contenu pour améliorer leur efficacité thérapeutique. Différents essais cliniques sont en cours. Leur utilisation clinique implique cependant la mise en place de nombreuses procédures standardisées concernant notamment leur production, leur caractérisation ou encore leur stockage. Nous avons traité ce sujet dans une revue de la littérature publiée récemment dans Frontiers in Bioengineering and Biotechnologies, intitulée « Mesenchymal Stem Cell-Derived Extracellular Vesicles : Opportunities and Challenges for Clinical Translation ». Je vous joints ici ce travail et discuterai par la suite des nouvelles données disponibles depuis la rédaction de cette revue concernant l'effet thérapeutique des EVs de CSMs dans les modèles pré clinique et en clinique.

- Article 2 : revue de la littérature

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REVIEW
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Mesenchymal Stem Cell-Derived Extracellular Vesicles: Opportunities and Challenges for Clinical Translation

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Extracellular vesicles (EVs), including exosomes and microvesicles, derived from mesenchymal stem/stromal cells (MSCs) exert similar effects as their parental cells, and are of interest for various therapeutic applications. EVs can act through uptake by the target cells followed by release of their cargo inside the cytoplasm, or through interaction of membrane-bound ligands with receptors expressed on target cells to stimulate downstream intracellular pathways. EV-based therapeutics may be directly used as substitutes of intact cells or after modification for targeted drug delivery. However, for the development of EV-based therapeutics, several production, isolation, and characterization requirements have to be met and the quality of the final product has to be tested before its clinical implementation. In this review, we discuss the challenges associated with the development of EV-based therapeutics and the regulatory specifications for their successful clinical translation.

Keywords: mesenchymal stem cells, extracellular vesicles, regenerative medicine, therapy, clinical translation

INTRODUCTION

Extracellular vesicles (EVs), including exosomes and microvesicles, are nanoscale vesicles that are released by all cell types and act as signaling/communication agents between adjacent or distant cells. The transmission of information to a multitude of cells and locations confers them important roles in both physiological and pathological processes. EVs derived from mesenchymal stromal/stem cells (MSCs) display similar functions as their parental cells and show therapeutic efficacy in many non-clinical models (Keshtkar et al., 2018). MSC-derived EVs (MSC-EVs) exert their functions through the transfer of their cargo (i.e., proteins, lipids, and nucleic acids such as mRNA, micro-RNAs, long non-coding RNAs, DNA, and metabolites) (Busatto et al., 2019; Qiu et al., 2019). They can be used as therapeutic tools either in naïve form, as substitutes of intact cells, or after modification for targeted drug delivery. However, for clinical applications, EV safe and effective production systems and rigorous quality control are needed before the release of clinical batches. The *International Society for Extracellular Vesicles* (ISEV) and the *European Network on Microvesicles and Exosomes in Health and Diseases* (ME-HaD) have highlighted a number of safety and regulatory requirements that must be considered for the clinical applications of EV-based

therapeutics (Lener et al., 2015). In the present review, we summarize the recent developments in EV production for pharmaceutical manufacturing, and discuss the regulatory issues associated with their clinical application.

DEFINITION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES

Extracellular vesicles are defined as particles that are delimited by a lipid bilayer, cannot replicate, and are released from the cell (Thery et al., 2018). At least three EV types can be characterized on the basis of their biogenesis pathway: (i) exosomes (small particles of endocytic origin with a diameter of 30–150 nm), (ii) microvesicles or microparticles (generated from the plasma membrane by direct budding; diameter of 150–500 nm), and (iii) apoptotic bodies (vesicles of 800–5000 nm in diameter, formed via membrane blebbing of apoptotic cells) (He et al., 2018). However, due to the overlapping sizes among EV subtypes and the frequent use of isolation techniques that rely on size-based separation, the ISEV recently recommended to define them as small vesicles (EVs $<$ 200 nm), and medium or large EVs (EVs $>$ 200 nm) (Thery et al., 2018). This definition is not comprehensive, particularly concerning EV biogenesis, but probably it represents the best option for classifying EVs that are mainly isolated according to their size. A more accurate definition will require the development of devices that allow EV isolation with high yields based on the presence of specific biomarkers and that are compatible with large-scale production.

Extracellular vesicles are secreted by all human and non-human cell types and can be divided into plant-derived EVs, bacterial/fungal/parasitic EVs, and animal product-derived EVs (Schuh et al., 2019). They are key components of the local environment through intercellular communication pathways, and also of the systemic environment through their release into the fluids of complex organisms. In animals and humans, they can be isolated from all body fluid types: blood, urine, breast milk, synovial liquid, amniotic fluid, cerebrospinal fluid, saliva,... (Schuh et al., 2019). EVs convey large numbers of molecules (e.g., proteins, mRNA, non-coding RNAs, and lipids) that mediate different functions, depending on the cells from which they originate. They transfer signals to recipient cells through different mechanisms: receptor-ligand interactions, direct membrane fusion, and endocytosis/phagocytosis (van Niel et al., 2018). They act in a paracrine and endocrine manner, and can be also taken up by their cells of origin. Therefore, EVs have important roles in both physiological and pathological processes.

POTENTIAL THERAPEUTIC USE OF MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES

Extracellular vesicles from various cell sources have different physiological functions and therefore, may have different therapeutic applications. They were first investigated as vaccines to enhance the antitumor response using antigen-presenting cells, primarily dendritic cells loaded with tumor antigens, and then as vaccines for infectious and allergic diseases (for review, see Markov et al., 2019; Zurita et al., 2019). Subsequently, the detection of EVs with increased concentrations and differential cargoes in body fluids from patients with different pathological conditions led to much research on the potential use of EV proteins and RNA molecules as biomarkers of different diseases (Lasser, 2015). Finally, due to their capacity to carry large numbers of active molecules, EVs can be exploited as drug delivery systems, and can be chemically or biologically engineered to deliver enhanced or broaden therapeutic agents. Indeed, EVs act as "logistics shuttles" that show high stability in the bloodstream, specific targeting capacities (like their parent cells), and capacity to pass through physiological barriers (the blood brain barrier, for example). Indeed, MSC-derived EVs were shown to strongly inhibit lymphocyte proliferation and antibody production by targeting B-cells in heart failure (van den Hoogen et al., 2019). Chemical modification of EVs by addition of RGD peptides conjugated onto EV surfaces led the EVs to pass the blood brain barrier and target brain cells after ischemic stroke (Tian et al., 2018). EVs can also be loaded with drugs by cell transfection and genetic expression of a candidate gene, or by drug encapsulation after their isolation [(Mao et al., 2019; He et al., 2020; Ou et al., 2020); for review, see Crivelli et al. (2017)]. MSC-EVs were extensively characterized as drug delivery platform and shown to have greater internalization capabilities than commercial liposomes (Le Saux et al., 2020). The interest of using MSC-EVs loaded with doxorubicin by electroporation to target murine breast cancer cells or osteosarcoma cells has been demonstrated (Gomari et al., 2019; Wei et al., 2019). Interestingly, MSCs can entrap drug-loaded nanoparticles and release EVs that contain the nanoparticles enabling to combine MSC-based regenerative therapy to pharmaceutical nanomedicine (Perteghella et al., 2017).

The interest of using MSC-EVs for clinical applications is related to the variety of molecules with therapeutic functions they can carry and the fact that their cargo is naturally protected from degradation in the circulation (Tsui et al., 2002). EVs isolated from autologous MSCs are non-immunogenic. Although they should be poorly immunogenic in the case of allogenic injection thanks to the immunomodulatory molecules they convey, it is still unclear whether EVs contain major histocompatibility complex (MHC) molecules that might elicit alloimmune responses (Crivelli et al., 2017; Lohan et al., 2017). Numerous reports have highlighted the functional properties of MSCs and MSC-EVs using *in vitro* assays, and identified many factors involved in their functions (for review, see Doorn et al., 2012; Maumus et al., 2013; Clark et al., 2014; Glenn and Whartenby, 2014; Burrello et al., 2016; Abbasi-Malati et al., 2018). The possibility of using MSC-EVs to eliminate or reduce the clinical symptoms of several diseases has been widely assessed in animal models. A recent review of the literature discussed the applications of EVs from umbilical cord-derived MSCs (UC-MSCs) in various diseases (Yaghoubi et al., 2019). EVs isolated from different MSC sources have shown efficacy in non-clinical models of neurological diseases, particularly epilepsy (Xin et al., 2013; Long et al., 2017), post-traumatic brain

injury (Zhang et al., 2015), brain damage in pre-term neonates (Ophelders et al., 2016; Drommelschmidt et al., 2017; Sisa et al., 2019), and stroke (Doeppner et al., 2015). In animal models, MSC-EVs have been used to treat myocardial infarction (Lai et al., 2010; Bian et al., 2014) and for ischemic injury prevention in chronic renal failure (Gregorini et al., 2017). MSC-EVs are also efficient for the management of acute conditions, such as acute renal failure (Bruno et al., 2009) and respiratory failure (Zhu et al., 2014; Monsel et al., 2015; Monsel et al., 2016). MSC-EVs can reduce clinical symptoms in murine models of osteoarthritis and rheumatoid arthritis (Cosenza et al., 2017, 2018). Finally, MSC-EVs are effective in liver regeneration, as well as in experimental infectious conditions and ophthalmic diseases (Li et al., 2013; Tan et al., 2014; Tan et al., 2016; Bai et al., 2017; Chen et al., 2017; Chang et al., 2018). In conclusion, many studies have demonstrated the therapeutic efficacy of MSC-EVs in animal models and their potential is now evaluated in human clinical trials.

As it is generally accepted that MSCs from different tissue sources and from different donors display qualitatively different functional capacities, EVs isolated from different MSCs also should present differences in their cargo and related properties (Baglio et al., 2015). However, only few studies compared EVs from MSCs isolated from different sources. It has been recently reported that EVs from adipose tissue-derived MSCs (AD-MSCs) and from cardiac MSCs exhibit more potent angiogenic capacities than bone marrow-derived MSCs (BM-MSCs) (Chance et al., 2020; Kang et al., 2020). In addition, the capacity of EV production and secretory profile are higher in BM-MSCs than AD-MSCs, supporting a differential activity of EVs from different MSCs (Villatoro et al., 2019). Similarly, the expression of surface markers and function vary in MSCs from different species. Indeed, although MSC immunosuppressive capacity may cross the species barriers, different mechanisms of action are reported: through soluble factors for human MSCs and through cell–cell contacts for rodent MSCs (Uder et al., 2018). To our knowledge, no study has compared EVs from different species yet. Nevertheless, it is obvious that EVs from different species are different. This implies that the conclusions of pre-clinical studies that use human EVs in animal models have to be taken with cautions.

Another important notion for EV therapeutic applications is the definition of the minimal dose for effective clinical outcome in patients. Like parental MSCs, the effective dose of EVs is dependent on the biological activity, which can be defined on the basis of the number of particles or the quantity of bioactive proteins or RNAs. However, the protein or RNA content may quantitatively and qualitatively vary in function of MSC culture conditions or activation status and EV production method. In general, a dose-dependent effect of MSC-EVs has been observed using different functional assays (Cosenza et al., 2018; Bari et al., 2019a; Dal Collo et al., 2020). For instance, 50 μg of MSC-EVs are needed to induce the proliferation and differentiation of neural stem cells to oligodendrocytes (Otero-Ortega et al., 2020), while 10 μg of placenta-derived MSC-EVs are sufficient to increase the migration and tube formation of placental microvascular endothelial cells (Salomon et al., 2013). These differences in the dose needed for bioactivity can be related to the MSC source, the method used for EV isolation, or the mechanism of action (MoA) of EVs. The definition of the MoA for a specific therapeutic indication should allow designing a reproducible and reliable functional *in vitro* assay to determine the EV protein or RNA effective concentration, as proposed in Dal Collo et al. (2020). However, *in vitro* potency assays do not necessarily predict the therapeutic effect *in vivo*, and even less the patients' outcome in clinical trials.

The minimal effective dose of EVs could be determined using a relevant pre-clinical model (ideally a large animal model) for a specific therapeutic application. The therapeutic dose of EVs is usually in the range of 10–100 μg of proteins in mouse models (Riau et al., 2019). For example, a dose of 50 μg of EVs was sufficient to enhance protection and brain repair in a rat model of subcortical ischemic stroke, compared with 100 and 200 μg of EVs (Otero-Ortega et al., 2020). This dose was also the smallest effective dose identified in a functional *in vitro* assay. However, the most efficient dose is not always the highest dose, as shown for MSCs in a model of systemic sclerosis (Maria et al., 2016). Interestingly, it was reported that EVs isolated from non-pigmented ciliary epithelium display enhanced pro-MMP9 activities at high doses, but significantly reduce β-catenin expression and GSK-3 phosphorylation only at low doses (Tabak et al., 2018). This concentration-dependent effect of EVs might be related to different interaction modes with the target cells (e.g., direct binding to cell membrane receptors or internalization).

Moreover, investigating different administration routes may help to reduce the effective dose, if the accessibility of the target tissue is increased. Indeed, as the route of administration determines EV biodistribution, increasing the uptake of exogenous EVs by a targeted organ can enhance their efficacy (Di Rocco et al., 2016). Unlike intravenous injection, EV administration by the intraperitoneal or subcutaneous route results in higher accumulation in pancreas and gastrointestinal tract and in lower concentrations in liver and spleen (Wiklander et al., 2015). In addition, EV uptake is potentiated by the concomitant presence of extracellular proteins, for instance albumin (Schneider et al., 2017). Moreover, EV dose also can affect their biodistribution, as indicated by the inverse correlation between intravenous injection of increasing EV concentrations and their accumulation in liver (Wiklander et al., 2015).

In conclusion, the minimal effective doses of EVs can be determined by *in vivo* studies and these findings can be extrapolated for human use. EV dose and also the route, timing, and frequency of administration need to be carefully investigated for optimal and safe EV delivery in patients, as discussed elsewhere (Bari et al., 2019b).

CLINICAL APPLICATIONS OF MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES

A total of nine clinical trials can be identified in the ClinicalTrials.gov database when using the keywords "exosomes"

and/or "extracellular vesicles" and focusing on MSC-EVs (**Table 1**). Six of them are still recruiting or are completed, two trials are not recruiting yet, and one has an unknown status.

The first phase I clinical trial was initiated in 2014 with the aim of evaluating the safety of EVs isolated from UC-MSCs in 20 patients with type 1 diabetes. Patients received a systemic injection of exosomes at day 0 and of microvesicles at day 7, and the effect on the total daily requirement of insulin was evaluated at 3 months. The status of the trial is unknown. In 2017, another phase I study enrolled patients with large and refractory macular holes (MH). This randomized and controlled study has included 44 patients who received 20 or 50 μg of UC-MSC-derived exosomes in the vitreous cavity close to the MH, after pars plana vitrectomy and internal limiting membrane peeling. This study is still recruiting. The treatment efficacy is evaluated by assessing MH closure by optical coherence tomography, at 24 weeks post-treatment. More recently, a safety and tolerability study was performed in pre-term neonates (born before gestational week 27) at high risk of bronchopulmonary dysplasia (BPD). This multi-center controlled double-bind trial included 3 to 14-day-old neonates (n = 18) who received 20, 60, or 200 pmol phospholipid of BM-MSC-EVs (UNEX-42)/kg body weight by intravenous injection. Safety was the primary endpoint, but BPD incidence and severity were also determined (secondary endpoints). A phase 1/2 multi-center randomized study to evaluate the effectiveness and safety of daily local injections of MSC-EVs (AGLE-103) in 30 patients with dystrophic epidermolysis bullosa was registered in November 2019. The primary endpoint is the safety and efficacy of wound closure at 8 months after treatment. The last phase 1/2 clinical study recorded in December 2019 will assess the alleviation of dry eye symptoms in 27 patients with chronic graft versus host disease (GVHD) after local treatment with UC-MSC-derived exosomes four times per day for 14 days. The treatment safety and efficacy will be evaluated at different time points by measuring the changes in the ocular surface disease index.

Two phase I clinical trials evaluate genetically engineered MSC-EVs. The first trial assessed EVs isolated from miR-124-overexpressing MSCs in five patients with acute ischemic stroke. Patients received 200 μg of total EV protein by stereotaxis, 1 month after the stroke. The incidence of adverse events was the primary outcome measure, but efficacy was also assessed using a Modified Rankin Scale after 12 weeks of treatment. The trial has recently been completed, but results are not available yet. The

TABLE 1 | Clinical trials evaluating MSC-EV therapies.

Disease	EV type	Administration route	Injected dose and time of injection	Cell source	Trial phase	Control group	Status	Number of patients	Ref/NTC
Type 1 diabetes	Exo and MV	IV	Eq of SN from 1.2-1.5 × 10^6 cells/kg body weight (day 0)	UC-MSCs	1	No	Unknown	20	NCT02138331
Macular holes	Exo	Local	20 μg or 50 μg Eq proteins (day 0)	UC-MSCs	1	Yes	Recruiting	44	NCT03437759
Bronchopulmonary dysplasia	Not indicated	IV	20, 60, or 200 pmol/kg (day 0)	BM-MSCs	1	Yes	Recruiting	18	NCT03857841
Dystrophic epidermolysis bullosa	Exo	Local	Not indicated (once a day for 60 days)	Not indicated	1/2	Yes	Not yet recruiting	30	NCT04173650
Dry eye (graft versus host disease)	Exo	Local	10 μg Eq proteins/drop, 4 times a day, 14 days	UC-MSCs	1	No	Recruiting	27	NCT04213248
Ischemic stroke	Exo	Local	200 μg Eq proteins (day 0)	miR-124 over expressing MSCs	1/2	No	Completed	5	NCT03384433
Pancreatic cancer	Exo	IV	Unspecified	MSCs loaded with KrasG12D siRNA	1	No	Not yet recruiting	28	NCT03608631
Graft versus host disease	Small size EVs	IV	4 units (1 unit = Eq of SN from 4 × 10^7 cells) (day 0)	BM-MSCs	NA	NA	Completed	1	Kordelas et al., 2014
Chronic kidney disease	Total EVs	IV (1st)/IA (2nd)	100 μg Eq proteins /kg (x2) (day 0)	UC-MSCs	2/3	Yes	Completed	40	Nassar et al., 2016

EVs, extracellular vesicles; Eq, equivalent dose; Exo, exosomes; IV, intravenous; IA, intra-arterial; MV, microvesicles; SN, supernatant; BM-MSC, bone marrow-derived mesenchymal stem cells; UC-MSC, umbilical cord-derived mesenchymal stem cells; NA, not applicable.

second clinical trial will evaluate MSC-derived exosomes loaded with small interfering RNAs against KRAS G12D (iExosomes) in patients with metastatic pancreatic cancer. Patients (n = 28) will receive the treatment by intravenous route at days 1, 4, and 10, and then every 14 days for up to three courses in the absence of adverse events or unfavorable disease outcome. The study aim is to identify the maximum tolerated dose and the dose-limiting toxicities of iExosomes, but has not recruited patients yet.

There are only two publications on the use of MSC-EVs in the clinic. The first article reported the case of one patient with therapy-refractory GvHD who received four units of BM-MSC-derived small EVs by intravenous injections (Kordelas et al., 2014). One unit of EVs was defined as the EV fraction recovered from the supernatant of 4×10^7 BM-MSCs conditioned for 48 h and isolated by filtration using 0.22 μm filter membranes, precipitation with polyethylene glycol, and a final ultracentrifugation at 100.000 g for 2 h. To reduce the potential side effects, the patient initially received one tenth of a unit, and then progressively increasing unit amounts every 2–3 days to a total of 4 units. No adverse event was observed and clinical symptoms were remarkably improved within 14 days after EV administration, suggesting the safety and potential efficacy of this EV-based treatment. The second article concerned a randomized placebo-controlled clinical trial that evaluated the safety and efficacy of UC-MSC-EVs in 40 patients with stage III and IV chronic kidney disease (Nassar et al., 2016). EVs were collected from UC-MSC conditioned supernatant using two ultracentrifugation steps at 100,000 g for 1 h. Patients received two injections of 100 μg EVs/kg body weight 1 week apart, the first one by the intravenous route and the second one through the intra-renal arteries. No adverse event was recorded. The overall renal function significantly improved during the 12-month follow-up period. Interestingly, TGFβ1 and IL10 levels significantly increased concomitantly with the clinical improvement, suggesting immune modulatory regulation. Although few clinical results are available, pre-clinical data and early clinical results on EV-based therapeutics are very encouraging. However, it is important to stress that these clinical trials are mostly phase 1 studies on the feasibility and safety of EV administration for different clinical applications. Only one phase 2/3 trial has been completed and showed the safety (primary endpoint) and efficacy (secondary endpoint) of UC-MSC-EVs in patients with chronic kidney disease, as indicated by the reduction of serum creatinine level by 50% and the twofold increase in eGFR (Nassar et al., 2016). Randomized, double-blinded phase 2 and 3 clinical trials are required to definitively demonstrate MSC-EV efficacy and therapeutic interest.

CHALLENGES FOR THE INDUSTRIAL PRODUCTION OF GMP-GRADE EXTRACELLULAR VESICLES

The main challenge linked to the industrialization of EV-based therapeutics for regenerative medicine is to define new manufacturing strategies under Good Manufacturing Practice (GMP) for EV scalable production and isolation. Standardized operating procedures (SOPs) using reproducible and standardized assays are mandatory to manufacture a defined and qualified EV product because each manufacturing procedure will generate a different product. To reach this goal, major questions have to be addressed early in the product development: (i) how to manufacture EVs; (ii) how to characterize and qualify the final product; and (iii) how to organize the product storage in order to maintain its stability.

Manufacturing

MSC Sources

Several tissue sources of MSCs can be used, such as BM, adipose tissue, synovial membrane, UC. These MSCs have been tested in various *in vitro* functional assays and in a large number of non-clinical disease models where MSC-EVs have shown therapeutic efficacy [for review see D'Arrigo et al. (2019)]. Nevertheless, no comparative study identified the most efficient MSC source for EV production, in terms of quantities or functional activities. Primarily two sources of MSCs (UC- and BM-MSCs) have been tested in clinical trials, but for different applications (**Table 1**). Therefore, the first question for EV manufacturing is the identification of the best MSC source(s) for a specific clinical application, and more data are necessary to answer this question. The best MSC source can be determined by identifying the most relevant MoA for the targeted therapeutic activity. For example, if an anti-inflammatory or pro-angiogenic function is envisioned, AD-MSCs or UC-MSCs might be preferred to BM-MSCs. It is nevertheless recommended to determine the best MSC source experimentally by comparing MSCs from different sources in a pre-clinical model relevant for the therapeutic application, by testing different batches of different MSC sources or by comparing pools of MSCs to avoid inter-donor variability. EV production is also influenced by the features of the producing cells. For instance, it has been shown that cell aging (replicative senescence and donor age-associated senescence) and cell–cell contacts (confluence and seeding density) affect EV production. Specifically, senescent MSCs secrete greater numbers of EVs than non-senescent MSCs (Huang et al., 2019; Fafian-Labora et al., 2020). Conversely, confluent MSCs produce lower amounts of EVs than proliferating MSCs (Patel et al., 2017). The impact of senescence on the production and functionality of MSC-EVs in different therapeutic applications has recently been reviewed (Boulestreau et al., 2020). EVs from aging MSCs did not exhibit the protective effect of EVs from young MSCs in an acute lung injury model (Huang et al., 2019). In consistency, intercellular transfer of EVs from young MSCs are more potent than EVs from aged MSCs to rejuvenate aged hematopoietic cells and restore their function through the uptake of autophagy-related mRNAs (Kulkarni et al., 2017). Production process will therefore have to include quality controls to evaluate the percentage of senescent cells and its impact on the functionality of EV batches.

Immortalized MSC lines could be used to ensure batch reproducibility, to avoid inter-individual donor variability, and

to maintain bioactivity during culture expansion. For instance, EVs released by embryonic stem cell-derived MSCs immortalized by transfection of a lentivirus carrying the c-Myc oncogene reduced the infarct size in a mouse model of myocardial injury (Chen et al., 2011). Of course, the immortalized MSC stability and absence of the transgene protein in the derived EVs must be demonstrated. Nevertheless, this strategy ensures an infinite supply of EVs with high inter-batch reproducibility.

EV Production

A second question is the choice of culture system (e.g., medium composition and cell-adhering support) for EV production. Indeed, several cell culture parameters influence EV production and cargo composition. For clinical purposes, the use of xeno- and EV-free culture media is recommended to remove any source of variability and animal-associated contaminations. It has been shown that xeno- and serum-free culture media support sustained MSC proliferation without loss of viability and promote the cell secretory functions (Lee et al., 2017; Mochizuki and Nakahara, 2018; Palama et al., 2020). Platelet lysates can be used at the place of fetal calf serum in GMP manufacturing conditions, although defined media are more appropriate (Pachler et al., 2017; Bari et al., 2018). To scale up EV production for industrialization, 3D-culture in bioreactors has been tested, such as multilayered cell culture flasks, hollow fiber bioreactors, stirred-tank bioreactors, and spheroidal aggregates of MSCs. Hollow fiber and stirred-tank bioreactors are the more promising approaches because they are closed and GMP-compatible scalable systems that provide a high surface-to-volume ratio for MSC growth (Mendt et al., 2018; Mennan et al., 2019; Vymetalova et al., 2020). EV production in bioreactors is increased at least by 40-fold compared with 2D culture systems (Watson et al., 2016). Furthermore, the duration of EV production and the frequency of medium collection have to be tested to determine the optimal parameters for cell proliferation, confluence and EV re-uptake by producing cells.

EV production can be stimulated using different biochemical or biophysical strategies. Among the biophysical strategies, hypoxia can be controlled and modulated. It has been reported that MSC culture and EV production in hypoxic conditions (1–5% O_2) increase the number of EVs released and their cargo composition (growth factors and miRNAs), thus enhancing their pro-angiogenic, immunomodulatory, cardioprotective and neuroprotective effects (Cui et al., 2018; Xue et al., 2018; Zhu et al., 2018). Another option is to take advantage of bioreactors to mechanically stimulate EV production by applying fluid shear stress or compression [for review, see Piffoux et al. (2019)]. Although, the underlying mechanisms are not known, one hypothesis is that in this condition, MSCs inhibit their own re-uptake of EVs. Recently, it has been reported that ultrasonication of ultracentrifuged MSC-EVs followed by regular centrifugation and filtration allows increasing the EV yield by 20-fold (Wang et al., 2019). Moreover, the authors demonstrated that these EVs are functional and promote wound healing in animal models. However, this technique might release a fraction of vesicles that normally remain tethered at the plasma membrane, or vesicles that have been recaptured by the producing cells, or even other components from secretory pathways (van Niel et al., 2018). Therefore, EVs isolated after ultrasonication need to be better characterized.

Different strategies have been considered to modulate EV content and biological activities, including biochemical stimuli and genetic modification of MSCs to overexpress specific proteins or miRNAs [for review, see Park et al. (2019)]. MSC activation with lipopolysaccharides before EV production does not change the number of released EVs, but influences their content. These EVs have been used to modify macrophage polarization, procoagulant properties, or the ability to support wound healing (Ti et al., 2015; Zeuner et al., 2016; Fiedler et al., 2018). The importance of miRNAs in MSC-EV therapeutic effects suggested that genetic engineering of MSCs to overexpress the miRNAs of interest might improve their efficacy. For example, miR-92a-3p overexpression in MSCs allowed producing EVs with higher protective effect against cartilage destruction in an osteoarthritis model (Mao et al., 2018).

In conclusion, all parameters that can influence EV number and content must be clearly identified to define the best balance between production conditions and EV functions. Improvement of EV functions can be obtained through genetic modification or pre-activation of MSCs. Because different manufacturing procedures and culture conditions can affect the characteristics and functionalities of EVs, the production process will have to be clearly defined for optimal use of EVs for specific clinical indications.

EV Isolation

There is no unique or standardized method to isolate EVs. This might explain the variability in EV characteristics and bioactivities among laboratories. For clinical applications, the challenge is to isolate EVs with high yield and purity, while preserving their structure and activity. In addition, the isolation method should be scalable, cost-effective, compatible with a high-throughput production process, and ideally, in a closed system. Differential ultracentrifugation-based techniques are the most common EV isolation methods in basic research, but they are not scalable, do not give pure EV preparations, may lead to EV aggregation, and are time-consuming. However, sequential centrifugation steps have been used for large-scale production of clinical-grade MSC-EVs (Mendt et al., 2018). Size-based fractionation methods that include tangential flow filtration and size exclusion chromatography are GMP-compliant and scalable systems for EV isolation [for reviews, see Agrahari et al. (2019); Paganini et al. (2019)]. Ultrafiltration also reduces the isolation times and costs compared with other techniques (Saxena et al., 2009; Bari et al., 2018, 2019b,c). In our opinion, currently, this is the method of choice for high-scale and GMP-compliant isolation of EVs. A comparative analysis of the secretome from BM- and AD-MSCs enriched by ultrafiltration or sequential ultracentrifugation indicated that ultrafiltration results in higher particle yield with higher protein content, in GMP-compliant conditions (Bari et al., 2019c). Finally, the choice of the isolation technique will have to be a compromise

between EV yield and cost. In addition, it is important to keep in mind that each variation in the production process generates a product modification that will require a new functional qualification.

Quality Controls

Quality controls concern MSC characterization and expansion, EV production and isolation, and the release criteria of EV batches [recently updated in Thery et al. (2018); Rohde et al. (2019)]. Attempts to standardize the methods of EV isolation and characterization are regularly discussed within ISEV. To measure the production yield, the number of isolated EV particles needs to be determined by Nanoparticle Tracking Analysis (NTA) or Tunable Resistive Pulse Sensing (TRPS). These methods allow measuring the number of particles in a solution. The production yield should be expressed as the particle number in cell equivalents because it takes into account the number of viable cells at harvest time and allows a better evaluation of the inter-batch reproducibility. Although there is no standard size for EV preparations, a size of $\leq$200 nm characterizes small EVs and could be defined as the standard to ensure better inter-batch reproducibility. EVs must also be profiled by flow cytometry or western blotting: expression of EV markers (CD9, CD63, TSG101, and CD81) and MSC markers (CD44, CD73, CD90, and CD105), and absence of signal for immune cell markers (CD14, CD34, and CD45). The presence of at least three different markers enriched in EVs should be a major criterion for batch release: CD9, CD63, CD81, Tsg101, Alix, and the ganglioside GM1, which has been described as an exosome marker (Tan et al., 2013). Finally, standard safety tests to exclude microbial impurities should be performed to determine the endotoxin levels, sterility, absence of mycoplasma, and absence of viral enrichment in the final product.

Additional information on protein and RNA concentration, which is not part of the released criteria, could be added to the quality control list. This information allows expressing the number of EVs as particles per μg of protein or RNA, and could be used to assess inter-batch reproducibility. In addition, specific microRNAs or proteins, known to be relevant for EV therapeutic effect, could be identified and quantified by quantitative PCR and ELISA assays to provide supportive data on EV functional properties. This will be relevant to define potency assays for EVs in relation to the dedicated clinical applications.

Storage and Stability

The preservation of EV biological activity during storage is both critical and challenging. Few studies have reported consistent data on EV storage and formulation. Siliconized vessels are recommended for EV storage to prevent their adherence to surfaces and their loss (Jeyaram and Jay, 2017). Phosphate buffered saline is habitually used for EV resuspension. Storage at −80°C is encouraged, although it can affect EV size, number and function (Lorincz et al., 2014; Cosenza et al., 2018). It has been reported that EV concentration (quantified by NTA) remains stable after 1 week of storage at +4, −20, and −80°C (Jeyaram and Jay, 2017). Nevertheless, storage at +4°C causes EV aggregation, and the amount of the associated proteins and miRNAs dramatically decreases at +4°C and −20°C. For clinical applications, EV products need to be suspended in sterile 0.9% NaCl and stored at −80°C. Moreover, they should be frozen and thawed rapidly to preserve their morphology and function. EV products should be formulated for single-use because it has been observed that their number decreases, and their morphology and content are altered after two cycles of freezing and thawing (Kusuma et al., 2018).

The possibility to freeze-dry the EV products for long-term storage at room temperature has been investigated. Freeze-drying preserves EV characteristics and function, and thus might represent a cost-effective storage strategy. It also reduce transport costs (Charoenviriyakul et al., 2018). The characteristics and functionality of peripheral blood mononuclear cell-derived secretomes remain stable for up to 6 months after lyophilization and high dose γ-irradiation when stored between −20 and +25°C (Laggner et al., 2020). However, such lyophilized secretomes contained albumin, cholesterol and triglycerides that might have preserved the sample bioactivity. Another study showed that the exosome number and size distribution and biological activity are not affected after storage at −80°C for 45 days or 6 months (Mendt et al., 2018). Disaccharide stabilizers could be added in the storage buffer to improve EV preservation. Trehalose is a natural, non-reducing disaccharide sugar used as a cryo-preservative for labile protein drugs, vaccines, and liposomes. Its safety and tolerance have been demonstrated in mice and humans after oral, gastric and parenteral administration (Sato et al., 1999; Richards et al., 2002). It has been reported that addition of trehalose to EV samples improves their stability when stored at −80°C and when lyophilized, by preventing EV aggregation and lysis (Bosch et al., 2016; Charoenviriyakul et al., 2018). Mannitol is another cryoprotectant that maintains the functionality of freeze-dried secretomes stored at −20°C for at least 2 months (Bari et al., 2019c). The addition of 5–10% dimethylsulfoxide (DMSO) also maintains EV integrity and function (Romanov et al., 2019). The possibility to develop an off-the-shelf lyophilized product is a huge strength compared with the parental cell product that must be frozen for preservation and must be transported fresh after revitalization and/or expansion, or frozen under stringent requirements. Finally, whatever the storage formulation and conditions, batch stability will have to be carefully examined and monitored during storage. Stability can easily be assessed by quantifying the particle number, the quantity of total RNA and proteins, and the MoA-associated bioactive factor at different times during storage.

REGULATORY ASPECTS FOR THE INDUSTRIAL AND CLINICAL USE OF EXTRACELLULAR VESICLES

The regulatory aspects for manufacturing and clinical applications of EVs as new therapeutics have to be implemented. In 2015, an ISEV position paper discussed the classification of EV-based products as biological medicine or biological drugs, and categorized EVs based on the anticipated active substance(s)

(Lener et al., 2015). It also provided a detailed discussion on the regulatory issues associated with EV-based therapeutics. According to the regulatory frameworks for manufacturing and clinical trials in Europe, United States and Australia, quality and safety control data must be provided, as underlined in the previous section. In addition, the existing guidelines require the identification, quantification and characterization of the main substance(s) of a biological drug to indicate the MoA. The active substance determines the pharmaceutical classification and the MoA, and will define the potency assay to be used (Rohde et al., 2019). However, we can expect that the MoA will not be limited to a single molecule, as it has been shown for MSCs, and it will be difficult to precisely define the active substance in EVs. We can also anticipate that the MoA, and associated bioactive factors, of a same MSC-EV batch may also depend on the targeted clinical application and the related therapeutic function. Interestingly, a review paper discussed the respective role of miRNAs and proteins as major factors in the MoA and in mediating EV therapeutic effect (Toh et al., 2018). A prerequisite for their potency is the presence of biologically relevant amounts of molecules. By analyzing the average quantity of miRNAs in MSC-EVs and the possible number of EVs taken up by a cell, the authors concluded that miRNAs are not likely to be in the right concentration or configuration to have a relevant biological activity. A similar analysis for proteins indicated that they were more likely to elicit a biologically relevant response, suggesting that proteins could be the main drivers of MSC-EV MoA. Nevertheless, it has been reported that several miRNAs are important actors, at least as mediators, of MSC-EV immunoregulatory effects (Martin-Rufino et al., 2019). More studies are needed to bring firm conclusions on the role of proteins and miRNAs in the MoA of MSC-EVs. Although not required in the early stages of clinical development, the definition of the active substance(s) that supports the MoA and the efficacy of EV-based treatments is a requirement to develop appropriate pharmaceutical control strategies. Indeed, in the early phases 1 and 2 of pharmaceutical development, the batch-to-batch consistency must be checked using biochemical, biophysical and functional assays (Rohde et al., 2019). Due to their complex nature, the specific MoA of EVs may be difficult to identify; however, in contrast to cells, it could be easier to set-up quality control tests for EV characterization and inter-batch homogeneity assays (Riazifar et al., 2017). The regulatory requirements will also be different for EV-based drugs derived from cells (genetically engineered or not) and for EVs used as drug-delivery systems [for review see Lener et al. (2015)]. Compliance with the regulatory frameworks is pivotal for the approval of EV-based therapies and their large-scale implementation.

CONCLUSION: CHALLENGES AND PERSPECTIVES

MSC-EVs exert comparable therapeutic functions as their parental cells, but have some advantages over MSCs because they lack nuclei and cannot abnormally proliferate or differentiate. Moreover, small-size EV preparations that are isolated using protocols including a filtration step through 0.22 μm membranes can be considered as sterile and do not require an additional sterilization step. However, there are still many challenges to be addressed concerning the scalable production, standardization, and characterization of EV products for the successful translation of EV-based therapeutics in the clinic (Agrahari et al., 2019). The heterogeneity of MSCs used for EV production (BM, adipose tissue, other tissues; non-manipulated or immortalized) and of the obtained EVs (production process; EV size; contents of EV fractions) makes difficult to select the EV drug with the highest therapeutic efficacy. Several companies have already developed EV- or secretome-based products for different clinical applications using diverse cell sources, and MSC-derived EVs represent around 40% of such products (Gimona et al., 2017). The best sources for reproducible, safe and cost-effective production must be identified using the relevant non-clinical models for each specific clinical applications. Large-scale processes to manufacture EV therapeutics in GMP conditions (mainly bioreactor technologies for EV production, and ultrafiltration technologies for EV purification) are being implemented using preferentially closed systems for higher safety to ensure robust production procedures. Several GMP-compliant processes for the production of MSC-EV or secretome products have been developed (Pachler et al., 2017; Bari et al., 2018; Mendt et al., 2018; Laggner et al., 2020). Importantly, although quality controls for cell production include cell viability and apoptosis rate measurements, they do not assess cell senescence. Yet, EV yield is higher when using senescent cells, and their cargo composition is altered (abnormal levels of some miRNAs) [for review, see Boulestreau et al. (2020)]. The proportion of senescent cells in the production batches and their effect on EV content should be taken into account in quality control procedures. Moreover, EV standardization (protein or RNA quantification, particle determination) should be improved. In addition, some undesirable miRNAs, such as miR-410 that promotes carcinoma cell growth and aging-associated miRNAs, should be quantified (Fafian-Labora et al., 2017; Dong et al., 2018). Analytical methods to accurately characterize EVs at the single-vesicle level are under development and are needed for reliable standardization. Lyophilization could be used for the long-term storage of EVs and to develop off-the-shelf products with high stability. This would represent a real advantage compared with MSCs because it would facilitate and reduce the costs of storage and transport (at room temperature). The formulation of EVs into a standardized biological drug has to be defined for each clinical application in terms of dosage, excipients (use and type of cryoprotectant, for example) and pharmaceutical forms (powder or liquid) (Bari et al., 2019b). The formulation may depend on the administration route. While liquid formulations can be used for systemic or parenteral injection of EVs, powder formulations might be preferred for oral or aerosolized administrations. The dosages for a specific application will determine the batch sizes for production (Rohde et al., 2019). The procedure for the characterization of the active substance(s), which can be localized in the inner and/or outer

part of EVs, and of the "excipient" non-biologically active moiety of the EVs will have to be established before the industrialization step. It is now crucial to address the challenges related to the production and the regulatory and clinical aspects of EV-based biological products in order to pave the way to their commercialization.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and writing of the manuscript. MM, PR, JB, and CJ proofread and given comments as well as suggestions. DN supervised and finalized the manuscript.

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Conflict of Interest: MM is an employee of Bauerfeind France.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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- Mise à jour des connaissances depuis la publication

Depuis la réalisation de ce travail, plusieurs études pré-cliniques ont été publiées, dont l'objectif était d'améliorer l'effet thérapeutique des EVs de CSMs en modulant le niveau d'expression de certains médiateurs. Par exemple, la sur-expression de let7i dans les EVs permet de limiter la progression du cancer du poumon chez des souris (608). En rhumatologie, la sur-expression de miR124a dans des EVs permet d'inhiber *in vitro* la prolifération et la migration de synoviocytes et favorise leur apoptose (609), ce qui en ferait un atout thérapeutique au cours de l'arthrose.

De nouveaux essais cliniques ont été recensés sur clinicaltrials.gov. Trois domaines, évaluant actuellement les EVs de CSMs, s'ajoutent à ceux discutés dans notre revue. Tout d'abord, un essai de phase I/II s'intéresse à la sécurité d'emploi des EVs au cours de la maladie d'Alzheimer après des pulvérisations intra-nasales (NCT04388982). Ensuite, un projet vise à évaluer la sécurité d'emploi ainsi que l'efficacité thérapeutique de l'injection intraveineuse d'EVs après une chirurgie de dissection aortique dans une approche préventive ou curative de la défaillance d'organe multiviscérale, fréquente dans les suites d'une telle chirurgie (NCT04356300). Enfin, les EVs font l'objet de plusieurs essais cliniques en cours pour le traitement du syndrome de défaillance respiratoire aiguë (SDRA) dans le contexte d'infection par le SARS-CoV-2. Un seul de ces essais thérapeutiques évalue l'utilisation des EVs par voie intraveineuse (NCT04657458). Les autres évaluent la sécurité et l'apport d'un traitement inhalé quotidien d'EVs chez des sujets sains (NCT04313647) et chez des sujets atteints de SDRA (NCT04602104 et NCT04544215 en Chine, NCT04602442 et NCT04491240 en Russie). Un de ces essais mené en Chine, qui évaluait l'efficacité de l'inhalation quotidienne d'un aérosol de $2x10^8$ particules d'EVs pendant 5 jours, est notifié comme « terminé » depuis le mois de septembre (NCT04276987), mais les résultats ne sont pas encore publiés. Ces différentes voies thérapeutiques utilisées soulignent l'importance des données connues à ce jour sur la biodistribution des EVs apportées par voie exogène.

- Biodistribution des EVs

Chez l'animal, différentes études montrent globalement que les EVs, après une injection systémique, semblent être localisées rapidement au niveau hépatique (610), puis splénique et pulmonaire. Tous les organes pourraient contenir des EVs injectées par voie intraveineuse, mais le rendement pour un organe donné pourrait être amélioré par l'utilisation d'une voie d'administration plus proche de l'organe à cibler (611). Par exemple, une administration intra-oculaire pourrait centraliser les EVs au niveau de l'œil et ainsi améliorer

leur efficacité thérapeutique dans cet environnement. Ces injections locales évitent ainsi le premier passage hépatique, inhérent à l'injection intraveineuse, qui est responsable de la capture d'une grande quantité de vésicules. Certaines études semblent montrer que les EVs auraient un tropisme pour un organe malade. Par exemple, après un accident vasculaire ischémique, les EVs pourraient se diriger préférentiellement vers la zone infarcie du cerveau (612). Ces données sont toutefois à prendre avec précaution, car les EVs sont visualisées grâce à l'utilisation d'un marqueur fluorescent lipophile qui s'incorpore à la membrane des vésicules. Cette incorporation à la membrane pourrait cependant modifier la biodistribution des EVs et favoriser notamment sa localisation cérébrale et splénique (613). Elle pourrait également allonger la demi-vie des EVs les rendant plus stables au cours du temps, du fait de la modification de leur composition membranaire. De plus, au-delà de la voie d'administration des EVs, leur biodistribution semble dépendre de la dose d'EVs injectées ainsi que du type de cellules d'où proviennent les vésicules (613,614).

3) Vésicules extracellulaires et ScS

a) *Rôle physiopathologique des EVs*

Comme nous l'avons évoqué précédemment, la ScS est une maladie au cours de laquelle de nombreux acteurs sont impliqués et dont leur interaction réciproque semble essentielle dans le développement de la pathologie : les EVs pourraient être impliquées dans ces échanges. Différentes études ont montré une augmentation des MV dérivées des CE, des plaquettes, des LT et des monocytes dans le sérum des patients (615,616). Ces MV induisent une majoration de l'activation des polynucléaires neutrophiles comparés aux MV issues de sujets sains, suggérant leur implication éventuelle dans les lésions microvasculaires et l'inflammation. Cet effet est associé à une expression différentielle du facteur de transcription HMGB1 (high–mobility group box 1) au sein des MV (617). Cependant, les patients qui souffrent d'ulcères digitaux ont des quantités plus faibles d'EVs circulantes suggérant un transfert d'EVs du sérum vers la peau (615). D'ailleurs, une autre étude a démontré qu'en comparaison à des sujets sains, l'expression de marqueurs endosomaux (CD9, CD63, CD81) est augmentée dans la peau des patients alors que la quantité d'exosomes dans le sérum de patients souffrant d'atteintes vasculaires (ulcères digitaux par exemple) est diminuée (618), suggérant ici aussi un transfert d'exosomes du sérum vers la peau. Enfin, l'étude des exosomes isolés du sérum de patients sclérodermiques montre une dérégulation de leur contenu en microARNs, notamment pro- et anti-fibrotiques. Ces exosomes induiraient l'acquisition d'un phénotype sclérodermique par des fibroblastes sains *in vitro* (619) et l'implication physiopathologique au cours de la ScS de certains microARNs contenus dans ces exosomes a été démontrée par ailleurs (423,620). L'origine tissulaire des exosomes présents dans le sérum des patients ScS n'est toutefois pas déterminée.

b) *Effet thérapeutique des EVs de CSMs au cours de la ScS*

Concernant le rôle thérapeutique potentiel des EVs libérées par les CSMs au cours de la ScS, il n'y avait aucune donnée disponible au démarrage de ma thèse. Mais leurs propriétés anti-fibrotiques, pro-angiogéniques et immunorégulatrices ont été démontrées par ailleurs, ce qui laisse penser qu'elles peuvent cibler les 3 axes principaux de dysfonctionnement de la maladie, constituant un espoir thérapeutique, tout comme leurs cellules mères, les CSMs, l'ont prouvé. Depuis, une étude a été publiée et les auteurs montrent que les CSMs améliorent l'ostéopénie de souris atteintes de ScS dans le modèle Tight Skin 1. Ils observent *in vitro* que les CSMs de donneurs transfèrent miR-151-5p aux CSMs des souris malades, grâce à des EVs et que ce microARN serait responsable de l'effet thérapeutique sur l'ostéopénie des souris. Aucune donnée n'est cependant rapportée sur les atteintes princeps de la ScS, que sont la fibrose cutanée, la fibrose pulmonaire, la vasculopathie et les signes d'auto-immunité.

HYPOTHESE DE TRAVAIL-OBJECTIFS

L'hypothèse principale de mon travail de thèse est que les EVs isolées à partir de CSMs peuvent reproduire l'effet thérapeutique des cellules parentales dans le traitement de la ScS. L'hypothèse secondaire est que les CSMs de patients et leurs EVs, peuvent perdre en partie leur capacité thérapeutique. Pour y répondre, mon projet s'est organisé autour de deux objectifs.

Le premier était d'évaluer l'effet thérapeutique des EVs isolées à partir de CSMs saines.
Dans ce premier axe de travail, j'ai d'abord évalué, dans le modèle murin de ScS induit par l'HOCl, l'effet thérapeutique des EVs isolées à partir de cellules murines et humaines ainsi que le rôle de miR-29a dans cet effet thérapeutique (article original 1 soumis).

Puis, j'ai souhaité améliorer l'efficacité thérapeutique des EVs, notamment en majorant la dose injectée ou en modifiant l'environnement dans lequel les EVs étaient produites (article original 2 en préparation).

Le deuxième objectif était d'évaluer *in vitro* l'effet thérapeutique anti-fibrotique et immunosuppresseur de CSMs de patients sclérodermiques et de leurs EVs (article original 3 en préparation).
Dans ce deuxième axe de travail, j'ai d'abord mis au point un modèle *in vitro* de fibroblastes malades à partir de fibroblastes sains stimulés par le TGFβ1 du fait de la rareté des cellules provenant de patients. Ensuite, j'ai comparé ce modèle de fibroblastes sains stimulés par le TGFβ1 à des fibroblastes de patients atteints de ScS. Enfin, j'ai étudié l'effet thérapeutique de CSMs (saines ou sclérodermiques) et de leurs EVs dans ce modèle *in vitro.* En parallèle, nous avons évalué les propriétés immunosuppressives des CSMs dans un modèle de coculture avec des PBMCs activés.

TRAVAUX PERSONNELS

1ère partie : Effet thérapeutique des vésicules extra-cellulaires provenant de cellules stromales mésenchymateuses dans un modèle murin de sclérodermie systémique : rôle de miR-29a.

Dans cette première partie de mon travail de thèse, nous avons voulu évaluer l'effet thérapeutique de EVs produites par des CSMs dans le modèle murin de ScS induite par l'HOCl et comprendre leur mécanisme d'action.

La première étape fut de reproduire le protocole de séparation et de concentration d' EVs produites par des CSMs murines qui avait été mis en place dans le groupe. Brièvement, nous collectons les EVs à l'aide de différentes ultracentrifugations pour séparer d'un côté, les grosses EVs après une ultracentrifugation à 18 000g pendant 1 heure et de l'autre, les petites EVs après une ultracentrifugation à 100 000g pendant 2 heures. Nous caractérisons ces EVs par leur taille et leur morphologie à l'aide d'une analyse au NTA et au cryoTEM ainsi que par leurs marqueurs de surface en cytométrie de flux. De même, nous nous sommes assurés de l'enrichissement en exosomes dans la population de petites EVs par la mise en évidence de marqueurs endosomaux en western blot.

Puis, nous avons reproduit le modèle murin à l'aide d'injections quotidiennes intradermiques d'HOCl, durant 6 semaines. Les souris ont bénéficié de leur traitement au 21ème jour après le début de l'induction de la maladie, dans une approche curative, à l'instar de ce qui avait été démontré précédemment par une injection systémique de 250 000 CSMs (621). D'ailleurs, nous avons choisi de traiter les souris avec la quantité d'EVs produites par 250 000 CSMs durant 48h de culture. Nous avons comparé l'effet de petites EVs à celui de grandes EVs produites par des CSMs provenant de moelle osseuse de souris et démontré qu'elles étaient tout aussi efficaces que les CSMs, pour stopper l'évolution de la maladie au niveau clinique, mais aussi à l'échelle histologique et moléculaire sur les prélèvements de peaux et de poumons.

Nous souhaitions également élucider les mécanismes d'action impliqués dans l'effet thérapeutique des CSMs et de leurs EVs, au cours de la ScS. Nous nous sommes tout particulièrement intéressés au rôle de miR-29a connu par ailleurs pour ses propriétés anti-fibrotiques et pour être présent dans les CSMs humaines. Nous avons d'abord évalué l'effet thérapeutique de CSMs murines qui sur-exprimaient miR-29a après transfection d'un pré-miR29a. Tous les groupes de souris traitées (CSMs non transfectées, CSMs transfectées par un pré-miR contrôle ou CSMs transfectées par un pré-miR-29a) ont vu leur maladie s'améliorer après le traitement, mais la sur-expression de plus de 300 fois de miR-29a n'augmentait pas l'effet thérapeutique des CSMs dans ce modèle. Nous avons, par la suite, évalué l'effet thérapeutique de CSMs murines dont l'expression de miR-29a était diminuée. Grâce à la transfection par un antagomiR-29a, nous obtenions 80% d'inhibition de l'expression de miR-29a dans les CSMs murines et cette expression plus faible de miR-29a est associée à la perte d'efficacité thérapeutique dans le modèle de ScS. Par la suite, nous avons mis en évidence miR-29a au sein des petites et des grandes EVs produites par les CSMs murines. L'effet de la transfection de ces CSMs par un antagomiR-29a ne persistait malheureusement pas au-delà de 24h après l'arrêt de la transfection. Or, les CSMs étant maintenues en culture 48 heures dans un milieu de production avant de collecter le surnageant, il était probable que l'effet de la diminution d'expression de miR-29a soit indétectable dans les EVs.

Nous avons alors travaillé avec des CSMs humaines d'origine adipocytaire (ASCs) dont le temps de doublement est plus long, ce qui permet à l'effet de la transfection de persister pendant 48 heures dans les cellules. Par ailleurs, les ASCs ont montré leur efficacité clinique dans le modèle murin de ScS (622). Nous avons donc mis au point les méthodes de concentration des EVs produites par les ASCs et caractériser ces EVs. Nous avons travaillé avec un pool d'EVs totales collectées après deux ultracentrifugations à 100 000 g durant 2 heures, n'ayant pas montré de différence d'efficacité entre les petites et les grandes EVs précédemment. Puis nous avons montré que l'injection systémique d'EVs isolées à partir d'ASCs, injectées à l'état frais ou congelé chez les souris sclérodermiques, stoppent l'évolution de la maladie. Après la transfection d'ASCs par un antagomiR-29a, les EVs isolées présentent une diminution d'expression de miR-29a et perdent en partie leur efficacité thérapeutique dans le modèle murin de ScS. Enfin, nous avons montré une corrélation inverse entre le taux d'expression de miR-29a dans les EVs et plusieurs de ses cibles dans la peau des souris sclérodermiques : collagène 1, Timp1, Pdgfr-bb, Dnmt3a ou encore Bcl2, Bax et Bclxl.

Au total, ce travail a permis de montrer pour la première fois dans un modèle systémique de SSc que les EVs isolées à partir de CSMs murines ou d'ASC humaines étaient tout aussi efficaces que les cellules sources pour stopper l'évolution de la maladie. L'effet thérapeutique des CSMs et de leurs EVs est médié par la molécule anti-fibrotique miR-29a, qui agit sur différentes cibles au niveau de la peau des souris. Ainsi, miR-29a agit probablement en favorisant l'apoptose au niveau des tissus fibrotiques, en modulant l'expression de gènes dérégulés épigénétiquement dans ces tissus, grâce à la diminution de la méthylase Dnmt3a et en réprimant Pdgfr-bb, facteur activateur d'une voie de signalisation pro-fibrotique.

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Mesenchymal stromal cells-derived extracellular vesicles alleviate murine systemic sclerosis via miR-29a-3p and regulation of DNMT3A and PDGFRBB.

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Running title: MSC-EV delivered miR-29a-3p alleviates SSc

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ABSTRACT

Objective. Systemic sclerosis (SSc) is a rare and potentially lethal disease with no curative treatment in current practice. Among the therapeutic strategies under investigation, mesenchymal stromal cells (MSCs) have proved efficacy in pre-clinical models of SSc. Here, we investigated the therapeutic interest of extracellular vesicles (EVs) isolated from MSCs in alleviating SSc clinical symptoms.

Methods. Small size (ssEVs) and large size EVs (lsEVs) were isolated from murine MSCs and total EVs from human adipose tissue-derived MSCs (ASCs). EVs were injected in the HOCl-induced systemic model of scleroderma (SSc) at day 21 and histological and molecular analysis was performed at day 42. Control antagomiR (Ct) or antagomiR-29a (A29a) were transfected in MSCs and in ASCs before EV production.

Results. SsEVs and lsEVs were as effective as the parental MSCs to slow-down the course of the disease. Anatomopathological features as well as markers of fibrosis, remodeling and inflammation were improved in skin and lungs. Down-regulation of miR-29a-3p in MSCs totally abolished their therapeutic efficacy. Furthermore, ASCs and ASC-EVs were shown to efficiently reduce clinical signs while ASC-EVs expressing lower amounts of miR-29a-3p did not. We identified Dnmt3a, Pdgfrbb, Bcl2, Bcl-xl as target genes whose regulation in fibrotic skin was associated with SSc improvement.

Conclusion. Our study highlights the therapeutic role of miR-29a-3p in SSc and revealed the importance of regulating methylation status and apoptosis in targeted tissues in the perspective of novel therapeutic options for human SSc.

Keywords: exosomes, extracellular vesicles, scleroderma, mesenchymal stem cells, miR-29a, DNMT3A, PDGFRBB

INTRODUCTION

Systemic sclerosis (SSc) is a rare, life-threatening autoimmune disease for which no curative treatment is available to date (1). Current treatments are essentially palliative and associate symptomatic support with organ transplantation or immunosuppressive drug therapy, which may cause severe side effects (2). Hematopoietic stem cell transplantation has proved to be a major step forward, but here again, at the expense of heavy side effects (3). Promising therapeutic options include mesenchymal stromal/stem cell (MSC)-based therapy, which has shown efficacy in several animal models of SSc. The therapeutic interest of bone marrow- and adipose tissue-derived MSCs has already been demonstrated in murine models (4, 5). Moreover, clinical studies report encouraging results (for review, see (5)).

The therapeutic effect of MSCs is mediated primarily through the secretion of soluble factors that can be conveyed within extracellular vesicles (EVs). EVs are classified into three major subtypes depending on their size and biogenesis. Apoptotic bodies are the largest vesicles (500-1500 nm) released by apoptotic cells, microvesicles/microparticles are large vesicles (120-500 nm) budding from the plasma membrane and exosomes are small vesicles (50-150 nm) originating from the endosomal compartment (6). However, because of overlapping sizes between subtypes, current isolation procedures generally lead to the isolation of small size vesicles (ssEVs) or large size vesicles (lsEVs) that cannot discriminate the vesicles by their origin (7). EVs are carriers of a variety of bioactive factors including proteins, lipids, DNA, mRNA and miRNAs that play important functions in intercellular communication. Recently, the therapeutic function of MSC-derived EVs (MSC-EVs) has gained much attention in various diseases, including autoimmune and rheumatic diseases (8), and in various animal models of diseases (for review, see (9)). However, to our knowledge, no data is available on the therapeutic function of MSC-EVs for skin fibrosis and more importantly, in a multi-organ fibrotic disorder such as SSc.

Among the mediators conveyed within MSC-EVs, miRNAs are reported to take a large part in their therapeutic effect. Numerous miRNAs could have functional relevance for fibrotic diseases by modulating the profile of mRNAs and consequently, proteins in target cells (for review, see (9)). One of these miRNAs, miR-29a gained interest in the context of scleroderma. Indeed, miR-29a was shown to be decreased in hair, serum and dermal fibroblasts from scleroderma patients (10-12). It was strongly down-regulated in fibroblasts and skin sections from SSc patients as well as in the bleomycin model of skin fibrosis and inverse correlation between miR-29a and type I collagen was demonstrated (13). In addition to type I collagen, miR-29a was found to target TGFβ activated kinase 1 binding protein 1 (TAB1), thereby reducing TIMP1 expression and increasing matrix metalloproteinase 1 (MMP1) (14, 15). Moreover, miR-29a has been described as a potent inducer of apoptosis in SSc fibroblasts by disrupting the production of anti-apoptotic factors (Bcl-2, Bcl-XL) (16). Therefore, miR-29a has been described as an anti-fibrotic and pro-apoptotic factor with potential therapeutic interest in SSc.

In the present study, we investigated the capacity of MSC-EVs to improve skin and lung fibrosis in a systemic fibrotic disorder, using the relevant HOCl-induced murine model of SSc. We determined the respective role of ssEVs and lsEVs from murine MSCs and that of EVs from human adipose tissue derived MSCs (ASC-EVs) in attenuating SSc clinical course. We focused our study on miR-29a as a potential anti-fibrotic factor conveyed within EVs, and its downstream targets to decipher its mechanism of action in SSc.

METHODS

Murine and human cell culture. Murine MSCs were isolated from the bone marrow of C57BL/6 mice and expanded in proliferative medium (DMEM, 10% fetal calf serum (FCS), 2 mmol/mL glutamine, 100 µg/mL penicillin/streptomycin). They were characterized by phenotyping and tri-lineage differentiation potential, as reported (17) and used at passage 12-18. Human ASCs were isolated from adipose tissue. All patients gave written consent *and a*pproval was obtained from the French Ministry of Higher Education and Research (DC-2009-1052). ASCs were cultured in αMEM medium containing

10% FCS, 100 µg/mL penicillin/streptomycin, 2 mmol/mL glutamine and 1 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems), characterized as in (18) and used at passage 1-3.

Cell Transfection. MSCs and ASCs were transfected with 50 nM of antagomiR-control (ACt) or antagomiR-29a-3p (A29a) overnight (ON), using Oligofectamine (ThermoFisher Scientific). Supernatants were collected 48h after transfection.

Production and isolation of EVs. MSCs and ASCs were seeded at $7x10^4$ cells/cm^2 and cultured for 24 hours. The medium was then replaced by the production medium consisting of DMEM or αMEM, for MSCs and ASCs respectively, containing 100 µg/mL penicillin/streptomycin, 2 mmol/mL glutamine and 3% EV-free FCS. This latter was obtained after ultracentrifugation of medium containing 20% FCS at 100,000g, ON. After 48h for MSCs or 60h for ASCs, conditioned supernatants were centrifuged at 300 g, 4°C for 10 min to eliminate cells and 2,500 g, 4°C for 25 min to remove debris and apoptotic bodies. Subsequently, media were centrifuged at 18,000 g, 4°C for 1 h to pellet lsEVs and, then at 100,000 g for 2h to pellet ssEVs. Pellets obtained at each step were submitted to a second round of centrifugation at 100,000g, 4°C for 2h and suspended in 100 µL of phosphate buffer saline (PBS). More than 95% viability and less than 15% apoptosis of producing cells, assessed by Annexin V/7AAD labelling and flow cytometry, were required before EV recovery. EV preparations were kept at 4°C ON and used as freshly prepared EV suspensions. Frozen EVs were kept at -80°C before use.

EV characterization. EVs were characterized as recommended by the International Society of Extracellular Vesicles (ISEV)(7). Total protein content of EV suspensions was quantified using the Micro BCA Protein Assay Kit (ThermoFisher Scientific). Number and size of particles were determined by nanotracking analysis (NanoSight NS300, Orsay). EV phenotype was determined by flow cytometry after EV incubation with 1 µL of aldehyde/sulfate latex beads (4 µm, 4% W/V, ThermoFisher Scientific) at 4°C ON and glycine (100 mM) addition for 30 min. EV-coated beads were labelled with fluorophore-

conjugated antibodies for murine CD9, CD11b, CD29, CD44, CD45, CD63, CD81, Sca-1 (BD Biosciences) for 30 min. For cryoTEM analysis, EVs were applied to glow discharged Lacey grid (Ted Pella inc.), blotted for 1s and then flash frozen in liquid ethane using a CP3 cryo-plunge (Gatan inc.). Cryo-EM was carried out on a JEOL 2200FS FEG operating at 200 kV under low-dose conditions in the zero-energy-loss mode with a slit width of 20 eV.

Western blot. Samples were lysed in 50 µL of RIPA containing 1/100 protease inhibitor (Sigma). Ten µg of proteins were loaded on 4-12% SDS polyacrylamadide gel (Life Technologies) and run at 200 V for 30 min. Protein transfer to nitrocellulose membrane was performed for 7 min using iBlot^{TM}2 Dry blotting system (ThermoFisher Scientific). Membranes were blocked with 5% skim milk in TBS and incubated ON at 4°C with primary antibodies: anti-Tsg101 (1/200), anti-Alix (1/100), anti-ApoA1 (1/200) from Santa Cruz and at room temperature for 2 h with anti-Actin (1/5000; Sigma). Blots were revealed using WesternBright Sirius HRP substrate (Advansta) and quantified with ChemiDoc MP imager and Image Lab Software (Bio-Rad).

Animal experimentation. HOCl-induced SSc murine model was approved by the Regional Ethics Committee on Animal Experimentation (APAFIS#535120160509190791 87). Six-week-old female BALB/cJRj mice (Janvier Labs) were cared for according to the European guidelines for the care and use of laboratory animals (2010/63/UE). Surgery and euthanasia were performed after anaesthesia with isoflurane gas, and all efforts were made to minimize suffering. Mice were housed in solid bottomed plastic cages in quiet rooms at 22° ± 1°C, 60% controlled humidity, and 12h/12h light/dark cycle. Animals were used after an adaptation period of 7 days and had free access to tap water and standard pelleted chow. Backs of mice were shaved the day before disease induction. Skin thickness was measured with a caliper and once a week thereafter. HOCl solutions were prepared extemporaneously by adding NaClO (9.6%) to KH2PO4 solution (100 mM, pH 6.2) to get the desired HOCl concentration, defined by optical density of 0.7-0.9 at 292 nm (Nanodrop, Thermoscientific). Daily prepared HOCl

(150 µL) was injected into two sites in the back of mice, 5 days a week for 6 weeks. Treatment groups were done at day 21 to homogenize the mean skin thickness in each group of 7-8 mice before NaCl (control group), cell or EV intravenous injections (100 µL). Animal number in each group was calculated using G Power software and data from previous experiments; no criteria for exclusion/inclusion was set and none was excluded. Treatment groups were mixed in cages to allow blinding and minimize bias. At day 42, mice were sacrificed and skin biopsies (6 mm punchs) and lungs were recovered. Samples were stored at −80°C for RT-qPCR or fixed in 4% formaldehyde for histology.

Histopathological and immunohistopathological analyses. Skin and lung samples were processed for routine histology. Masson trichrome staining or immunostaining were performed using antibodies specific for αSma (1/100; Abcam), TGFβ (1/200; Abcam) or DAPI (1/1000; Sigma). Histological slides were scanned with Nanozoomer (Hamamatsu) and dermal thickness was measured with NDP.view2 software. Images of immunostaining slides were acquired on a confocal laser scanning microscope (TCS SP5-II, Leica Microsystems). Image stacks were analyzed with Image J software.

RNA extraction and RT-qPCR. Total RNA was extracted from skin and lung samples after mechanical dissociation and using the RNeasy Mini Kit (Qiagen, Courtaboeuf). Reverse transcription was performed on 0.5 µg RNA using 100 U of M-MLV reverse transcriptase (ThermoFisher Scientific). Real time PCR was performed on 20 ng cDNA using specific primers (Supl. Table) and SYBR Green I Master mix (Roche Diagnostics). Values were normalized to TATA binding protein (*Tbp*) housekeeping gene and expressed as relative expression or fold change using the respective formulae $2^{-\Delta CT}$ or $2^{-\Delta\Delta Ct}$.

MiRNA extraction was performed using the miRNeasy Micro Kit (Qiagen). Reverse transcription was performed on 150 ng RNA using TaqMan® MicroRNA Reverse Transcription Kit (ThermoFisher Scientific). Real time PCR was performed on 10 ng cDNA using Taqman microRNA hsa-miR29a-3p (Life Technologies) and Taqman Master Mix II no UNG (Life Technologies). Values were normalized to Ct for MSCs, ASCs and EVs.

Statistical analysis. Statistical analyses were performed using GraphPad 8 Prism Software. Data were analyzed by comparing a single group with the control group using the Student t-test for values with a normal distribution or the Mann-Whitney test when values did not show normal distribution, as evaluated using the Shapiro-Wilk normality test. For normalized values, a one sample t-test or Wilcoxon test were performed when values were parametric or non-parametric, respectively. Data are presented as mean ± SEM.

RESULTS

Both MSC-EV subtypes stop disease progression in murine HOCl-induced SSc. Before investigating the therapeutic effect of MSC-EVs in SSc, we first characterized the two EV subtypes recovered by differential ultra-centrifugation. SsEVs with a mean size of 158 nm were isolated at 100,000g while a more heterogenous population of lsEVs with a mean size of 191 nm was isolated at 18,000g (Fig. 1A). The median size of ssEVs (145 nm) was significantly lower than that of lsEVs (165 nm). The number of particles produced by MSCs was significantly higher for ssEVs than lsEVs (1.5-2 fold more) when reported to 10^6 cells or 1 µg of total proteins (Fig. 1A). The two subtypes were characterized by a bilayer membrane as visualized by cryoTEM (Fig. 1B). They were negative for hematopoietic markers (CD11b, CD45), positive for MSC markers (CD29, Sca-1, CD44) and markers of the endosomal compartment (CD9, CD63, CD81). All endosomal markers were expressed at higher levels on ssEVs (Fig. 1C). Western blot analysis revealed absence of ApoA1, indicative of low contamination by serum proteins while expression of Alix and Tsg101 was detected with ssEVs and only Alix with lsEVs (Fig. 1D-E). These data support that ssEV and lsEV populations released by MSCs exhibit slightly different characteristics.

We then compared the course of HOCl-induced SSc between a group of control mice and three groups that were treated with $2.5x10^5$ MSCs or 250 ng of ssEVs or lsEVs (the approximate quantity of particles produced by $2.5x10^5$ MSCs for 48 h). Skin thickness increase was stopped and thickness was significantly lower in all treated groups than in control group at sacrifice (Fig. 2A). Clinical data were

confirmed by histology as shown by representative skin sections and dermal thickness measures that were significantly lower in all treated groups (Fig. 2B-C). Mice treated with ssEVs exhibited less severe involvement with lower dermal thickness than other treated groups. Immunohistological analysis revealed lower cellularity and lower expression of *Tgfβ1* and *αSma* (Fig 2C). At the molecular level, expression of several fibrotic (*Col1a1, Tgfβ1, TgfβR2, αSma*) or inflammatory (*IL1β*, *Tnfα*) markers was significantly decreased and markers of remodeling (*Mmp9, Mmp1, Timp1*) were improved (Fig. 2D). In the lungs, cellular infiltrates were smaller and low amounts of fibrotic tissue were observed in the pulmonary parenchyma, regardless of treatment (Fig. 2E). The molecular analysis confirmed improvement with lower expression of fibrotic, inflammatory and remodeling markers (Fig. 2F). Altogether, the data indicated that both types of EVs attenuated disease symptoms.

miR-29a-3p is responsible for the therapeutic effect of MSCs in SSc. The next step was to investigate the role of miR-29a in the therapeutic effect of MSCs. The more predominant mature form of miR-29a is miR-29a-3p (19). We therefore transfected MSCs with premiR-29a-3p and showed that transfected MSCs expressed 300-fold more miR-29a-3p than control MSCs. However, the therapeutic effect of miR-29a-3p-over-expressing MSCs was similar to control MSCs in the model of HOCl-induced SSc (data not shown). We therefore down-regulated miR-29a-3p in MSCs using antagomiR-29a-3p (MSC-A29a). Expression of miR-29a-3p was down-deregulated by 80% as compared to MSCs transfected with control antagomiR (MSC-ACt) at day 0 (Fig. 3A). Interestingly, injection of MSC-A29a did not stop disease progression, by contrast to MSC-ACt or non-transfected MSC (MSC-NT) (Fig. 3B). Consistently, skin thickness at sacrifice was lower in the MSC-NT and MSC-ACt groups than in control HOCl group but similar in the MSC-A29a and control groups. Similar observations were obtained by histological analysis of skin sections and measures of dermal thickness (Fig. 3C-D). At the molecular level, expression of fibrotic, remodeling and inflammatory markers was not improved in MSC-A29a group compared to HOCl control group, except for *Col3a1* and the *TGFβRII/TGFβ1* and *Mmp1/Timp1* ratios (Fig. 3E). Moreover, all markers were significantly worsened in MSC-A29a group compared to

MSC-ACt group. High expression of *Tgfβ1* and *αSma* proteins was detected in skin sections from MSC-A29a and control groups while low expression was detected in MSC-ACt and MSC-NT groups (Fig. 3F). Altogether, our results demonstrated that miR-29a-3p mediates the therapeutic effect of MSCs in SSc skins.

Human ASC-EVs improve SSc clinical signs and convey miR-29a-3p. We thereafter investigated the possibility that miR-29a-3p could be delivered by EVs that are released by MSCs. We detected miR-29a-3p in both ssEVs and lsEVs isolated from MSCs (Fig. 4A). To explore the role of miR-29a-3p in MSC-EVs, EVs were recovered from two days-conditioned supernatants of MSC-A29a. Unfortunately, similar levels of miR-29a-3p were quantified in MSC-A29a and MSC-ACt (data not shown). This was attributed to the dilution of transfected A29a in the highly proliferating MSCs. We therefore decided to use human ASCs, which display a lower proliferative rate. ASCs expressed miR-29a-3p at similar levels as murine MSCs and total EVs isolated from ASCs also contained miR-29a-3p (Fig. 4A). Importantly, after transfection of antagomiR-29a-3p in ASCs (ASC-A29a), expression of miR-29a-3p was inhibited by 80% at day 0 and by 50% at day 2. We therefore isolated total EVs from ASCs using the same conditions as those used for MSC-EVs but suppressing the 18,000g step. ASC-EVs displayed a heterogeneous size profile with a median and modal size of 129.2 ± 0.88 nm and 106.9 ± 10.9 nm, respectively (Fig. 4B). Production of ASC-EVs was estimated at 307 ± 15 particles/ASC and 1.74 particles/µg total proteins. Particles with a bilayer membrane were observed by CryoTEM (Fig. 4C). ASC-EVs expressed markers of endosomal compartment (CD9, CD63, CD81) and parental cells (CD29, CD44, CD73, CD90) and did not express CD45 or CD11b confirming the phenotype of ASC-EVs (data not shown).

We then evaluated the efficacy of ASC-EVs in the SSc model. ASC-EVs (4×10^7 particles) were injected either as freshly isolated suspension (EV-fresh) or as thawed suspension after preservation at -80°C (EV-frozen). This amount of particles was the quantity produced by 2.5×10^5 ASCs during 48h. Both EV-fresh and EV-frozen efficiently stopped disease progression and reduced skin thickness as measured at sacrifice (Fig. 4D). They also reduced fibrosis as shown on histological sections and

improved markers of fibrosis, remodeling and inflammation (Fig. 4E-F). The data indicated that ASC-EVs were efficient to attenuate SSc symptoms and short-term preservation of ASC-EVs at -80°C did not alter their functional properties.

MiR-29a-3p delivered by ASC-EVs contributes to their therapeutic effect. EVs were then recovered from ASCs transfected with antagomiR-29a-3p (EV-A29a). They contained lower levels of miR-29a-3p (78% less) than EVs from antagomiR-control ASCs (EV-ACt) (Fig. 5A). In SSc, EV-A29a did not reverse the disease course and did not prevent the skin thickness increase as measured at day 42 (Fig. 5B-C). Higher collagen deposition and fibrotic lesions were observed on skin histological samples in the EV-A29a group compared to EV-ACt (Fig. 5D). In accordance, expression levels of *TGFβ1, αSma, Mmp9*, *IL1β* did not improve and expression of all markers was higher in EV-A29a group compared to EV-ACt group, even though not all reached statistical significance (Fig. 5E). In lungs, expression of *αSma* and *TGFβ1* was significantly higher in EV-A29a treated mice (Fig. 5F).

Finally, we focused on the potential mechanism of action of miR-29a-3p in SSc fibrotic tissues and investigated the expression of already validated targets. We confirmed an increased expression of *Col1a1, Col3a1* and of *Mmp9, Timp1* in the skin of SSc mice treated with MSC-A29a and EV-29a (Fig. 3E; 5E). We also detected that anti-apoptotic markers *Bcl2* and *Bcl-XL* were significantly up-regulated while the pro-apoptotic marker *Bax* was not significantly modulated (Fig. 6A). In addition, we found out that the DNA demethylase Ten-Eleven translocation methylcytosinedioxygenase 1 (*Tet1*), the DNA methyltransferase 3A (*Dnmt3a*) and platelet-derived growth factor receptor B (*Pdgfrbb*) were significantly down-regulated in MSC-NT, MSC-Act and EV-ACT-treated mice (Fig. 6B). By contrast, these factors were expressed at higher levels in skin from MSC-A29a and EV-29a treated mice compared to MSC-ACt or EV-ACT-treated mice. These data argued that miR-29a-3p mediates in a large part the therapeutic effect of ASC-EVs in the murine model of SSc by modulating the expression of multiple markers that are deregulated in SSc.

DISCUSSION

This study reports the first demonstration that EVs isolated from murine MSCs and human ASCs exert a therapeutic effect in a relevant systemic model of scleroderma, by halting the clinical symptoms, limiting collagen fiber accumulation in skin and lungs as well as improving the molecular markers of fibrosis, remodeling and inflammation. We also provide evidence that miR-29a-3p is one key mediator, responsible for EV-mediated improvement of SSc by targeting several genes deregulated during the course of the disease.

Previous studies have reported that either total EVs or ssEVs, frequently called exosomes, can reduce fibrosis in different organs (for review, see (9)). Nevertheless, few studies have investigated the therapeutic role of EVs in SSc, most of them focusing on EVs as diagnosis and follow-up tools (for review, see (20)). They reported an increased secretion of exosomes by SSc dermal fibroblasts and a higher number of particles in the serum of SSc patients, even though patients with cutaneous ulcers exhibited a significantly reduced number of vesicles (21). A possible role of EVs in the pathogenesis of the disease was therefore suggested. After internalization by target cells, EVs might induce a pro-fibrotic molecular program and be involved in the disease propagation/maintenance (22). However, only one study has reported the therapeutic interest of EVs in SSc. Indeed, exosomes isolated from bone marrow-derived MSCs were shown to rescue the osteopenic phenotype of tight-skin (*Tsk/+*) mutant mice, a relevant genetic murine model of SSc, characterized by osteopenia and skin fibrosis, through the transfer of miR-151-5p (23). Our data are in line with their findings although the authors did not test the direct effect of exosomes on skin and lung fibrosis but reported the beneficial role of adenovirally expressed miR-151-5p on these organs. Here, we showed that both ssEV and lsEV subtypes were equally efficient to protect mice from skin and lung fibrosis through the modulation of fibrotic, remodeling and inflammatory genes.

We also found that total EVs from human ASCs exert similar therapeutic effect as murine EV subtypes. Similar findings were previously reported comparing murine BM-MSCs and human ASCs or BM-MSCs in the HOCl-induced murine model of SSc (4). This suggests similar mechanisms of action

exerted by MSCs from the two species and mainly through the release of EVs. Of note, both freshly prepared and frozen EVs were efficient to stop the course of the disease. This result was of interest for manufacturing and clinical application of EVs because we previously showed that cryopreservation of MSC-EVs impacts their immunosuppressive properties (24). We found that the median size of frozen EVs was lower compared to fresh EVs suggesting a loss of the largest size EVs and/or destabilization and reorganization of EV structure (24). Formation of multilamellar EV structures was clearly identified by cryoTEM analysis (data not shown) and impact of storage-induced changes on bioactivity still remains unclear (25). Upon freezing-thawing of EV suspensions, proteins can be released either after leakage or membrane shedding, which can lead to altered bioactivity of EVs (26). Bioactivity of manufactured cryopreserved EV products will need to be checked on the long term and the HOCL-induced SSc model is a reliable model for evaluation in this therapeutic indication.

Several miRNAs whose expression are altered in SSc have been proposed to be implicated in the regulation of fibrotic genes (20). Of those, the expression of let-7a and miR-29a was shown to be down-regulated in fibroblasts or skin of SSc patients while miR-146a downregulation was detected in the peripheral blood mononuclear cells of patients (13, 27, 28). Both let-7a and miR-29a down-regulate the expression of type I and type III collagens and miR-29a was further shown to target TAB1 resulting in increased expression of MMP1 and reduced expression of TIMP1 (13, 14, 27). All these factors were modulated in skin and lungs of mice treated with EVs, we therefore focused our attention on miR-29a-3p (the mature form of miR-29a). We here demonstrated its key role in the therapeutic effect of MSCs in the HOCl-induced SSc model through its release within EVs. In line with previous studies, we showed that down-regulation of miR-29a-3p in MSCs and in ASC-EVs resulted in higher levels of type I and type III collagens and a lower MMP1/TIMP1 ratio in the skin of SSc mice. Therefore, miR-29a-3p appears as a major factor involved in the regulatory function of ASC-EVs in SSc.

Other targets of miR-29a-3p have been shown to be modulated in different models of fibrosis. Indeed, an inverse correlation between miR-29a-3p and MMP9 has been demonstrated in patients with lumbar spinal stenosis and MMP2 was found to be a direct target of miR-29a-3p in an

experimental model of renal fibrosis (29, 30). MiR-29a is also a pro-apoptotic factor in SSc fibroblasts by disrupting the production of anti-apoptotic factors (Bcl2, BclXL) (16). Another direct target of miR-29a-3p is Tet1, a DNA demethylase, which is deregulated in the transformation of vascular smooth muscle cells (31) and in SSc (32). The expression of Tet1 was higher in SSc fibroblasts and increased with hypoxia, a common characteristic of SSc. Actually, global DNA hypomethylation was observed in skin suggesting an aberrant DNA methylation in the pathogenesis of SSc. Another methylase, Dnmt3A, is also targeted by miR-29a-3p (33). In the fibrotic skin of SSc patients, the induction of Dnmt3A by TGFβ leads to the silencing of suppressor of cytokine signaling 3 (Socs3) by promoter hypermethylation and the downregulation of Socs3 promotes the fibroblast-to-myofibroblast transition and fibrosis (34). Finally, miR-29a-3p was reported to target Pdgfrbb, fibrillin 1, follistatin-like 1, laminin subunit gamma 2 and other genes of the basement membrane including type 4 collagen, perlecan and osteonectin in other disease models (35-37). Of interest, Sunitinib, an inhibitor of Pdgfrbb and vascular endothelial growth factor receptor (Vegfr) phosphorylation, reverses fibrosis in the HOCl-induced model of SSc (38). Here, we demonstrated for the first time an inverse correlation between EV-mediated miR-29a-3p treatment and expression of *Tet1, Dnmt3a*, *Pdgfrbb,* in the skin of SSc mice. These data indicate that miR-29a-3p plays a critical role in regulating a variety of cellular processes and balancing numerous factors whose expression is altered in SSc.

In conclusion, the present study presents evidence that MSCs can regulate skin and lung fibrosis in SSc through the release of EVs that exert pleiotropic functions acting on different molecular pathways that are deregulated in the disease. One of the mechanisms of action displayed by MSC- and ASC-EVs is the release of miR-29a-3p that can downregulate the expression of several pro-fibrotic, remodeling and anti-apoptotic factors as well as demethylases. This study highlights the role of miR-29a-3p in the murine HOCl-induced SSc and encourages research focusing on this miRNA and its targeted genes, especially *Dnmt3a* and *Pdgfrbb*, in the perspective of novel therapeutic options for human SSc.

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AUTHOR CONTRIBUTIONS

Study design: DN, PG, CJ; Experimental work and data analysis: PR, MM, AM, KT, JL, DN; Writing original draft: PR, DN; Manuscript revision and final approval: PR, MM, AM, KT, JL, CJ, PG, DN

COMPETING INTERESTS

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Characterization of extracellular vesicles isolated from murine mesenchymal stromal cells. (A) Size distribution of small size and large size extracellular vesicles (ssEVs and lsEVs) assessed by NanoTracking Analysis. EV concentration is expressed as particle numbers produced by 10^6 cells or per µg of total proteins. Mean and median sizes of EVs are indicated (n=3). (B) Cryo-transmission electron microscopy images of ssEVs and lsEVs (scale bar, 200 µm). (C) Immunophenotyping of ssEVs (red), lsEVs (blue) and isotype control (black). Expression of markers from parental cells (CD29, Sca-1, CD44), hematopoietic cells (CD11b) and exosomes (CD9, CD63, CD81) is analyzed by flow cytometry. (D) Western blot analysis of endosomal (Alix, Tsg101) and serum (ApoA1) markers in ssEV and lsEV protein extracts. (E) Quantification of proteins shown in (D). *: $p<0.05$

Figure 2. Therapeutic effect of MSC-EVs in the murine model of HOCl-induced SSc. (A) Skin thickness increase in control mice (HOCl) and mice that have been injected with MSCs, small size or large size EVs (ssEVs or lsEVs, respectively) on day 21 (arrow). Mean skin thickness in the different groups of mice at day 42 (right panel). (B) Mean dermal thickness on histological sections from the different groups of mice. (C) Representative histological sections of skin after Masson's trichrome staining (double arrow delineates the dermis; scale bar, 250 µm; upper panels) and after immunostaining with antibodies specific for Tgfβ1 (green), αSma (red) and DAPI (blue) in skin (scale bar, 100 µm; enlargement x2; lower panels). (D) Gene expression in skin as expressed as fold change. (E) Representative histological sections of lungs after Masson's trichrome staining (scale bar, 250 µm). (F) Gene expression in lung samples as expressed as fold change (n=13 to 18 per group from 2 separate experiments; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 3. Loss of the therapeutic effect of MSCs expressing low amounts of miR-29a-3p in the murine model of HOCl-induced SSc. (A) Expression of miR-29a-3p in MSCs transfected with 50 nM of antagomiR-control (MSC-ACt) or antagomiR-29a-3p (MSC-A29a). (B) Skin thickness increase in control mice (HOCl) and mice that have been injected with non-transfected MSCs (MSC-NT), MSC-ACt or MSC-A29a on day 21 (arrow). Mean skin thickness in the different groups of mice at day 42(right panel). (C) Mean dermal thickness on histological sections of skin from the different groups of mice. (D) Representative histological sections of skin after Masson's trichrome staining (the double arrow delineates the dermis; scale bar, 250 µm). (E) Gene expression in skin samples as expressed as fold change in treated versus HOCl control mice. (F) Immunostaining with antibodies specific for Tgfβ1 (green), αSma (red) and DAPI (blue) in representative skin samples (scale bar, 100 µm). (n=8 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 4. Therapeutic efficiency of human ASC-EVs in HOCl-induced SSc. (A) Expression of miR-29a-3p in murine MSCs (MSC), ssEVs and lsEVs (left panel). Expression of miR-29a-3p in human ASCs (ASC) and EVs (middle panel) and in ASCs transfected with antagomiR-control (ASC-ACt) or antagomiR-29a-3p (ASC-A29a) (right panel) (n=3). (B) Size distribution and mode-median sizes of EVs assessed by NanoTracking Analysis (left panels). EV concentration is expressed as particle numbers produced by 10^6 ASCs or per µg of total proteins (n=3).(C) Cryo-transmission electron microscopy photograph of EVs (scale bar, 200 µm). (D) Skin thickness increase in control mice (HOCl) and mice that received $4x10^7$ particles of freshly prepared EVs (EV-fresh) or frozen EVs (EV-frozen) on day 21. Mean skin thickness in the different groups of mice at day 42 (right panel) (E) Representative histological sections of skin after Masson's trichrome staining (the double arrow delineates the dermis; scale bar, 250 µm). (F) Gene expression in skin samples as expressed as fold change. (n=7-8 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 5. Therapeutic efficiency of human ASC-EVs in HOCl-induced SSc. (A) Expression of miR-29a-3p in murine MSCs (MSC), ssEVs and lsEVs (left panel). Expression of miR-29a-3p in human ASCs (ASC) and EVs (middle panel) and in ASCs transfected with antagomiR-control (ASC-ACt) or antagomiR-29a-3p (ASC-A29a) (right panel) (n=3). (B) Size distribution and mode-median sizes of EVs assessed by NanoTracking Analysis (left panels). EV concentration is expressed as particle numbers produced by 10^6 ASCs or per µg of total proteins (n=3).(C) Cryo-transmission electron microscopy photograph of EVs (scale bar, 200 µm). (D) Skin thickness increase in control mice (HOCl) and mice that received $4x10^7$ particles of freshly prepared EVs (EV-fresh) or frozen EVs (EV-frozen) on day 21. Mean skin thickness in the different groups of mice at day 42 (right panel) (E) Representative histological sections of skin after Masson's trichrome staining (the double arrow delineates the dermis; scale bar, 250 µm). (F) Gene expression in skin samples as expressed as fold change. (n=7-8 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 6. Regulation of several target genes of miR-29a-3p in the skin of SSc mice. (A) Gene expression of apoptotic markers in skin samples from mice that received non-treated MSC (MSC-NT), MSC transfected with antagomiR-control (MSC-ACt) or antagomiR-29a-3p (MSC-A29a), EVs isolated from ASCs transfected with antagomiR-control (EV-ACt) or antagomiR-29a-3p (EV-A29a). (B) Gene expression of demethylases (*Tet1, Dnmt3a*) and *Pdgfrbb* in skin samples from same groups of mice as in A (n=7-8 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group pf mice).

Figure 1

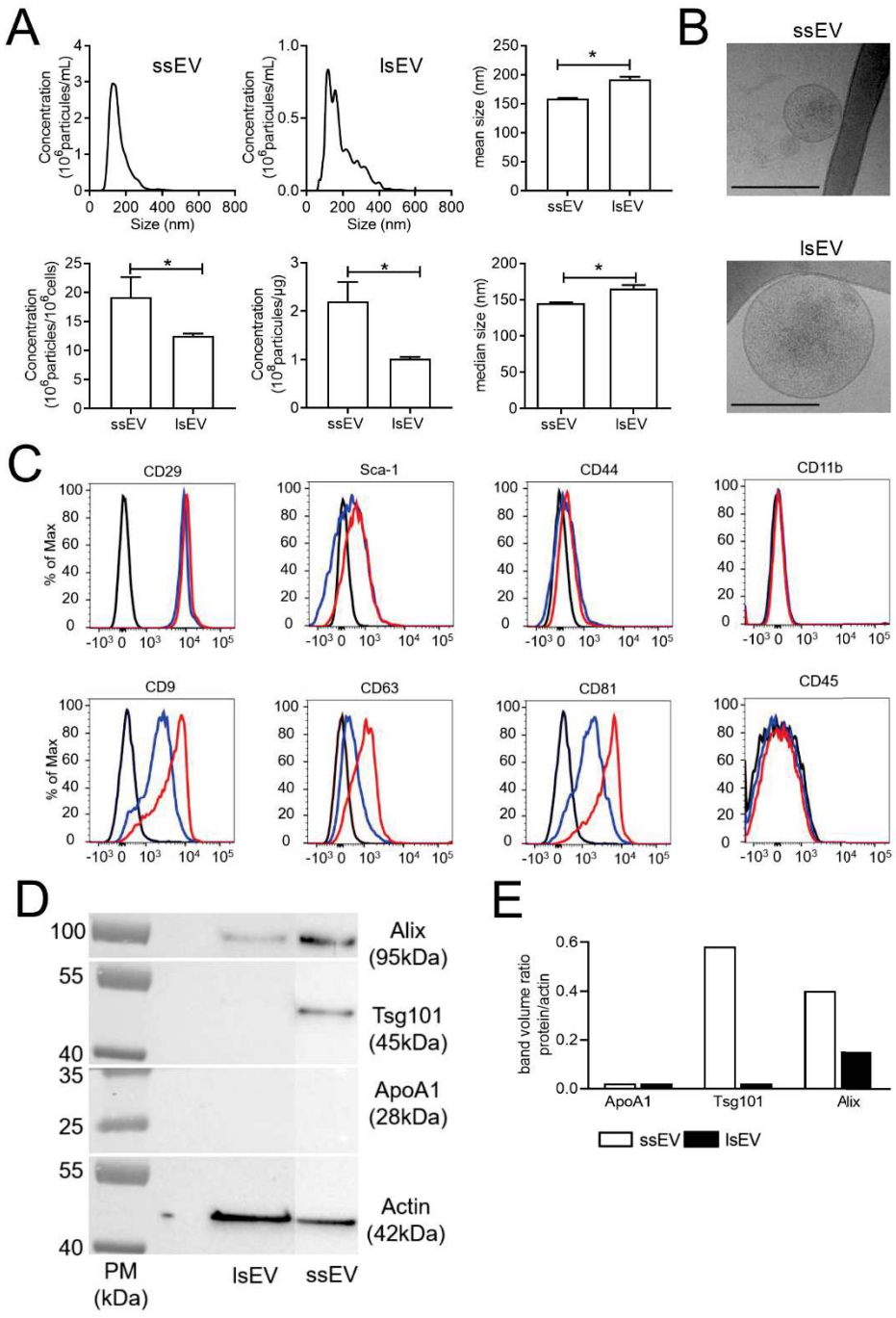

A
ssEV
lsEV
Concentration (10^6particules/mL)
Size (nm)
mean size (nm)
Concentration (10^6particles/10^6cells)
Concentration (10^8particules/µg)
median size (nm)
B
ssEV
lsEV
C
CD29
Sca-1
CD44
CD11b
CD9
CD63
CD81
CD45
% of Max
D
Alix (95kDa)
Tsg101 (45kDa)
ApoA1 (28kDa)
Actin (42kDa)
PM (kDa)
E
band volume ratio protein/actin
ApoA1
Tsg101
Alix

Figure 2

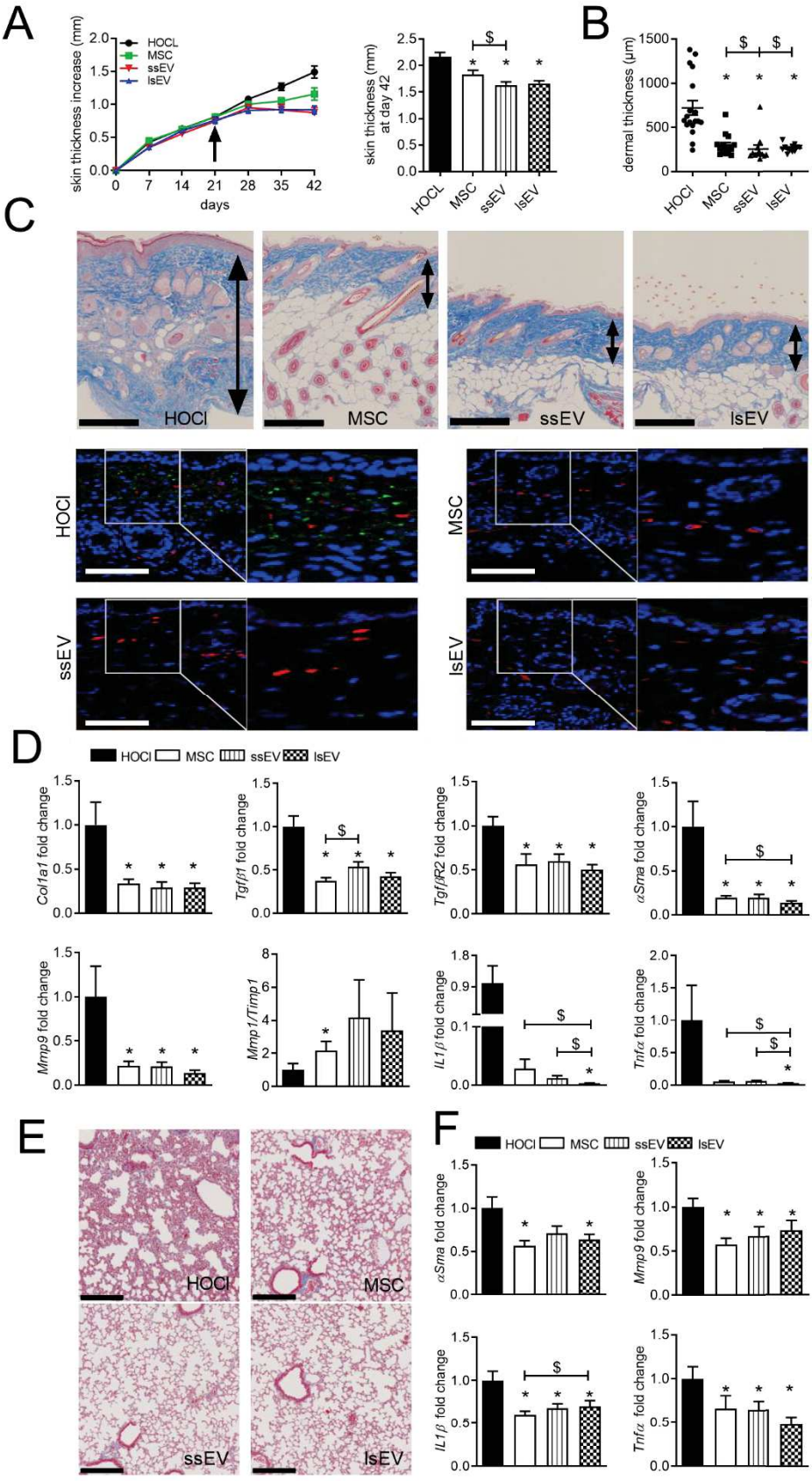

A
skin thickness increase (mm)
HOCL
MSC
ssEV
lsEV
days
skin thickness (mm) at day 42
B
dermal thickness (µm)
C
HOCl
MSC
ssEV
lsEV
D
Col1a1 fold change
Tgfβ1 fold change
Tgfβ R2 fold change
αSma fold change
Mmp9 fold change
Mmp1/Timp1
IL1β fold change
Tnfα fold change
E
F

Figure 3

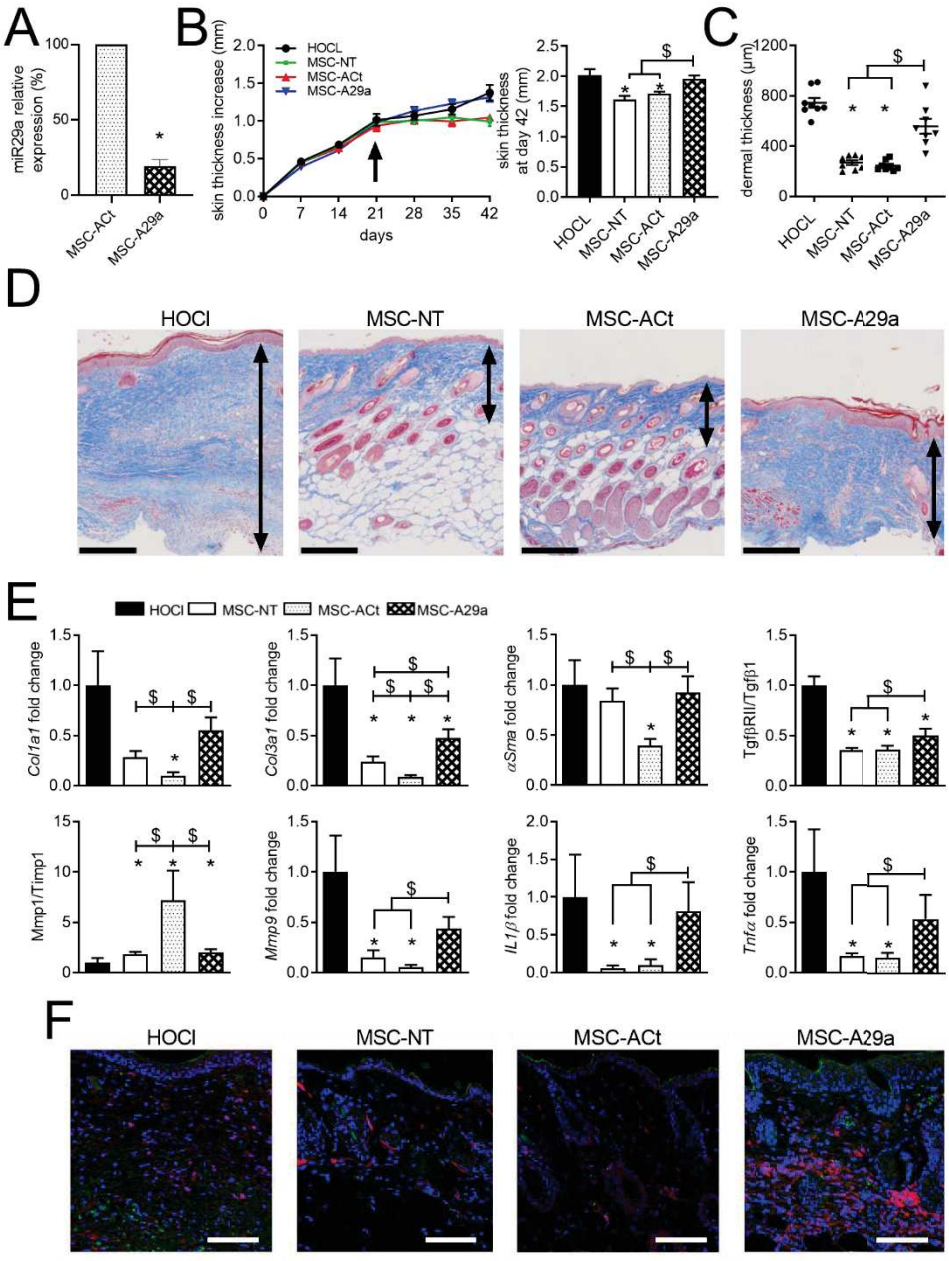

A
miR29a relative expression (%)
MSC-ACt
MSC-A29a
B
skin thickness increase (mm)
HOCL
MSC-NT
MSC-ACt
MSC-A29a
days
skin thickness at day 42 (mm)
C
dermal thickness (µm)
D
HOCl
MSC-NT
MSC-ACt
MSC-A29a
E
Col1a1 fold change
Col3a1 fold change
αSma fold change
TgfβRII/Tgfβ1
Mmp1/Timp1
Mmp9 fold change
IL1β fold change
Tnfα fold change
F

Figure 4

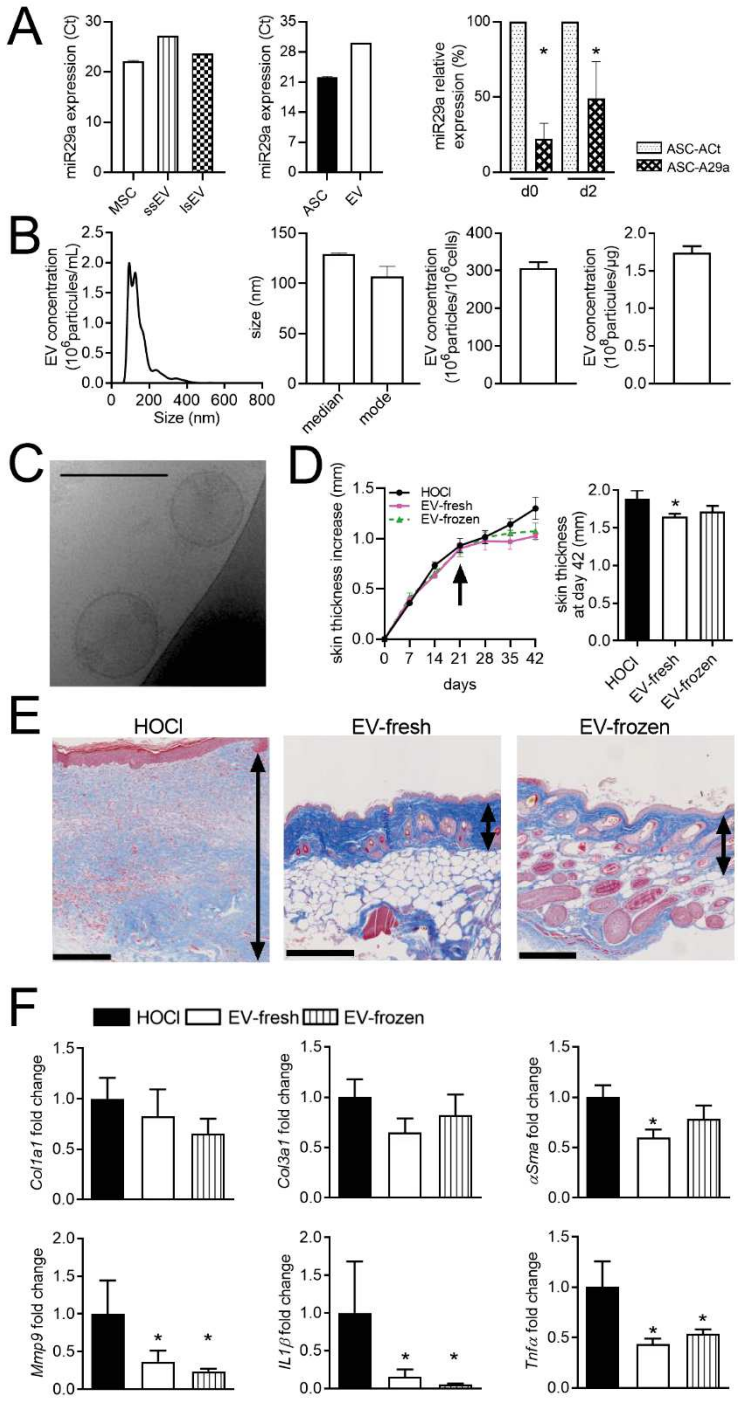

A
miR29a expression (Ct)
MSC
ssEV
lsEV
ASC
EV
miR29a relative expression (%)
ASC-ACt
ASC-A29a
d0
d2
B
EV concentration (10^6particules/mL)
Size (nm)
size (nm)
median
mode
EV concentration (10^6particles/10^6cells)
EV concentration (10^8particules/µg)
C
D
HOCl
EV-fresh
EV-frozen
skin thickness increase (mm)
days
skin thickness at day 42 (mm)
E
HOCl
EV-fresh
EV-frozen
F
HOCl
EV-fresh
EV-frozen
Col1a1 fold change
Col3a1 fold change
αSma fold change
Mmp9 fold change
IL1β fold change
Tnfα fold change

Figure 5

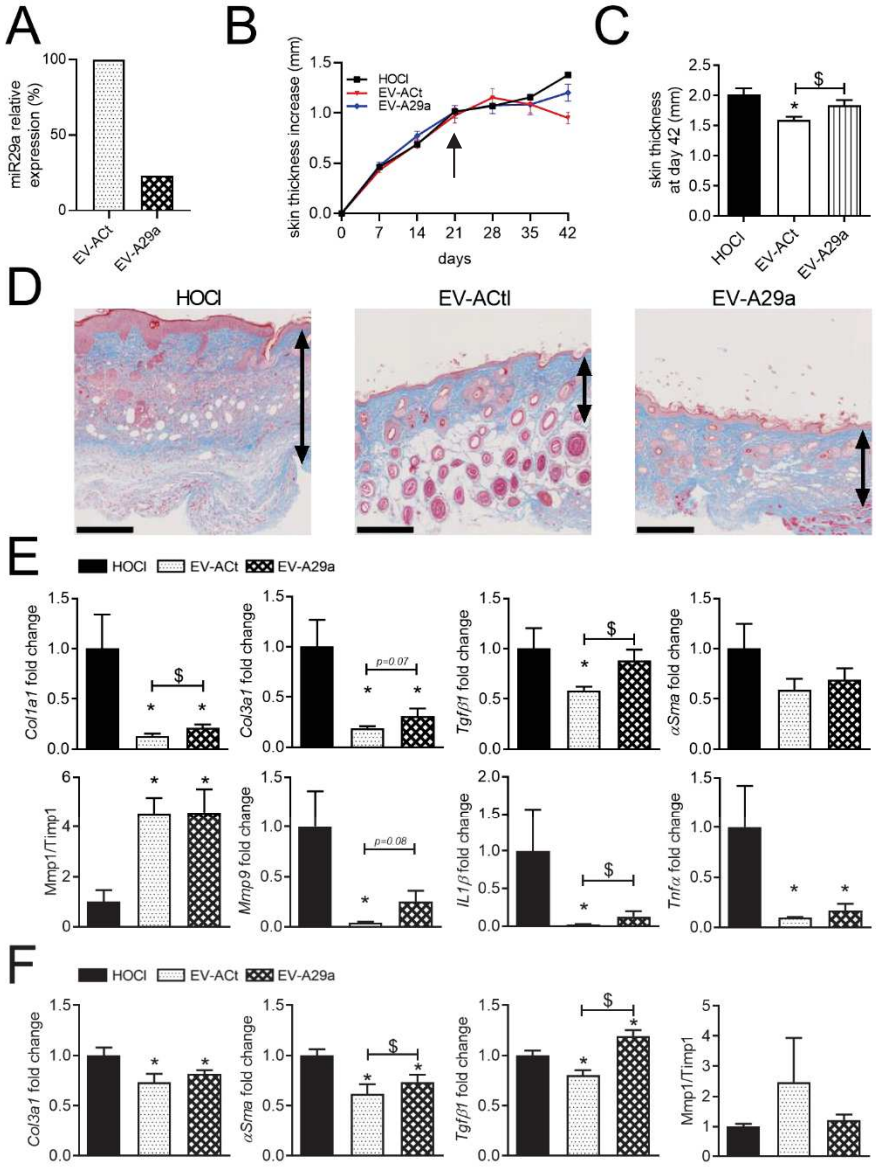
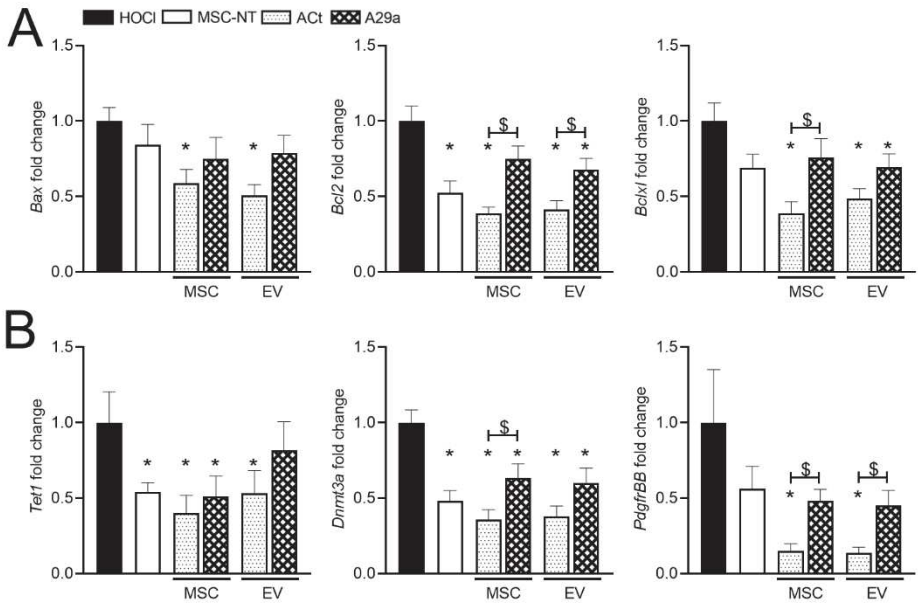

A
miR29a relative expression (%)
EV-ACt
EV-A29a
B
skin thickness increase (mm)
HOCl
EV-ACt
EV-A29a
days
C
skin thickness at day 42 (mm)
HOCl
EV-ACt
EV-A29a
D
HOCl
EV-ACtl
EV-A29a
E
HOCl EV-ACt EV-A29a
Col1a1 fold change
Col3a1 fold change
Tgfβ1 fold change
αSma fold change
Mmp1/Timp1
Mmp9 fold change
IL1β fold change
Tnfα fold change
p=0.07
p=0.08
F
HOCl EV-ACt EV-A29a
Col3a1 fold change
αSma fold change
Tgfβ1 fold change
Mmp1/Timp1

Figure 6



Supl Table 1

Gene	sequence forward	sequence reverse
Tbp	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT
Col1a1	TGTTCAGCTTTGTGGACCTC	TCAAGCATACCTCGGGTTTC
Col3a1	CGGTGAACGGGGCGAAGCTGGTT	GACCCCTTTCTCCTGCGGCTCCT
Acta2(αSma)	AAGGCCAACCGGGAGAAAAT	AGCCAAGTCCAGACGCATGA
Tgfβ1	TGCGCTTGCAGAGATTAAAA	CTGCCGTACAACTCCAGTGA
TgfβR2	CGACCCCAAGCTCACCTACC	CAACAACAGGTCGGGACTGC
Mmp1	TTCAAAGGCAGCAAAGTATGGGCT	CCAGTCTCTTCTTCACAAACAGCAGCA
Mmp9	TCCAGTTTGGTGTCGCGGAGCACG	CAGGGGGAAAGGCGTGTGCCAGA
Timp1	CTCCGCCCTTCGCATGGACATT	GGGGGCCATCATGGTATCTGCTCT
IL1β	TTTGACAGTGATGAGAATGACCTGTTC	TCATCAGGACAGCCCAGGTCAAAG
TNFα	AGCCCACGTCGTAGCAAACCA	TGTCTTTGAGATCCATGCCGTTGGC

2ème partie : La pré-activation des cellules stromales mésenchymateuses améliore l'effet thérapeutique de leurs vésicules extracellulaires dans le modèle murin de sclérodermie.

Dans cette seconde partie de mon projet de thèse, nous avons souhaité améliorer l'efficacité thérapeutique des EVs, notamment en majorant la dose injectée ou en modifiant l'environnement dans lequel les EVs étaient produites.

Pour cela, nous avons collecté des petites EVs et des grosses EVs produites par des CSM murines selon le protocole décrit dans la 1ère partie de mes travaux personnels. Nous avons collecté des EVs produites à partir de CSMs murines, qui étaient non activées (NA) ou pré-stimulées par l'IFNγ adjoint au milieu de production à 1 ng/mL (A1) ou 20 ng/mL (A20). Le modèle murin utilisé était induit par des injections quotidiennes d'acide hypochloreux et les souris bénéficiaient de leur traitement au 21ème jour. L'efficacité du traitement était évaluée cliniquement toutes les semaines par la mesure du pli cutané ainsi qu'à l'échelle histologique et moléculaire sur des prélèvements de peaux et de poumons réalisés au 42ème jour. Dans le même temps, nous avons étudié l'effet des EVs sur le stress oxydant présent dans le sérum des souris, recueilli à partir de sang prélevé quelques minutes avant le sacrifice des animaux.

Tout d'abord, nous avons évalué l'effet thérapeutique d'une injection de 1500 ng d'EVs chez les souris sclérodermiques. L'augmentation de la dose d'EVs n'a apporté aucun bénéfice à la dose de 250 ng qui avait prouvé son efficacité dans la première partie de ce travail.

Ensuite, nous avons évalué l'effet de la pré-activation des CSMs par l'IFNγ sur l'efficacité thérapeutique des EVs dans le modèle murin de ScS. En effet, l'IFNγ est connu pour activer les CSMs en culture afin de mimer l'effet de l'environnement dans lequel elles sont soumises au cours de pathologies, notamment inflammatoire, dans les suites d'une lésion tissulaire par exemple. De plus, le contenu des EVs varie en fonction de la cellule dont elles sont issues et notamment de leur état d'activation : de ce fait, les propriétés des EVs peuvent être modifiées. Nous avons observé que la pré-activation des CSMs par l'IFNγ augmente l'efficacité thérapeutique des EVs au niveau des marqueurs moléculaires de fibrose et d'inflammation, dans les poumons.

Enfin, nous avons observé, *in vitro*, que la pré-activation des CSMs par l'IFNγ entraîne une augmentation, dans les cellules et dans les EVs, de l'expression d'ARNm codant pour des molécules immunosuppressives (iNos, IL1ra, IL6), tandis que la majoration de l'expression de molécules anti-fibrotiques comme l'Hgf est uniquement observé dans les EVs. L'expression de miR-29a n'était pas drastiquement modulé par l'IFNγ, ni dans les CSMs ni dans leurs EVs, ce qui est concordant avec les récentes données rapportées dans la littérature.

Au total, ce travail a permis de montrer que la pré-activation des CSMs par l'IFNγ améliore l'effet thérapeutique des EVs dans les poumons de souris sclérodermiques en modulant l'expression des marqueurs anti-inflammatoires et anti-fibrotiques.

Ce travail sera soumis très prochainement à la revue Frontiers in Immunology dans un numéro spécial intitulé « Extracellular Vesicles as Immunomodulatory Mediators in Inflammatory Processes". L'abstract a d'ores et déjà été validé pour la soumission du manuscrit dans ce numéro spécial.

Extracellular vesicles from IFNγ-primed mesenchymal stromal cells improve lung fibrosis in murine systemic sclerosis

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ABSTRACT

Objective: Systemic sclerosis (SSc) is a severe autoimmune disease for which no curative treatment is available in routine care. Mesenchymal stromal cells (MSCs)-based therapy is a promising strategy that was reported to reduce SSc-related symptoms in pre-clinical studies. Recently, we have shown that extracellular vesicles released by MSCs (MSCs-EVs) mediated their systemic therapeutic effect in the HOCl-induced murine model of SSc. Here, we aimed at improving their efficacy by testing increased dose of MSC-EVs and IFNγ-priming of MSCs.

Methods: Small size (ssEVs) and large size EVs (lsEVs) were recovered from supernatants of murine MSCs. MSCs were previously pre-activated using 1 or 20 ng/mL of IFNγ (A1 or A20) or not. In the SSc HOCl-induced model, mice were treated with EVs at day 21 and sacrificed at day 42. Lung and skin samples were collected for histological and molecular analyses and blood for oxidative stress analysis.

Results: Increasing the dose of MSC-EVs did not add benefit to the dose previously reported efficient in the SSc murine model. By contrast, IFNγ pre-activation improved MSC-EVs-based treatment as shown by improvement of molecular markers in lungs. Low dose of IFNγ decreased the expression of fibrotic markers while high dose IFNγ improved remodeling and anti-inflammatory markers. In vitro, IFNγ pre-activation resulted in the upregulation of *iNos, IL1ra and Il6 transcripts* in both MSCs and ssEVs while *Hgf* was detected in higher amounts only in ssEVs.

Conclusion: IFNγ-pre-activation improved the therapeutic effect of MSC-EVs in lungs of SSc mice by modulating the expression of anti-inflammatory and anti-fibrotic markers.

Keywords: mesenchymal stromal cell, scleroderma, extracellular vesicles, exosomes, microvesicles, therapy

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease with a severe prognosis due to generalized fibrosis and vasculopathy (1). Currently, symptomatic management is the only strategy available to relieve patients and no curative treatment can reverse the disease. For some patients, immunosuppressive drugs and hematopoietic stem cell transplantation could be efficient to stop disease course but these are also responsible of severe side effects (2). More recently, mesenchymal stromal cells (MSCs) have demonstrated therapeutic benefit in preclinical models of SSc thanks to their pleiotropic properties. Currently, MSCs are being evaluated in the clinics and promising results have been reported (3).

MSCs exert their therapeutic function through the release of many soluble mediators that are secreted in the extracellular milieu and/or within extracellular vesicles (EVs). EVs are a heterogeneous population of vesicles that are characterized by their size and biogenesis (4). Apart from apoptotic bodies that are released by apoptotic cells, the two main EV subtypes are exosomes, which are below 150 nm in diameter and generated via the endosomal pathway inside multivesicular bodies, and microvesicles or microparticles, whose size is above 120 nm and form by budding of plasma membrane. Because of overlapping sizes, current isolation procedures allow to isolate small size EVs (ssEVs) and large size EVs (lsEVs), which are enriched in exosomes and microvesicles, respectively (5). Both types of EVs contain a cargo of proteins, lipids, nucleic acids (including DNA, mRNA, miRNA) that mediate the functions of parental cells.

Using the HOCl-induced murine model of SSc, we previously reported that murine and human MSCs from bone marrow (BM) and adipose tissue (AT) are efficient to stop the course of the disease and prevent skin and lung sclerosis (6, 7). Recently, we demonstrated that EVs isolated from murine BM-MSCs and human AT-MSCs reproduce the therapeutic effect of parental cells (Rozier et al, submitted). We showed that both ssEVs and lsEVs powerfully stop disease progression and regulate expression of fibrotic and remodeling markers in HOCL-induced murine SSc. They also down-regulate inflammation in the skin and lungs of treated mice. The beneficial effect has been associated with the presence of

miR-29a-3p in murine ssEVs and lsEVs as well as in total EVs from human AT-MSCs. MiR-29a-3p was shown to act via the regulation of type I and III collagens, apoptotic factors (Bax, Bcl2, Bcl-xl), methylation-regulating genes (Tet1, Dnmt3a) and Pdgfrbb in the skin of SSc mice.

In the present study, we investigated the possibility to enhance the therapeutic effect of ssEVs and lsEVs isolated from murine BM-MSCs (mMSC) in the murine model of SSc, by testing two doses of EVs. Because pre-activation of MSCs by IFNγ is reported to stimulate their immunosuppressive effect (8, 9, 10), we tested the possibility that IFNγ could up-regulate the production of immunosuppressive factors and their release within EVs, thereby enhancing their therapeutic effect in SSc.

MATERIALS AND METHODS

Mesenchymal Stem Cell expansion

C57BL/6 mice-derived mMSC were cultured in proliferative medium containing DMEM, 10% fetal calf serum (FCS), 100 µg/mL penicillin/streptomycin and 2 mmol/mL glutamine. Their characterization by phenotyping and tri-lineage differentiation potential has been reported before (11). We used mMSC between passage 12 and 18 for the following experiments.

Production and isolation of EVs

EV subtypes were produced from mMSCs and characterized according to ISEV recommendations as previously described (12)(Rozier et al, submitted). Briefly, mMSCs were seeded at 7,000 cells/cm^2 in proliferative medium for 24 hours. After a wash with phosphate buffer saline (PBS), the production medium (DMEM containing 3% EV-free FCS) was added for 48 hours. When necessary, recombinant mouse IFNγ (1 or 20 ng/mL) was added (R&D system, Lille). After removing cells and debris thanks to low speed centrifugation, lsEVs were pelleted by a first ultracentrifugation at 18 000 g, 4°C, for 1 h and ssEVs were pelleted by a second ultracentrifugation at 100,000 g, 4°C, for 2 h. Both pellets were then washed in PBS and submitted to another ultracentrifugation round at 100,000 g for 2 h. EV subtypes

were characterized according to their morphology and their size thanks to cryoTEM and nanotracking analysis. Protein content was evaluated thanks to cytometry and western blot experiments, as reported elsewhere (Rozier et al, submitted).

Animal model and histopathological analysis

Mice were raised in the conditions required by the European guidelines for the care and use of laboratory animals (2010/63/UE). This project was approved by the Regional Ethics Committee on Animal Experimentation (APAFIS#53512016050919079187). SSc was induced onto six-week-old female BALB/cJRj mice (Janvier Labs) using daily HOCl intradermic injections for 42 days (150 µL into two sites at the base of the tail). Once a week, skin thickness was measured thanks to a caliper. At day 21, groups of 7-8 mice were formed to homogenize the mean skin thickness between groups. Mice received one intravenous injection (100 µL) of either NaCl 0.9% (control group), 250,000 mMSC or 250 ng EVs (or 1500 ng depending on the experiment). Mice were split in the cages to minimize bias between cages. At day 42, blood, lungs and skin biopsies (6 mm punchs) were recovered. Blood was allowed to coagulate at room temperature (RT) at least 30 min and was then subjected to centrifugation at 1,000 g, RT for 10 min to recover the serum, which was stored at -80°C. Skin or lung samples for molecular analysis were stored at -80°C. Samples dedicated to histology were directly fixed in 4% formaldehyde before paraffin embedding and routine histology. Histological slides (5 µm thick) were stained by Masson's trichrome staining and analyzed with Nanozoomer (Hamamatsu) and NDP.view2 software to measure dermal thickness.

RNA extraction and RT-qPCR

Skin and lung samples were grinded in RLT-buffer containing 1% β-mercaptoethanol and processed using the RNeasy Mini Kit for total RNA extraction, according to manufacturer's recommendations (Qiagen, Courtaboeuf). RNA (500 ng) was reverse transcribed by M-MLV reverse transcriptase (ThermoFisher Scientific). Using specific primers (Table 1), real time PCR was performed on 20 ng cDNA

using the Master SYBR Green I mix (Roche Diagnostics). TATA binding protein (*Tbp*) was used as the housekeeping gene and data were expressed as *Tbp* relative expression and as fold change compared to control non-treated group using the formulae $2^{-\Delta\Delta Ct}$.

MSC and EV samples were treated with Qiazol reagent (ThermoFisher Scientific) using miRNeasy Mini or Micro Kit, respectively (Qiagen, Courtaboeuf). Reverse transcription was performed using M-MLV Reverse Transcriptase kit (ThermoFisher Scientific), followed by real-time PCR on 10 ng cDNA using specific primers (Table 1) and SYBR Green I Master mix (Roche Diagnostics). Data were normalized to the expression of D-glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) housekeeping gene for mMSCs and expressed using the formulae $2^{-\Delta CT}$. Reverse transcription of miRNA from 10 ng total RNA followed user guide's recommendations of TaqMan® MicroRNA Reverse Transcription Kit (ThermoFisher Scientific). Real-time PCR was performed on 2 ng cDNA using Taqman microRNA hsa-miR29a-3p (Life Technologies) and Taqman Master Mix II no UNG (Life Technologies). Data were normalized to the expression of U6 snRNA (Life Technologies) for mMSCs and data were expressed as 100/Ct for EVs.

Measure of Advanced Oxidation Protein Products

Sera or a range of 200 µL of standard chloramine T solution (0 to 1000 µM) were incubated with 20 µL acetic acid and 10 µL potassium iodide (1.16 M) at RT. Optical densities were measured at 340 nm on a microplate reader (Varioskan, ThermoFisher Scientific), before incubation and each minute for 10 min. AOPP concentrations were expressed as chloramine T equivalents (µM).

Measure of anti-oxidant capacity

Anti-oxydant capacity (AOC) was measured on sera diluted to 1/10 or a standard range of Trolox using the Antioxidant Assay Kit (Cayman Chemical, Interchim). The absorbance was read at 750 nm and AOC was expressed as mM Trolox equivalents.

Statistical analysis

Statistical analyses were performed using GraphPad 8 Prism Software. Data distribution was evaluated using the Shapiro-Wilk normality test. Each single group was compared to the control group using the Student t-test or the Mann-Whitney test when values were parametric or non-parametric, respectively. For values normalized to 1, a one sample t-test or Wilcoxon test were performed when values assumed or not a normal distribution, respectively. Data are presented as mean ± SEM.

RESULTS

3.1. High dose of MSC-EVs impair their therapeutic effect in SSc

Isolation and characterization of ssEVs and lsEVs from murine BM-MSCs have been described previously (Rozier et al, submitted). After systemic injection of these MSC-derived ssEVs and lsEVs, we showed that they can efficiently reduce clinical symptoms and histopathological alterations in SSc. With the aim to improve treatment efficacy, we tested whether a higher dose of lsEVs might be beneficial. By comparison with the dose of 250 ng used in the previous study, the high dose of lsEVs (1500 ng) did not stop disease progression as shown by a continuous skin thickness increase, which is similar to non-treated mice, and higher skin thickness measured at day 42 (Fig. 1A-B). On histological sections, dermal thickness was similar in non-treated mice and mice receiving high dose of lsEVs and significantly higher than in mice receiving low dose of lsEVs (Fig. 1C-D). Contrary to low dose, high dose of lsEVs did not significantly improve expression of several fibrotic (*Col1a1),* remodeling *(Mmp9)* and inflammatory *(Il1β, Tnfα)* markers compared to non-treated mice (Fig. 1E). Expression of *αSma* and *Il1β* was higher in mice treated with high dose versus low dose of lsEVs while anti-oxidant marker *Sod2* was lower. Similar results were observed on histological sections of lungs (Supl Fig. 1A). No improvement in pulmonary fibrosis was noticed as indicated by a dense and infiltrated parenchyma in mice injected with high dose lsEVs. Expression of fibrotic markers (*Col1a1, Col3a1, αSma*), did not improve although the expression of *Mmp9* and *Il1β* was reduced to similar levels as the low dose of lsEVs (Supl Fig. 1B). Of note, mRNA levels of *Mmp1 (*as illustrated by the ratio *Mmp1/Timp1), Hmox1,*

Nfe2l2 and *Sod2* increased after the injection of high dose of lsEVs. Altogether, the results indicated that high dose of lsEVs did not improve the clinical and histological features of SSc, even though remodeling and anti-oxidative capacity improved in the lungs.

3.2. Low dose IFNγ pre-activation improves the anti-fibrotic effect of MSC-EVs in lungs.

We then evaluated whether pre-activation of MSCs by a low dose of IFNγ (1 ng/mL) could improve the therapeutic effect of ssEVs and lsEVs, notably their anti-inflammatory effect (13). Although disease progression was stopped or slowed down in all treated groups, pre-activated EV subtypes (ssEV A1 and lsEV A1) were less efficient than non-activated EV subtypes (ssEV NA and lsEV NA) (Fig. 2A). At day 42, skin thickness of mice that received pre-activated EVs was significantly thicker as compared to non-activated EVs (Fig. 2B). Measure of dermal thickness on skin histological sections indicated lower thickness in all groups and revealed a heterogeneity of response in the ssEV A1 group (Fig. 2C-D). All the fibrotic and inflammatory markers were significantly lower in treated groups, even though lsEV A1 were less efficient than lsEV NA to reduce the expression of *Tgfβ1, αSmα* and *Tnfα* (Fig. 2E). As expected, the gelatinase *Mmp9* was reduced in all treated groups and the *Mmp1/Timp1* ratio, which is representative of matrix remodeling, was increased. We also measured the advanced oxidized protein products (AOPP) concentration in serum as a marker of oxidative stress. Interestingly, AOPP levels were significantly lower in all treated groups (Fig. 2F). Again, lsEV A1 were less efficient than lsEV NA to decrease AOPP levels. Anti-oxidative capacity (AOC) in the sera of treated groups was not increased as compared to that of control group and was even decreased in ssEV A1 group (Fig. 2F). As a result, the AOPP/AOC ratio was significantly reduced in all treated groups but to a lesser extend in the lsEV A1 group.

In the lungs, analysis of histological sections showed fewer collagen deposits in treated groups, regardless of EV subtype (Fig. 3A). At the molecular level, both ssEV A1 and lsEV A1 decreased the expression of fibrotic markers (*Col3a1, Tgfβ1, Tgfβr2)* as compared to ssEV NA and lsEV NA or control group (Fig. 3B). Nevertheless, ssEV A1 and lsEV A1 did not reduce the expression of *Mmp9* and did not

impact the *Mmp1/Timp1* ratio. The inflammatory markers *Il1β* and *Tnfα* were lower in all treated groups. Overall, MSC pre-activation did not improve the efficacy of EV subtypes on cutaneous fibrosis but improved at least partially lung fibrosis by reducing *Il1β* and expression of all fibrotic markers.

3.3. High dose IFNγ pre-activation improves remodeling and anti-inflammatory effect of MSC-EVs in lungs. With the hope to increase these anti-fibrotic and anti-inflammatory effects of MSC-EVs in the lungs and possibly in skin, we tested a higher dose of IFNγ (20ng/mL) to pre-activate MSCs. Both pre-activated MSC-EV subtypes (ssEV A20 and lsEV A20) stopped the disease course and reduced the skin thickness to similar levels as non-activated EV subtypes, but lsEV A20 were more efficient than ssEV A20 (Fig. 4A-B). Histological sections of skin revealed lower dermal thickness in all treated groups (Fig. 4C). Most of the molecular markers improved in the skin of treated groups except in the ssEV-A20 group for *Col1α1*, *Tgfβr2*, *Mmp1/Timp1*, *Il1β, Tnfβ* (Fig. 4D). In the skin, MSC pre-activation did not improve efficacy of lsEV A20 as compared to unprimed lsEV NA but improved efficacy as compared to ssEV A20.

In the lungs, improvement of several fibrotic, remodeling and inflammatory markers was observed in all treated groups even though statistical significance was not reached for some markers in this specific experiment (Fig. 4E). Pre-activated ssEVs A20 and lsEVs A20 seemed to be more potent than non-activated EVs to improve *αSmα, Tgfβr2* as well as remodeling and inflammatory mediators (Fig. 4E). No further beneficial effect was observed with lsEV A20 as compared to ssEV A20.

In summary, pre-activation of MSCs did not improve clinical efficacy of MSC-EV subtypes in skin while pre-activation with a low or high dose of IFNγ exert beneficial effect in lungs. Interestingly, lsEV A20 were more efficient than ssEV A20 to reduce all SSc-associated markers.

3.4. IFNγ pre-activation up-regulates anti-inflammatory factors in MSCs and MSC-EVs.

To better understand the mechanism of action of MSC-EVs, we quantified the expression of known immunosuppressive and anti-fibrotic markers in MSCs. Both low and high dose of IFNγ up-regulated

iNos, Il1ra, Il6, Cox2, Tsg6 mRNAs while expression of *Hgf* and *TGFβ1* was not modulated (Fig. 5A). Expression of miR-29a-3p, which was shown to be involved in the beneficial effect of MSC-derived EVs in SSc (Rozier et al, submitted), was also slightly up-regulated in *IFNγ* pre-activated ssEVs but did not reach significance (Fig. 5A). Those markers were then quantified at the mRNA level in the subtypes of EVs. Higher levels of *iNos, Il1ra, Il6 and Hgf* were detected in ssEV A20 as compared to ssEV NA, lsEV NA or lsEV A20 5fig. 5B). By contrast, expression of *Tgfβ1* was downregulated in both ssEV A20 and lsEV A20. In summary, *IFNγ* pre-activation up-regulated expression of several anti-inflammatory and anti-fibrotic factors in mMSCs and EVs, which may participate to improve symptoms in SSc.

DISCUSSION

In the present study, we report that IFNγ pre-activation of MSCs did not improve the therapeutic efficacy of EVs in skin of SSc-induced mice but improved the beneficial effect of EVs in lungs.

We first showed that the therapeutic efficacy of MSC-EVs was dose-dependent. Interestingly, increasing the dose of lsEVs by a 6-fold factor abolished the beneficial effect of EVs. This is consistent with our previous study that demonstrated that increasing the quantity of injected MSCs from $2.5x10^5$ cells to $1x10^6$ cells reversed the beneficial role of cell therapy (6). The reason for this is not known. This might be related to the local accumulation of EVs that might contribute to a dysregulated crosstalk with surrounding endogenous cells in a context of oxidative stress and inflammation related to SSc. This might also be related to the raise of a number of factors in serum, including inflammatory cytokines or profibrotic factors, to levels that can induce unwanted effects on organs or immune cells and counteract the beneficial effects. The only markers that improved following infusion of high lsEV doses were anti-oxidative stress genes in lungs. Whether this reflects an adaptive response to the elevation of oxidative stress in lungs of mice receiving high doses of lsEVs remains to be elucidated.

Nevertheless, the data indicate that the optimal dose will have to be determined in larger preclinical models before clinical translation.

One important finding of the study is the interest of using EVs from IFNγ pre-activated MSCs to enhance their beneficial effect in lungs. Improvement of fibrotic, remodeling and inflammatory markers was observed by comparison with EVs from non-activated MSCs. Few studies have investigated the interest of IFNγ pre-activation of MSCs before EV isolation. Nevertheless, EVs isolated from IFNγ pre-activated human umbilical cord-derived MSCs were shown to increase survival of rats with *Escherichia coli*-induced pneumonia and to reduce lung injury compared with EVs from naïve MSCs (14). EVs from IFNγ pre-activated human BM-MSCs reduced experimental autoimmune encephalomyelitis (EAE) via the generation of Treg cells (15). By contrast, EVs from primed human cord blood-derived MSCs were not able to protect kidney from ischemia-reperfusion injury or displayed similar efficacy as naïve EVs in experimental spinal cord injury (16, 17). In vitro, EVs isolated from human MSCs, whether pre-activated or not with IFNγ, exerted similar ability to inhibit T-cell proliferation although only pre-activated EVs contained IDO mRNA (18) and a number of anti-inflammatory RNAs and proteins (15). In addition to IDO and iNOS in human and mouse MSCs, respectively, up-regulation of PDL1 or CD200 was also proposed to sustain the enhanced immunosuppressive function of IFNγ pre-activated MSC-EVs (8). In our study, up-regulation of several known immunosuppressive markers was found both in pre-activated MSCs and in derived EVs, which likely explains the higher therapeutic effect observed in the lungs of SSc mice. Indeed, EVs can transfer mRNAs to target tissues where they are translated into effector proteins regulating intrinsic pathways in the recipient cells. We did not observe higher content of miR-29a-3p in EVs, which is consistent with the limited effect of IFNγ pre-activation on the miRNA landscape of human MSC-EVs (19). Nevertheless, IFNγ pre-activation did not enhance the beneficial effect of EVs in skin. To our knowledge, no differential effect of pre-activated MSC-EVs in different organs has been described so far and better understanding of their mechanism of action is needed. One possibility is that the therapeutic effect of EVs is already maximal on skin and increasing the production of therapeutic

factors did not increase their effect. Another hypothesis is the up regulation of specific soluble and membrane markers, which could impact their biodistribution and/or act differentially on different organs.

Beside functional differences between EVs isolated from naïve and pre-activated MSCs, we also observed a slightly but constant higher therapeutic effect of lsEV A20 compared to ssEVs A20 at lest in the skin of treated mice. SsEVs have been previously shown to outperform lsEVs in collagen-induced arthritis, kidney injury or in delayed-T hypersensitivity mouse models (20, 21, 22). Although not pointed out in another study, the proportion of small size EVs versus larger EVs was decreased after IFNγ pre-activation and loss of therapeutic efficiency was observed (16). This observation might suggest than IFNγ pre-activated lsEVs were more efficient than ssEVs. Differences in protein, mRNA and miRNA content were proposed to explain the differential regenerative capacities of EV subtypes in a model of acute kidney injury (20). The proproliferative effect of ssEVs was ascribed to the presence of many factors playing a role in maintenance of cell cycle while p53, a negative regulator of cell cycle, was found in lsEVs. Differences in membrane markers expressed by EV subtypes could also explain differential targeting of injured or diseased tissue since MSC-EVs rapidly localized in the injured organs and remained up to 7 days after systemic administration (23). Further studies are needed to decipher the respective roles of ssEVs and lsEVs keeping in mind that the current isolation protocol does not allow isolation of pure populations of exosomes or microvesicles, which might explain differential cargos according to EV biogenesis.

In conclusion, we showed that IFNγ pre-activation of MSCs enhanced the beneficial effect of ssEVs and lsEVs by regulating several markers whose expression is altered in SSc. We further observed that IFNγ pre-activated lsEVs might be more efficient than ssEVs in this specific disease application.

CONFLICT OF INTEREST

The authors disclose any financial or personal conflict of interest.

AUTHOR CONTRIBUTIONS

DN, PG, CJ designed the experiments. Experimental work was performed by PR, MM, KT and data were analyzed by PR, MM, AM. PR and DN prepared the manuscript. All authors have contributed to revising the manuscript and final approval.

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FIGURE LEGENDS

Figure 1. Dose effect of lsEVs isolated from MSCs in the murine model of HOCl-induced SSc. **(A)** Measures of the skin thickness increase in control mice (HOCl) and mice that have been injected with 250 or 1500 ng of large size extracellular vesicles ($lsEV_{250}$ or $lsEV_{1500}$, respectively) on day 21 (arrow). **(B)** Mean skin thickness in the different groups of mice at day 42. **(C)** Mean dermal thickness on

histological sections of skin from the three groups of mice. **(D)** Photographs of representative histological sections of skin after Masson's trichrome staining (the double arrow delineates the dermis; scale bar, 250 µm). **(E)** Gene expression in skin samples as expressed as fold change in treated versus HOCl control mice. Data are presented as mean ± SEM (n=8 to 12 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 2. Therapeutic effect of EVs isolated from MSCs pre-activated with low dose of IFNγ in skin from HOCl-induced SSc. (A) Measures of the skin thickness increase in control mice (HOCl) and mice that have been injected with small size or large size extracellular vesicles isolated from non-activated MSCs (ssEV NA or lsEV NA) or MSCs pre-activated by 1 ng/mL IFNγ (ssEV A1 or lsEV A1) on day 21 (arrow). **(B)** Mean skin thickness in the different groups of mice at day 42. **(C)** Mean dermal thickness on histological sections of skin from the different groups of mice. **(D)** Photographs of representative histological sections of skin after Masson's trichrome staining (the double arrow delineates the dermis; scale bar, 250 µm). **(E)** Gene expression in skin samples as expressed as fold change in treated versus HOCl control mice. **(F)** Quantification of advanced oxidation protein products (AOPP) and of anti-oxidant capacity (AOC) in the serum of mice as expressed as chloramine T and Trolox equivalents, respectively and, expression of the AOPP/AOC ratio. Data are presented as mean ± SEM (n=12 to 18 per group from 2 separate experiments (except for data in f); *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

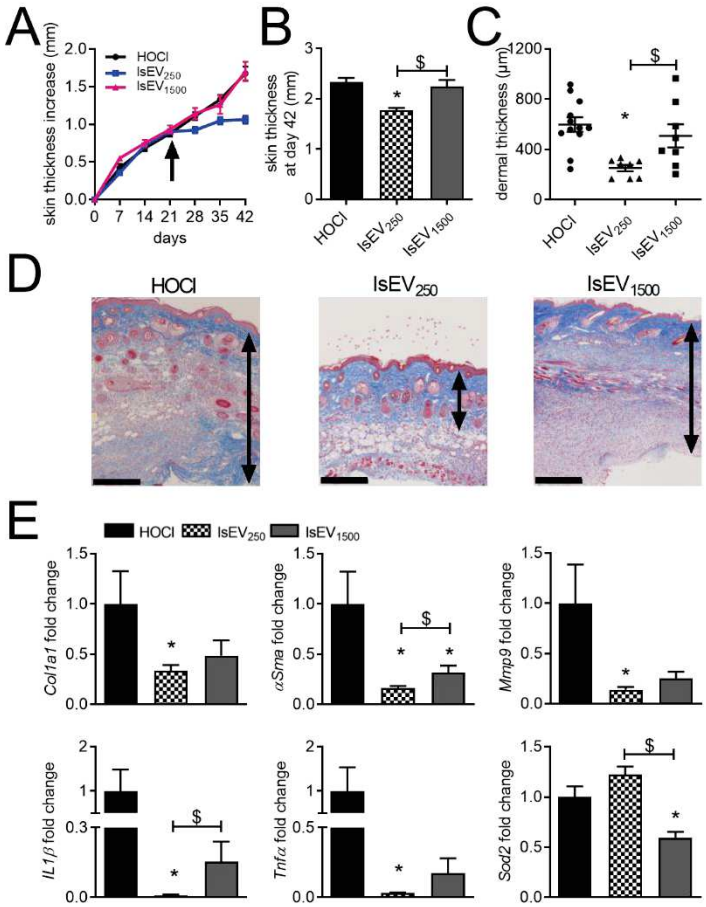
Figure 3. Therapeutic effect of EVs isolated from MSCs pre-activated with low dose of IFNγ in lungs from HOCl-induced SSc. (A) Photographs of representative histological sections of lungs after Masson's trichrome staining in control mice (HOCl) and mice that have been injected with small size or large size extracellular vesicles isolated from non-activated MSCs (ssEV NA or lsEV NA) or MSCs pre-activated by 1 ng/mL IFNγ (ssEV A1 or lsEV A1) (scale bar, 250 µm). **(B)** Gene expression in lung samples as expressed as fold change in treated versus HOCl control mice. Data are presented as mean ± SEM (n=12 to 18 per group from 2 separate experiments (except for data in f); *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 4. Therapeutic effect of EVs isolated from MSCs pre-activated with high doses of IFNγ in the murine model of HOCl-induced SSc. (A) Measures of the skin thickness increase in control mice (HOCl) and mice that have been injected with small size or large size extracellular vesicles isolated from non-activated MSCs (ssEV or lsEV NA) or MSCs pre-activated by 20 ng/mL IFNγ (ssEVs or lsEVs A20) on day 21 (arrow). **(B)** Mean skin thickness in the different groups of mice at day 42. **(C)** Mean dermal thickness on histological sections of skin from the different groups of mice. **(D)** Gene expression in skin samples as expressed as fold change in treated versus HOCl control mice. **(E)** Gene expression in lung samples as expressed as fold change in treated versus HOCl control mice. Data are presented as mean ± SEM (n=6 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 5. Immunosuppressive factors up-regulated in MSCs and in MSC-EVs after pre-activation with IFNγ. (A) Gene expression of known immunosuppressive and anti-fibrotic markers in non-activated MSCs (NA) or MSCs pre-activated with 1 (A1) or 20 ng/mL (A20) IFNγ. Data are presented as mean ± SEM (*: $p<0.05$ versus NA mMSCs; n=3) **(B)** Gene expression of known immunosuppressive and anti-fibrotic markers in non-activated ssEVs and lsEVs (ssEV NA and lsEV NA) and EVs derived from mMSCs pre-activated with 20 ng/mL IFNγ (ssEV A20 and lsEV A20) (n=1).

Supl Figure 1. Dose effect of lsEVs isolated from MSCs in the murine model of HOCl-induced SSc. (A) Photographs of representative histological sections of lungs after Masson's trichrome staining in control mice (HOCl) and mice that have been injected with 250 or 1500 ng of large size extracellular vesicles ($lsEV_{250}$ or $lsEV_{1500}$, respectively) (scale bar, 250 µm). **(B)** Gene expression in lung samples as expressed as fold change in treated versus HOCl control mice. Data are presented as mean ± SEM (n=8 to 12 per group; *: $p<0.05$ versus control or $: $p<0.05$ versus the indicated group of mice).

Figure 1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IsEV1500
days
B
skin thickness at day 42 (mm)
C
dermal thickness (µm)
D
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IL1β fold change
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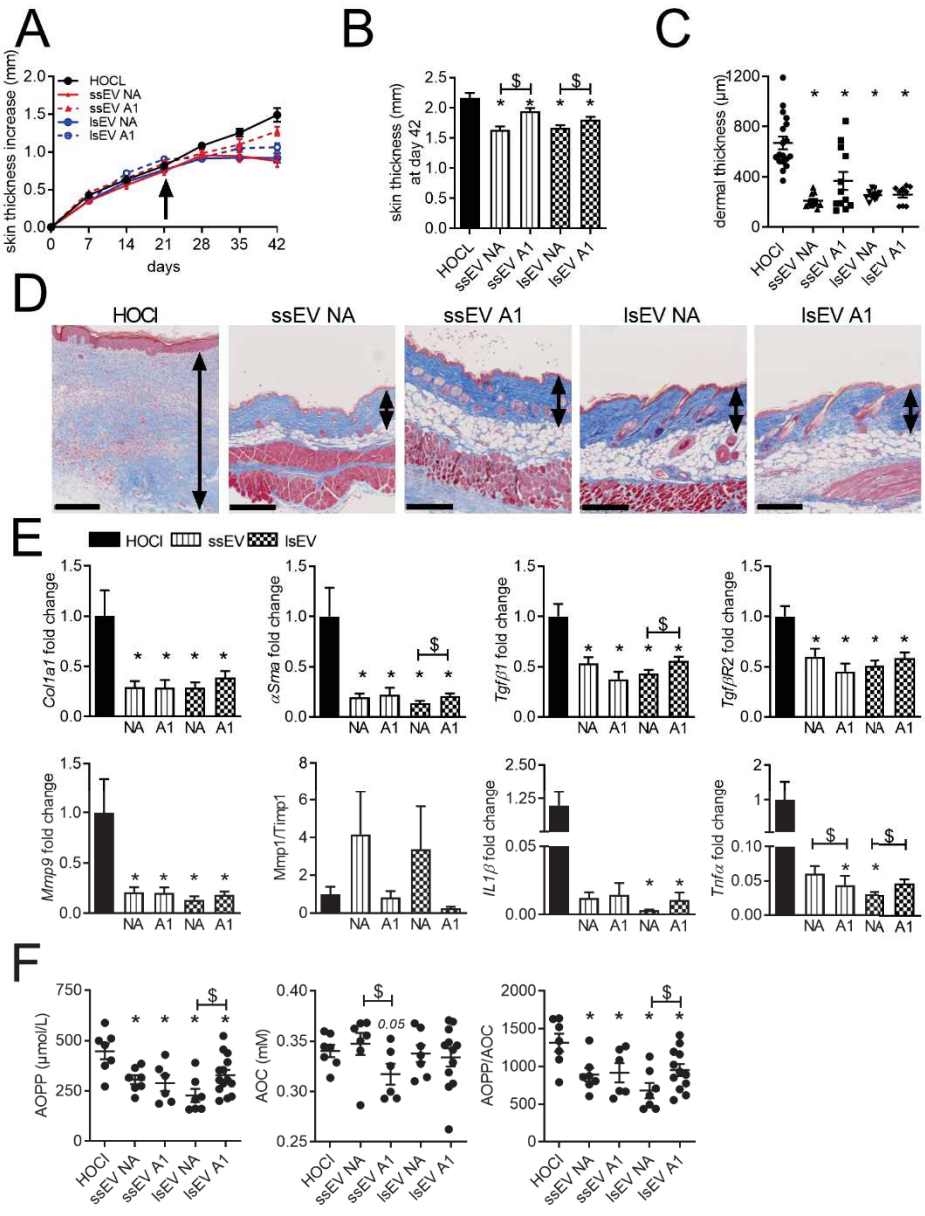
Figure 2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days
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dermal thickness (µm)
ssEV
lsEV
Col1a1 fold change
αSma fold change
Tgfβ1 fold change
TgfβR2 fold change
Mmp9 fold change
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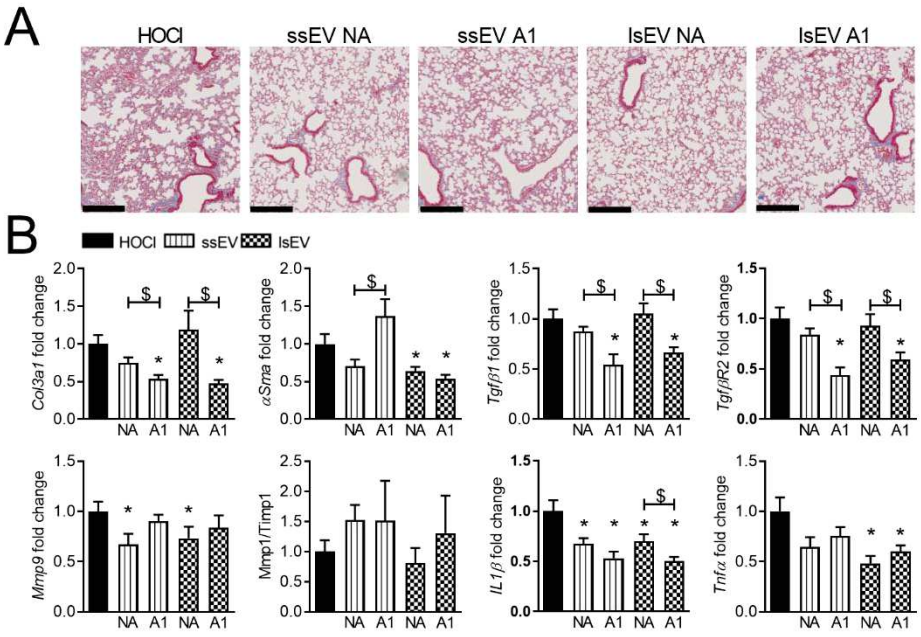
AOPP (µmol/L)
AOC (mM)
AOPP/AOC
NA
A1

Figure 3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αSma fold change
Tgfβ1 fold change
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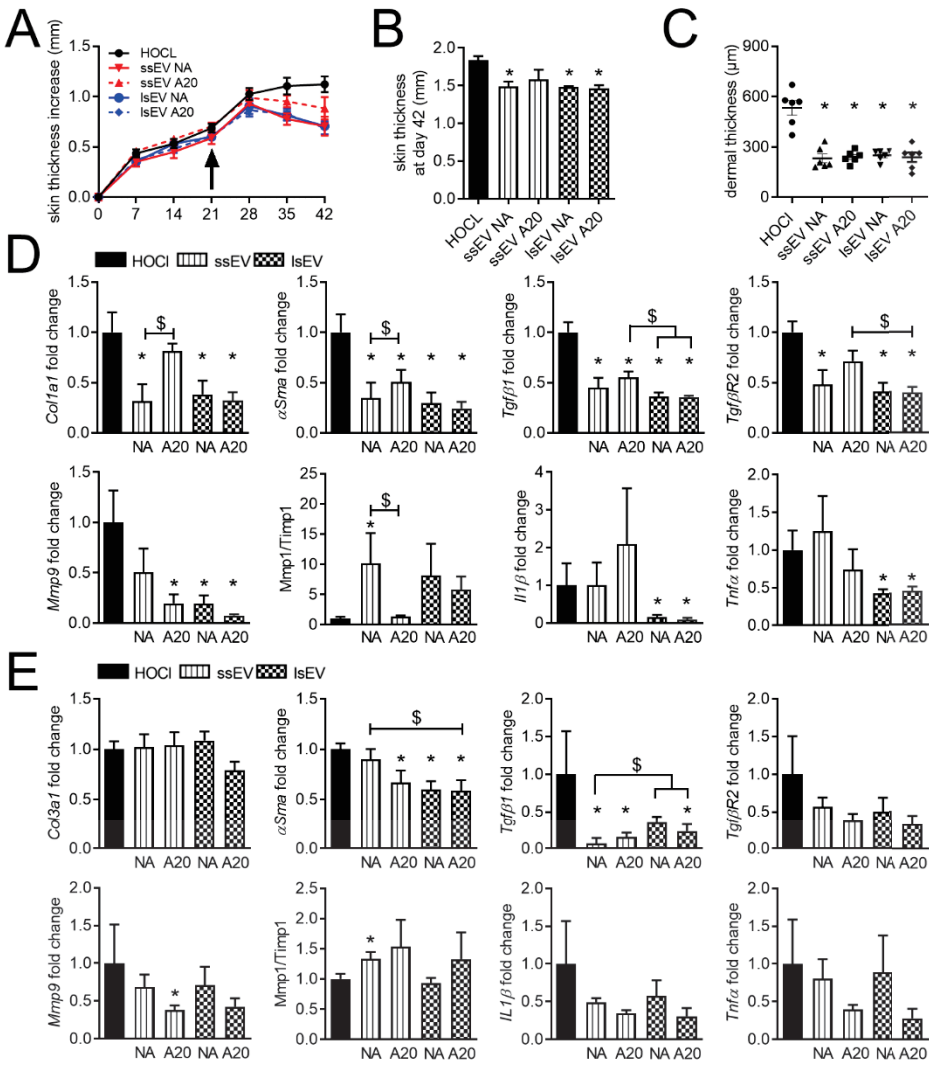
Mmp9 fold change
Mmp1/Timp1
Il1β fold change
Tnfα fold change
NA
A1

Figure 4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B
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dermal thickness (µm)
D
HOCl ssEV lsEV
Col1a1 fold change
αSma fold change
Tgfβ1 fold change
TgfβR2 fold change
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Il1β fold change
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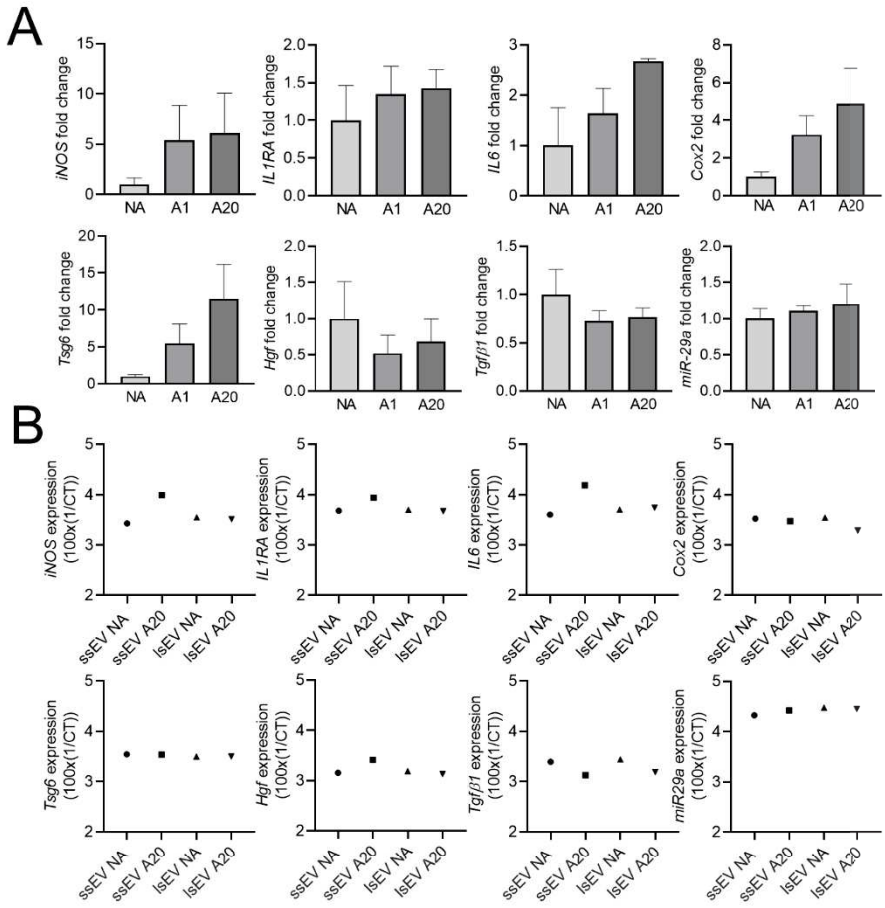
NA A20 NA A20
E
Col3a1 fold change
IL1β fold change

Figure 5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IL6 fold change
Cox2 fold change
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Hgf fold change
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miR-29a fold change
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A1
A20
B
iNOS expression (100x(1/CT))
IL1RA expression (100x(1/CT))
IL6 expression (100x(1/CT))
Cox2 expression (100x(1/CT))
Tsg6 expression (100x(1/CT))
Hgf expression (100x(1/CT))
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miR29a expression (100x(1/CT))
ssEV NA
ssEV A20
lsEV NA
lsEV A20

Supl Figure 1

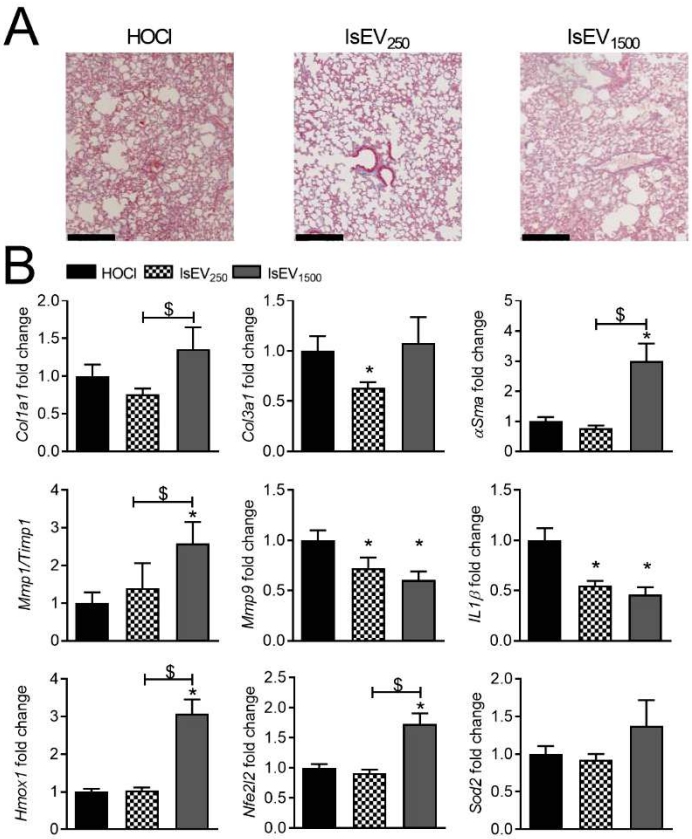

Table 1

Gene	sequence forward	sequence reverse
Acta2(αSma)	AAGGCCAACCGGGAGAAAAT	AGCCAAGTCCAGACGCATGA
Col1a1	TGTTCAGCTTTGTGGACCTC	TCAAGCATACCTCGGGTTTC
Col3a1	CGGTGAACGGGGCGAAGCTGGTT	GACCCCTTTCTCCTGCGGCTCCT
Cox2	GCATTCTTTGCCCAGCACTT	AGACCAGGCACCAGACCAAAGA
Gapdh	GGTGCTGAGTATGTCGTGGA	GTGGTTCACACCCATCACAA
Hgf	TGCCCTATTTCCCGTTGTGA	CGCTTCTCCTCGCCTCTCTC
Hmox1	GCAGAGCCGTCTCGAGCATA	GCATTCTCGGCTTGGATGTG
IL1RA	AGGCCCCACCACCAGCTTTGA	GGGGCTCTTCCGGTGTGTTGGT
IL1β	TTTGACAGTGATGAGAATGACCTGTTC	TCATCAGGACAGCCCAGGTCAAAG
IL6	TGGGACTGATGCTGGTGACA	TTCCACGATTTCCCAGAGAACA
iNOS	CCTTGTTCAGCTACGCCTTC	GCTTGTCACCACCAGCAGTA
Mmp1	TTCAAAGGCAGCAAAGTATGGGCT	CCAGTCTCTTCTTCACAAACAGCAGCA
Mmp9	TCCAGTTTGGTGTCGCGGAGCACG	CAGGGGGAAAGGCGTGTGCCAGA
Nfe2l2	CGCCAGCTACTCCCAGGTTG	ACTTTCAGCGTGGCTGGGGA
Sod2	TCAGGACCCATTGCAAGGAA	TGTGGCCGTGAGTGACGTTT
Tbp	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT
Tgfβ1	TGCGCTTGCAGAGATTAAAA	CTGCCGTACAACTCCAGTGA
TgfβR2	CGACCCCAAGCTCACCTACC	CAACAACAGGTCGGGACTGC
Timp1	CTCCGCCCTTCGCATGGACATT	GGGGGCCATCATGGTATCTGCTCT
Tnfα	AGCCCACGTCGTAGCAAACCA	TGTCTTTGAGATCCATGCCGTTGGC

3ème partie : Effet thérapeutique des cellules stromales mésenchymateuses de patients sclérodermiques et de leurs vésicules extracellulaires.

Dans la troisième partie de ce travail, nous souhaitions évaluer l'effet anti-fibrotique et immunosuppresseur de CSMs de patients atteints de ScS (et de leurs EVs) dans des modèles *in vitro*.

Afin de surseoir aux difficultés d'accès à des fibroblastes de patients, nous avons mis en place, dans notre laboratoire, un modèle *in vitro* de fibroblastes stimulés par du TGFβ1 afin de reproduire une partie des caractéristiques de la maladie présentée par les fibroblastes de patients. Différentes données étaient disponibles dans la littérature et nous avons comparé plusieurs conditions. Finalement, la stimulation de fibroblastes, cultivés dans un milieu contenant seulement 1% de sérum, à l'aide de 5ng/mL de TGFβ1 pendant 24 heures, permet d'induire un phénotype myofibroblastique aux cellules, exprimant plus d'α-sma et de collagènes, en comparaison à des fibroblastes sains. De même, la stimulation au TGFβ1 diminue l'expression, dans les fibroblastes, de MMP1 et de MMP3 et majore celle des TIMPs et de la gélatinase MMP2 : la modulation de ces marqueurs est en défaveur du remodelage de la MEC. Enfin, le TGFβ1 induit l'expression de cytokines impliquées dans l'inflammation, notamment l'IL6 et COX2. Par la suite, nous avons eu accès à des fibroblastes de deux patients sclérodermiques ce qui nous a permis de démontrer que les fibroblastes stimulés aux TGFβ1 se comportent comme des fibroblastes de patients dans nos conditions de culture, ce qui a confirmé la robustesse de notre modèle d'étude.

Nous avons ensuite évalué l'effet anti-fibrotique d'ASCs de donneurs sains sur des fibroblastes qui avaient été au préalablement stimulés au TGFβ1. Nous avons montré que la coculture de ces fibroblastes avec les ASCs saines permet de réverser en partie le phénotype pathologique des fibroblastes. En l'absence de stimulation par le TGFβ1 des fibroblastes sains, les ASCs n'ont pas d'effet mais nous observons une tendance à la diminution de certains marqueurs de fibrose sur les fibroblastes de patients : ces données doivent être confirmées sur un plus grand nombre d'échantillons. Nos données préliminaires montrent que l'addition d'EVs produites par des ASCs saines sur des fibroblastes stimulés au TGFβ1 ne semble pas reproduire l'effet anti-fibrotique des ASCs alors que le SN de culture a un puissant pouvoir anti-fibrotique.

Enfin, nous avons évalué l'effet thérapeutique des ASCs de patients sclérodermiques dans le modèle de fibroblastes de donneurs sains stimulés au TGFβ1 et nous avons observé qu'elles ont la même efficacité que des ASCs saines. En outre, contrairement aux ASCs saines, les ASCs sclérodermiques réduisent l'expression de l'α-sma et du collagène 1 et augmentent celle de MMP1 sur des fibroblastes sains non stimulés, suggérant que ces ASCs pré-conditionnées par l'environnement pathologique chez le patient auraient conservé une fonction anti-fibrotique après culture *in vitro*.

Parallèlement, nous avons comparé l'effet immunosuppresseur des ASCs de patients sclérodermiques à celui d'ASCs de donneurs sains. Pour cela, nous avons utilisé le test de prolifération lymphocytaire en utilisant des PBMCs de donneurs sains. Ainsi, nous avons montré que les ASCs de patients ont un effet anti-prolifératif sur les PBMCs qui est dépendant de la quantité de cellules utilisées. Nous avons également montré que cet effet immunosuppresseur des ASCs sclérodermiques est similaire à celui des ASCs saines.

En résumé, cette partie de mon travail de thèse a permis de mettre en place, dans notre laboratoire, un modèle d'étude in vitro de la fibrose, indépendamment de l'accès aux prélèvements extrêmement limités de patients. De plus, dans les limites attribuables à l'utilisation d'un modèle *in vitro*, ce travail a permis de montrer que les ASCs de patients sclérodermiques présentent un effet anti-fibrotique et immunosuppresseur semblable à celui des ASCs de donneurs sains. Il reste à évaluer le mécanisme d'action de ces ASCs notamment en évaluant l'effet des EVs produites par les ASCs dans ces deux modèles in vitro. Enfin, pour des questions techniques, nous n'avons pas pu évaluer, dans le temps imparti à ce travail de thèse, l'effet thérapeutique des ASCs de patients sclérodermiques dans le modèle murin de sclérodermie, comme nous l'aurions souhaité. Ces expériences *in vivo* dans un environnement complexe de ScS permettra de compléter cette évaluation et de mieux appréhender les fonctions multiples des ASCs sclérodermiques.

Article original 3 en cours de préparation

Adipose mesenchymal stromal cells from patients with systemic sclerosis maintain anti-fibrotic and immunosuppressive capacities

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ABSTRACT

Objectives. Systemic sclerosis (SSc) is a complex disorder resulting from dysregulated interactions between the three main pathophysiological axis: fibrosis, immune dysfunction and vasculopathy, with no specific treatment available to date. Adipose tissue-derived mesenchymal stromal cells (ASCs) and extracellular vesicles (EVs) isolated from their conditioned medium have proved efficacy in pre-clinical evaluation using ASCs isolated from healthy donors. However, in the perspective of clinical translation, the use of autologous ASCs is still discussed. Here, we evaluated the functional properties of ASCs from scleroderma patients (SSc-ASCs) with regards to their anti-fibrotic and immunosuppressive effect.

Methods. Human fibroblasts from healthy (H-Fb) and SSc donors (SSc-Fb) were stimulated with 5 ng/mL of TGFβ1 for 24 h to generate SSc-like fibroblasts (Tβ-Fb) and TGFβ1-stimulated SSc-Fb. Coculture experiments were performed by seeding ASCs from SSc (SSc-ASCs) or healthy (H-ASCs) donors in culture inserts on top of fibroblasts or by adding different doses of ASC-derived EVs for 24 h. Expression of myofibroblastic markers was measured by RT-qPCR. For T cell proliferation assay, different ASC:CTV-labelled PBMC ratios were cocultured for 96 h and T cell proliferation assessed by flow cytometer analysis.

Results. We used a model of SSc-like fibroblasts (Tβ-Fb) that display the main features of SSc-Fb, including hypertrophic morphology and myofibroblastic phenotype. We found out that H-ASCs were able to regulate the expression of most myofibroblastic markers on H-Fb and SSc-Fb, only when pre-stimulated with TGFβ1, whereas SSc-ASCs could modulate the myofibroblastic phenotype of H-Fb pre-stimulated or not by TGFβ1. Of note, the conditioned medium of ASCs, but not their EVs, was effective in this assay. Finally, SSc-ASCs and H-ASCs were equally effective in suppressing T cell proliferation.

Conclusion. H-ASCs and SSc-ASCs exert similar anti-fibrotic and immunosuppressive functions *in vitro* suggesting their functionality although additional validation step using *in vivo* models is likely required.

Keys words. Systemic sclerosis, mesenchymal stem cells, TGFβ1, anti-fibrotic, immunosuppression

INTRODUCTION

Systemic sclerosis (SSc) is a severe disease characterized by generalized dysfunctions, including diffuse fibrosis, general vasculopathy and immune system dysfunction[1]. The knowledge of the mechanisms involved in the pathology is even more complex as SSc is a heterogeneous disease, both clinically and biologically. This certainly explains the obstacles encountered in developing a curative treatment, which is still not available today. Immunosuppressive drugs and hematopoietic stem cell transplantation are two current options to stop the disease course of some selected patients, but these strategies are associated with heavy side effects.

Fibroblasts are widely involved in the physiopathology of SSc and diffuse fibrosis is the main cause of organ dysfunction. In the pathological environment, fibroblasts actively proliferate, accumulate because of reduced apoptosis and, differentiate into myofibroblasts responsible of exaggerated and uncontrolled production of collagens and extracellular matrix (ECM). Transforming Growth Factor β1 (TGFβ1) plays a major role in fibrogenesis[2]. TGFβ1 is an immunosuppressive and pro-fibrotic cytokine secreted in a latent form notably by immune cells and sequestered by components, such as fibrillin-1, in the ECM[3]. Once activated by fibroblast-mediated release of integrins or thrombospondin-1, TGFβ1 activates canonical and non-canonical intracellular pathways that induce biological responses, including pro-fibrotic activity[4]. As a consequence, a strong expression of TGFβ1-responsive gene signature is observed in the skin of patients with severe diffuse cutaneous SSc[5,6]. In addition to fibrosis, dysregulation of the immune system plays also a major role[7], particularly in the initial phase of the disease. Macrophages, lymphocytes or mast cells infiltrate the affected tissues. B lymphocytes seem to be highly activated, as shown by the presence of various autoantibodies in patients. Finally, the endothelium is largely involved in vasculopathy[8]. Endothelial cells are apoptotic while no compensation by neovascularization is possible, and many mediators of endothelial function are deregulated, such as endothelin 1, which is produced in excess.

To overcome the limitations of current therapeutic options, mesenchymal stromal/stem cells (MSCs) are an attractive alternative approach due to their low immunogenicity and immunosuppressive function. Their additional anti-fibrotic and pro-angiogenic properties make them a promising treatment for SSc patients, thus targeting the three main axes of disease dysfunction. Consequently, several pre-clinical studies have reported their therapeutic effect in murine models of SSc and clinical trials are in progress[9]. MSCs can reduce fibrosis and improve the inflammatory and remodeling molecular signature in the skin and lungs of SSc-induced mice[10]. Interestingly, MSCs from human bone marrow (BM-MSCs) and adipose tissue (ASCs) were shown to be equally effective in a murine model of SSc[11]. MSCs exert their pleiotropic effect by contact with target cells but mainly through soluble mediators released in the extracellular environment or contained within extracellular vesicles (EVs). MSC-derived EVs display the main functions of parental cells and have therefore aroused considerable attention as alternative therapeutic strategies in many diseases[12]. They are also of interest for SSc treatment and we have recently demonstrated their beneficial effect in the HOCl-induced murine model of SSc (Rozier et al, submitted manuscript). In a clinical perspective, the debate on the use of autologous versus allogenic MSCs is still ongoing while studies have reported that MSCs from SSc patients may lose part of their functionality[9]. SSc MSCs were reported to retain their immunosuppressive function while they were less prone to differentiate into osteoblasts[13,14]. By contrast, decreased immunosuppressive properties of MSCs have been correlated with the oxidative stress in SSc patient sera[15]. Overall, the contradictory results from the literature underline the need to investigate the impact of MSCs from SSc patients on relevant models of fibrosis or immune response[9].

A better understanding of the functional properties of SSc MSCs is particularly relevant for therapy because the exact mechanism of action of MSCs in the context of SSc is not known to date. In the present study, we evaluated the anti-fibrotic effect of ASCs, and derived EVs, from healthy and SSc subjects using an in vitro model of coculture with TGFβ1-induced SSc-like fibroblasts. We also challenged the immunosuppressive function of SSc ASCs.

MATERIALS & METHODS

Cell isolation and expansion

Adipose tissue-derived mesenchymal stromal cells and fibroblasts obtained from healthy subjects, called H-ASCs and H-Fb respectively, were isolated from surgical residues obtained after aesthetic liposuction or abdominoplasty. ASCs from SSc patients (SSc-ASCs) were isolated from adipose surgical surplus recovered after routine lipotransfer and dermal SSc fibroblasts (SSc-Fb) were isolated from skin microbiopsies collected at the same time from the cannulation site[16]. H-Fb from three donors and SSc-Fb from two donors were used in the study. H-ASCs and SSc-ASCs were isolated 7 distinct donors, respectively. All procedures were approved by the French Ministry of Research and Innovation and the Personal Data Protection Ethics Committee (CPP) (approval AC-2010-1200) and patients gave written informed consent.

Isolation and characterization of ASCs and Fb have been reported previously[16,17]. ASCs were expanded in α-MEM medium containing 10 % fetal calf serum (FCS), 100 µg/mL penicillin/streptomycin, 2 mmol/mL glutamine and 1 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems). ASCs were used between passages 1 to 3. Fibroblasts were cultured in DMEM medium containing 10% FCS, 100 µg/mL penicillin/streptomycin, 2 mmol/mL glutamine and sub-cultured when reaching sub-confluency. Fibroblasts were used between passages 3 to 5.

Production and isolation of EVs

ASCs were seeded at $2x10^4$ cells/cm^2 and cultures for 96 h. After a wash step with phosphate buffer saline (PBS), ASCs were cultured in production medium for 72 h. αMEM containing 20% EV-free FCS was first obtained by recovering the medium after overnight ultracentrifugation at 100,000g. This medium was then diluted to get the production medium containing 3% EV-free FCS. After 72 h, cells were eliminated from the conditioned supernatant by centrifugation at 300 g, 4°C for 10 min whereas debris and apoptotic bodies were discarded by centrifugation at 2 500 g, 4°c for 25 min. Total EVs were then pelleted by ultracentrifugation at 100,000 g, 4°C for 2 h. EVs were characterized by their size and

concentration (by nanotracking analysis), their structure (by cryo-TEM) and their protein content (by flow cytometry and western blot) as described elsewhere (Rozier et al, submitted manuscript). EVs were used freshly prepared for functional assays.

***In vitro* model of TGFβ1-induced SSc-like fibroblasts**

H-Fb were seeded at $5x10^4$cells/cm^2 in DMEM medium containing 10% FCS for 8 h and then (day-2), they were cultured with DMEM plus 1% FCS for 24 h for cell synchronization. At day-1, inductive medium consisting of DMEM with 1% FCS and 5 ng/mL TGFβ1 (R&D Systems) was added for an additional 24 h. In the meantime, ASCs were seeded onto polyethylene terephthalate (PET) culture inserts with pore size of 0.4 µm (BD, Corning, Boulogne-Billancourt) in proliferative medium for 12 h. The following day (day 0 of treatment), ASCs in culture inserts or different doses of EVs were added in DMEM medium containing 1% FCS to the TGFβ1-induced fibroblasts for 24 h (Fig. 1A).

Proliferation and apoptosis quantification

Cultured fibroblasts were washed once with PBS. Proliferation and apoptosis rate were evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay and the Caspase-Glo® 3/7 Assay System, respectively, following manufacturer's instructions (Promega, France). White microplates (Cellstar®; Greiner Bio-one) were used to measure the luminescent signal on a microplate reader (Varioskan, ThermoFisher Scientific).

RNA extraction and RT-qPCR

Total RNA was extracted using 350 µL RLT buffer from the RNeasy Mini Kit according to supplier's recommendations (Qiagen, Courtaboeuf). Reverse transcription of 300 ng RNA was obtained by M-MLV reverse transcriptase (ThermoFisher Scientific). Real-time PCR was done on 10 ng cDNA using SYBR Green I Master mix (Roche Diagnostics) and specific primers (Table 1). Values were normalized to the Ribosomal Protein S9 (*RPS9*) housekeeping gene and expressed as relative expression or fold change using the respective formulae $2^{-\Delta CT}$ or$2^{-\Delta\Delta CT}$.

T cell proliferative assay

Ficol-Hypaque density gradient was used to isolated peripheral blood mononuclear cells (PBMCs) from six buffy coats. The PBMC pool was kept at -80°C until use. After thawing, PBMCs were labelled with Cell-Trace Violet (CTV) according to manufacturer's instructions (Molecular Probes) and suspended in IMDM supplemented with 10% inactivated FCS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM Hepes, 100 U penicillin, 100 µg streptomycin, 0.25 mM β-2-mercaptoethanol. They were then activated by addition of 2.5 µg/mL phytohemaglutinin (Sigma) before addition at the concentration of $2x10^5$cells/well in 96 well plates. Different amounts of ASCs were plated the day before in order to test different ASC:PBMC ratios (1:40, 1:20, 1:10, 1:5). Cocultures were maintained for 96 h. Lymphocyte proliferation was calculated by evaluating the number of CTV^+ cells that have divided more than once, by flow cytometry (Canto II; BD Biosciences). Results were expressed as the mean percentage of PMBC proliferation in absence of ASCs, normalized to 100%.

Statistical analysis

Statistical analyses were performed using GraphPad 8 Prism Software. Data distribution was assessed using the Shapiro-Wilk normality test. A one sample t-test or Wilcoxon test were done when values displayed a normal distribution or not, respectively. When indicated, statistical analysis between two groups were compared using the Student t-test or the Mann-Whitney test when values were parametric or non-parametric, respectively. Data are presented as mean ± SEM.

RESULTS

TGFβ1-induced SSc-like fibroblasts display similar phenotype as fibroblasts from SSc patients.

Because fibroblasts from SSc patients are rare and difficult to obtain in large quantities, we set up a model of SSc-like fibroblasts by TGFβ1-mediated stimulation of dermal fibroblasts from healthy donors (H-Fb). H-Fb were starved 24 h before TGFβ1 stimulation for 24 h and analysis was performed at day 0 and day 1 (Fig. 1A). TGFβ1 activation of H-Fb (Tβ-Fb) resulted in a myofibroblast-like flattened cell shape (Fig. 1B) and cytoskeleton modification as also supported by the significant increase in *α-SMA*

expression at day 0 and 1 (Fig. 1C). Tβ-Fb expressed higher levels of *COL1A1* and lower quantities of the matrix metalloproteinases *MMP1* and *MMP3* compared to non-stimulated H-Fb while *MMP2* (gelatinase A) was significantly increased at day 1 (data not shown). When *MMP* isoforms were correlated to their respective inhibitors (*TIMP1* or *TIMP2*), similar regulation was observed (Fig. 1C). TGFβ1 stimulation increased significantly the expression of *IL6* and slightly *COX2*, while *IL1β* expression was decreased, as soon as day 0 till day 1. This phenotype was no more observed at day 2 (data not shown). Interestingly, the proliferation of Tβ-Fb was significantly increased from day 0 to day 2 compared to H-Fb while the apoptosis rate was similar in Tβ-Fb and H-Fb (Fig. S1). We also observed a high induction of apoptosis in both cells at day 2.

In parallel, similar conditions were used using SSc-Fb from two patients. The morphology of SSc-Fb was altered as shown by their flattened shape before TGFβ1 stimulation and further alterations resembling senescence induction were seen at day 0 and day 1 (Fig. 1D). Basal expression of *αSMA, COL1A1, COL3A1, IL6* was higher in SSc-Fb than in H-Fb while expression of *MMP1, MMP3* was lower in SSc-Fb (Fig. 1E). TGFβ1 stimulation of SSc-Fb resulted in additional up-regulation of *αSMA, COL1A1, COL3A1, IL6,* and down-regulation of *MMP1*, *MMP3* as in Tβ-Fb. Moreover, TGFβ1-stimulated SSc-Fb and Tβ-Fb expressed significantly lower amounts of *IL1β* than non-stimulated SSc-Fb and H-Fb while expression of *MMP2* and *COX2* was not modulated whatever the sample. Altogether, TGFβ1 stimulation regulated the expression of factors characterizing the myofibroblastic phenotype in SSc, both in H-Fb and SSc-Fb. Importantly, Tβ-Fb displayed the main features of SSc-Fb from patients and therefore could be used as a relevant model of SSc-like fibroblasts.

ASCs partly reversed the myofibroblastic phenotype of TGFβ1-stimulated fibroblasts.

We then investigated the effect of H-ASCs on the myofibroblastic phenotype of fibroblasts cocultured under different conditions for 24 h. In a preliminary experiment, we evaluated the ratio of ASC:Fb that was the most efficient in regulating the expression of myofibroblastic markers in Tβ-Fb. No significant difference was seen at the ASC:Fb ratio 1:1, 1:3 or 1:10 and we therefore used the 1:3 ratio in further

experiments (Fig. S2). Addition of H-ASCs on Tβ-Fb and TGFβ1-induced SSc-Fb reversed the myofibrobastic phenotype of both cells as shown by the down-regulation of *αSMA and COL1A1* and up-regulation of *MMP1, MMP2, MMP3* (Fig. 2A). No regulation of *COL3A, IL6, COX2* was observed with Tβ-Fb while *IL6, COX2* were significantly up-regulated in TGFβ1-induced SSc-Fb. By contrast, no regulation of gene expression was observed in H-Fb or SSc-Fb that were not pre-stimulated with TGFβ1 (Fig. 2B). Data indicated that TGFβ1 stimulation conditioned fibroblasts to respond to H-ASC treatment by improving the expression of fibrosis and remodeling markers.

The secretome of ASC but not EVs improved the myofibroblastic phenotype.

Since MSCs are known to act primarily through their secretome and EV production, we tested the effect of EVs on Tβ-Fb. Different doses of EVs, from 8 to 150 ng of total protein amounts, were tested. Those doses correspond to the quantity of EVs produced by $8x10^3$ to $1.5x10^5$ H-ASCs for 72 h and the dose of 25 ng of EVs corresponds to the ASC:Fb ratio of 1:3 used in the experiments described above. Surprisingly, addition of EVs did not significantly modulate the expression of the different markers, except for *COL1A1* and *COX2*, which were up-regulated and *MMP1*, which was down-regulated at the dose of 25 ng (Fig. 3A). We then evaluated the effect of secretome using 6 mL of conditioned medium on Tβ-Fb. The conditioned supernatant drastically reversed the myofibroblastic phenotype, as shown by reduced expression of *αSMA, COL1A1, COL3A1, IL6, MMP2 whereas MMP1, MMP3, COX2* were increased (Fig. 3B). The effect of conditioned supernatant was even much higher than that of ASCs. These results suggested that the anti-fibrotic effect of H-ASCs is mediated through the release of soluble mediators that are not conveyed within EVs.

SSc- and H-ASCs shared similar anti-fibrotic and immunosuppressive properties.

In the context of SSc, MSCs were reported to display dysregulated functions[9]. We therefore evaluated the effect of SSc-ASCs on H-Fb and Tβ-Fb. SSc-ASCs down-regulated the expression of *αSMA, COL1A1, COL3A1* and up-regulated that of *MMP1, MMP2, MMP3, IL6, COX2* in both H-Fb and Tβ-Fb (Fig. 4A).

They were even more efficient than H-ASCs for a number of markers (Fig. 4B). Finally, we compared the immunosuppressive function of H- and SSc-ASCs in a T cell proliferative assay. Both ASCs significantly reduced the proliferation of activated PBMCs when cocultured at the ASC:PBMC ratio of 1:5, 1:10 and 1:20 (Fig. 4C). No significant difference between H- or SSc-ASCs was observed. Altogether, our data provided evidence that ASCs from SSc patients display similar anti-fibrotic and immunosuppressive properties as their healthy counterparts.

DISCUSSION

In the present study, we implemented an *in vitro* model of SSc-like fibroblasts to overcome patient cell shortage. We provided evidence that Tβ-Fb displayed similar myofibroblastic phenotype as SSc fibroblasts stimulated by TGFβ1, suggesting that a pro-fibrotic environment is required for *in vitro* maintenance of myofibroblastic phenotype. We further established that both H- and SSc-ASCs exert similar anti-fibrotic and immunosuppressive functions *in vitro*.

TGFβ1 stimulation of healthy dermal fibroblasts to mimic key characteristics of myofibroblasts has already been reported. Different doses of TGFβ1 in the range of 5-20 ng/mL for 24-48h result in similar up-regulation of α-SMA at mRNA and protein levels [18–21]. Even though 48h incubation is largely used[22–24], some markers are no more deregulated in stimulated fibroblasts, suggesting that 24h stimulation is more relevant for a larger set of markers[21]. Modulated expression of other ECM components (collagens, MMPs, TIMPs, fibronectin)[21–28], as well as other factors involved in fibroblast activation such as connective tissue growth factor (CTGF), platelet-derived growth factor receptor (PDGFR), thrombospondin (TSP) or IL6 is also reported[20–24,27,29,30]. Overall, we reproduced here the main characteristics of previous models and further demonstrated that Tβ-Fb and TGFβ1-stimulated SSc-Fb share the same phenotype indicating the relevance of Tβ-Fb model for mimicking SSc-Fb. Such in vitro model does not perfectly reproduce the in situ phenotype of SSc-Fb, since the protein content of TGFβ1-stimulated H- or SSc-Fb is different[31]. Nevertheless, it allows to partly reflect the disease

phenotype and is helpful in investigating the impact of treatments and clarifying their mechanism of action, in absence of rare fibroblast samples from SSc patients.

Here, we demonstrated that ASCs can regulate several markers associated with fibrosis, extracellular matrix remodeling and inflammation and, partly reverse the myofibroblastic phenotype of Tβ-Fb or TGFβ1-stimulated SSc fibroblasts. Prevention of TGFβ1-induced fibroblast-myofibroblast transition was also reported using murine and human BM-MSCs[32]. Other studies have reported the anti-fibrotic role of ASCs or BM-MSCs on TGFβ1-stimulated fibroblasts from different sources indicated by reduced expression of type I and type III collagens, α-SMA and higher levels of MMPs[33–37]. This is also in accordance with our previous results demonstrating that human ASCs were able to stop the progression of the disease in HOCl-induced mice and improve histological and molecular parameters in skin and lungs[11]. Interestingly, we report that TGFβ1 stimulation of both H-Fb and SSc-Fb is required to see a regulatory effect of ASCs on the cells. This suggests that TGFβ1 modulates signaling pathways in fibroblasts, which activate the expression of mediators that are released in the extracellular environment. These signals are sensed by ASCs, which respond by producing anti-fibrotic factors. This is relevant to the in vivo situation where ASCs are primed by the pathologic environment and adapt their response via the production of appropriate counteracting mediators (for review see[38]). In vitro, HGF was proposed to be one of these anti-fibrotic factors[33,34,39,40]. This also suggests that the TGFβ1-related environmental foot printing is lost in fibroblasts upon in vitro culture. This supports the use of TGFβ1-stimulated H-Fb as a relevant in vitro model, at least to investigate the fibroblast-myofibroblast transition. Our results further demonstrate that this transition is reversible and that ASCs are key actors in this process.

To our surprise, we did not observe a therapeutic benefit when using EVs isolated from H-ASCs on Tβ-Fb. This was unexpected since we have recently demonstrated their *in vivo* efficacy in the HOCl-induced model of SSc when injected systemically (Rozier et al, submitted). This may suggest that the main effect of EVs is not a direct action on the fibroblast-myofibroblast transition but likely on other cellular components dysregulated in SSc. However, two studies have reported that EVs from different

sources of MSCs can down-regulate the expression of α-SMA and type I collagen in TGFβ1-stimulated fibroblasts[41,42]. Too few data are available to definitively conclude. This raises also the question about the requirement of ASC priming to produce proper mediators that are then directed to EVs for optimizing their cargo for a dedicated function. However, it could not be required since we showed that conditioned supernatants from naïve ASCs were powerful in reversing the myofibroblastic phenotype of Tβ-Fb. These are even more effective than ASCs, suggesting that Tβ-Fb may secrete factors that interact with ASCs during the co-culture and attenuate their anti-fibrotic properties. However, we cannot exclude the possibility that the quantity of active soluble mediators was higher in the conditioned supernatants than in EVs. A dose-response experiment would help to resolve this question.

The other important result of the study is that SSc-ASCs display similar anti-fibrotic and immunosuppressive functions as H-ASCs. Likewise, recent data reported identical anti-fibrotic effect of SSc-ASCs on SSc-Fb[16] and similar immunosuppressive function of BM-MSCs from SSc patients[13,14]. Indeed, although controversial studies exist on the altered properties of MSCs from SSc patients, none report alterations on their capacities to reverse the fibrotic phenotype or suppress immune cell proliferation[9]. In these studies, SSc-ASCs that have been expanded from one or up to three passages were used. It can be hypothesized that the pathological foot printing of the SSc environment may have been lost during cultures[43]. TGFβ1 pre-stimulation of SSc-ASCs should be done to determine whether the activation of pro-fibrotic pathways could impact their function. On the contrary, the results may indicate that SSc-ASCs maintain their regenerative potential even in a pathological context. Beyond the mechanism understanding, this would be of interest for therapeutic applications, since both autologous and allogeneic ASCs could be functional and therefore interchangeable.

CONFLICT OF INTEREST

The authors disclose any financial or personal conflict of interest.

AUTHORS CONTRIBUTION

DN, PG, CJ designed the experiments. Experimental work was performed by PR, MM, CB and data were analyzed by PR, MM, DN. PR and DN prepared the manuscript. All authors have contributed to revising the manuscript and final approval.

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LEGENDS OF FIGURES

Figure 1 - *In vitro* model of TGFβ1-stimulated Fibroblasts. (A) Experimental scheme. Dermal fibroblasts from healthy donors (H-Fb) were starved in DMEM medium containing 1% FCS (day-2) for 24 h before stimulation with 5 ng/mL TGFβ1 for another 24 h (day-1). At day 0, treatment was applied for 24 h and samples analyzed (day 1). **(B)** Representative pictures of H-Fb at day 0 or day 1 ± TGFβ1 stimulation (x40 objective). **(C)** Fold change of gene expression for myofibroblast (*αSMA*), collagen (*COL1A1, COL3A1*), remodeling (*MMP1, MMP2, MMP3, TIMP1, TIMP2)* and inflammatory (*IL1β, IL6, COX2*) markers normalized to day-1 (n=6 with 2 replicates of 3 biological replicates). **(D)** Representative pictures of SSc-Fb at day 0 or day 1 ± TGFβ1 stimulation (x40 objective). **(E)** Fold change of gene expression of markers as in (C) (n=4 with 2 replicates of 2 biological replicates). *: $p<0.05$; unpaired t-test.

Figure 2 – ASCs reversed the myofibroblastic phenotype of SSc fibroblasts. (A) Fold change of gene expression in healthy fibroblasts (H-Fb) and fibroblasts from SSc patients (SSc-Fb) for myofibroblast (*αSMA*), collagen (*COL1A1, COL3A1*), remodeling (*MMP1, MMP2, MMP3, TIMP1, TIMP2)* and inflammatory (*IL1β, IL6, COX2*) markers normalized to non-treated Fb (n=8-14 with replicates from 3 H-Fb, 2 SSc-Fb, 5 H-ASCs) (**B)** Fold change of gene expression of markers as in (A) using TGFβ1-stimulated H-Fb (Tβ-Fb) or SSc-Fb (n=4-10 with 3 H-Fb, 2 SSc-Fb, 5 H-ASCs). *: $p<0.05$; unpaired t-test.

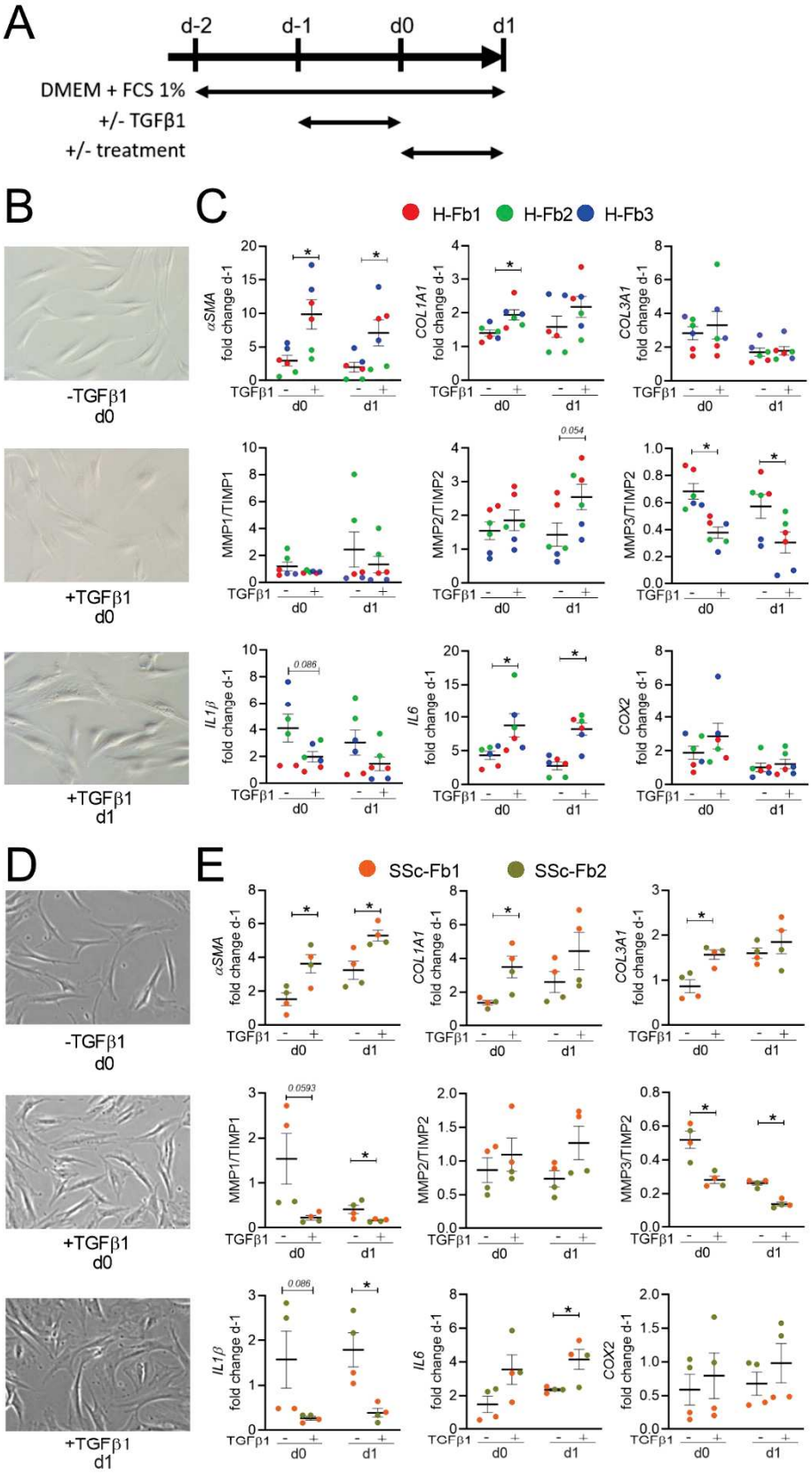
Figure 3 – ASC secretome reversed the myofibroblastic phenotype of SSc-like fibroblasts. (A) Fold change of gene expression in healthy fibroblasts (H-Fb) and fibroblasts from SSc patients (SSc-Fb) for myofibroblast (*αSMA*), collagen (*COL1A1, COL3A1*), remodeling (*MMP1, MMP2, MMP3, TIMP1, TIMP2)* and inflammatory (*IL1β, IL6, COX2*) markers normalized to non-treated Fb (n=18 with 3 replicates from 3 Tβ-Fb, 2 EV preparations; *: $p<0.05$; one sample wilcoxon t test). **(B)** Fold change of gene expression of markers as in (A) using conditioned supernatants (SN) from healthy ASCs (n=2 replicates).

Figure 4 – Anti-fibrotic and immunosuppressive effect of SSc-ASC. (**A**) Fold change of gene expression in healthy fibroblasts (H-Fb) and TGFβ1-stimulated H-Fb (Tβ-Fb) for myofibroblast (*αSMA*), collagen (*COL1A1, COL3A1*), remodeling (*MMP1, MMP2, MMP3, TIMP1, TIMP2)* and inflammatory (*IL1β, IL6, COX2*) markers normalized to non-treated Fb (n=10-21 with 2 replicates from 2 H-Fb, 3 Tβ-Fb, 5-7 SSc-ASCs; *: $p<0.05$; one sample wilcoxon t test). **(B)** Fold change of gene expression of markers as in (A) normalized to non-treated Tβ-Fb (n=18 with replicates from 3 Tβ-Fb, 4 H-ASCs, 3 SSc-ASCs; *: $p<0.05$; one sample wilcoxon t test) **(C)** Proliferation of peripheral blood mononuclear cells (PBMC) cultured in presence of H-ASCs or SSc-ASCs and expressed as percentage of PHA-activated PBMCs (aPBMCs) (n=3 for H-ASC, n=5 for SSc-ASC; *: $p<0.05$, one sample t-test)

Figure S1 – TGFβ1 stimulation induced proliferation of H-Fb. (A) Proliferation of healthy fibroblasts (H-Fb) or TGFβ1-stimulated H-Fb (Tβ-Fb) estimated using the CellTiter-Glo® Luminescent Cell Viability Assay (CTG). Data were normalized to H-Fb at day-1 (n=8-12 from 3 H-Fb; *: $p<0.05$ compared to H-Fb at day-1 using one sample t test and $: $p<0.05$ compared to non-stimulated H-Fb at the same day, using One-way Anova. **(B)** Apoptosis of H-Fb or Tβ-Fb estimated using the Caspase-Glo® 3/7 Assay System and expressed as the caspase:CTG ratio (n=8-12 from 3 H-Fb; *: $p<0.05$ compared to H-Fb at day-1 using one sample Wilcoxon test and $: $p<0.05$ compared to non-stimulated H-Fb at the same day, using Kruskall-Wallis test).

Figure S2 – Dose effect of ASCs on TGFβ1-stimulated fibroblasts. Fold change of gene expression in healthy fibroblasts (H-Fb) and TGFβ1-stimulated H-Fb (Tβ-Fb) cultured with healthy ASCs (H-ASCs) at different ratios. Expression for myofibroblast (*αSMA*), collagen (*COL1A1, COL3A1*), remodeling (*MMP1, MMP2, MMP3, TIMP1, TIMP2)* and inflammatory (*IL1β, IL6, COX2*) markers was normalized to non-treated Tβ-Fb at day1 (n=8-14 with 3-4 replicates from 3 H-Fb, 3 H-ASCs; *: $p<0.05$ using one sample or Wilcoxon test).

Figure 1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MMP3/TIMP2
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IL6
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Figure 2

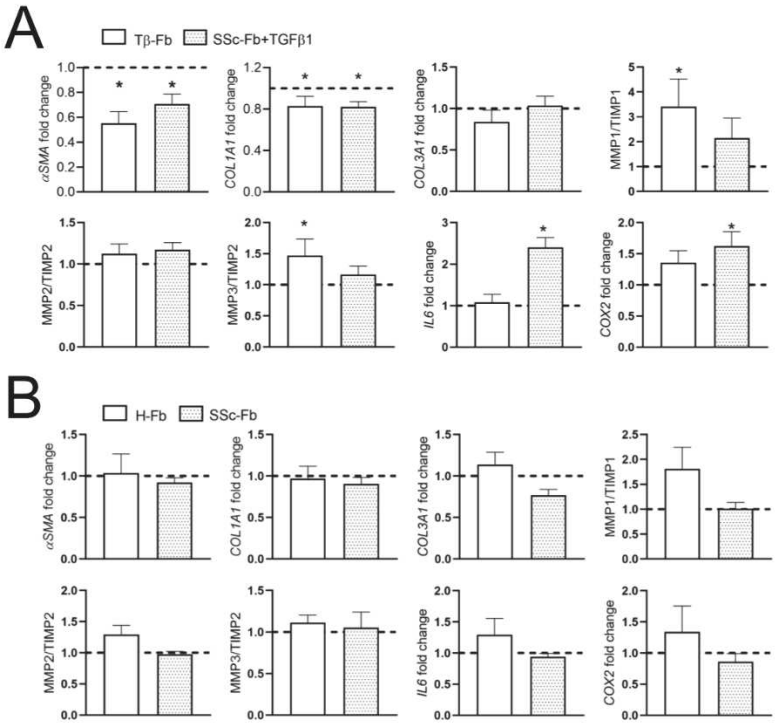

Figure 3

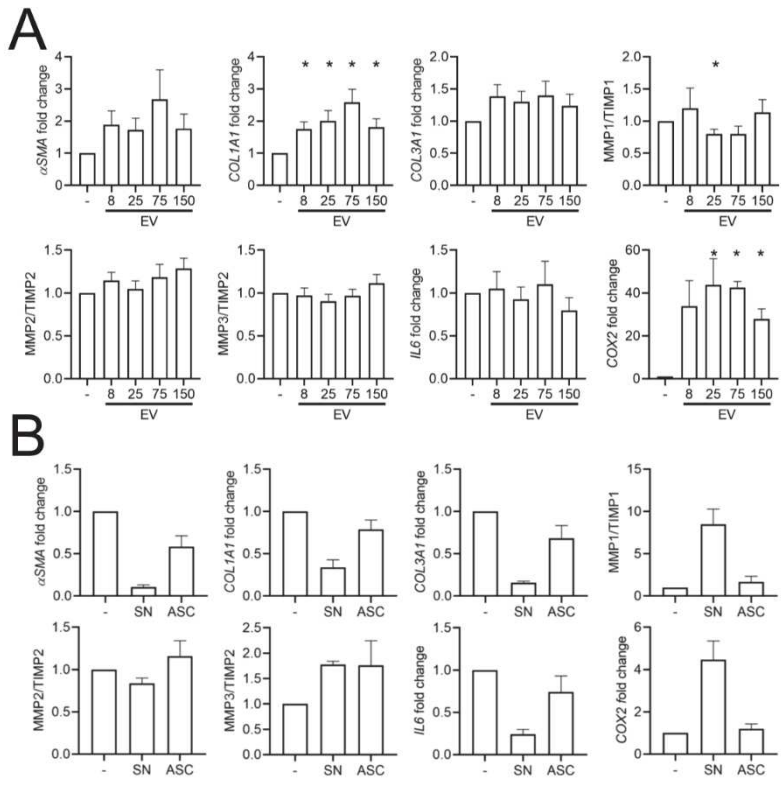

Figure 4

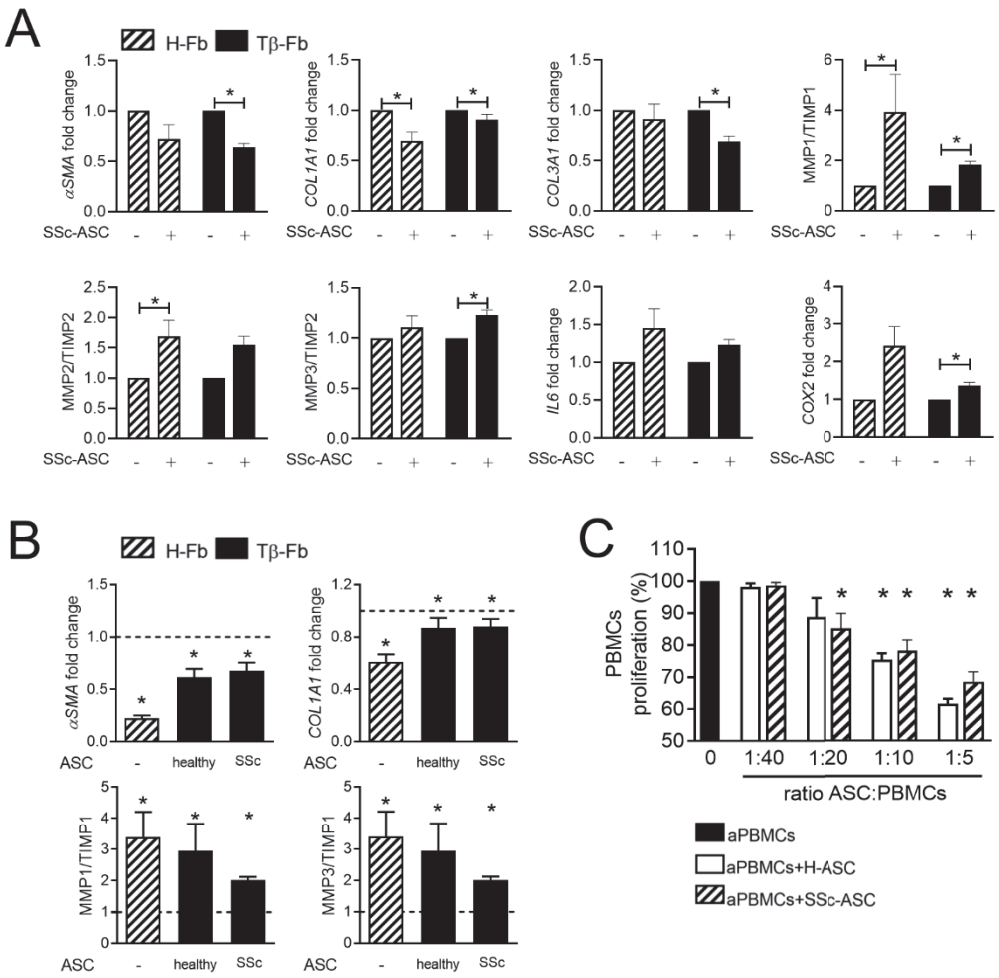

Figure Suppl 1

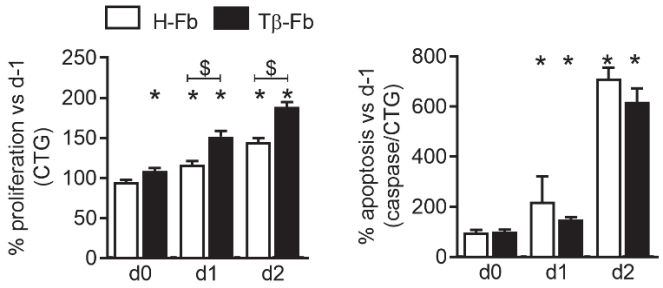

Figure Suppl 2

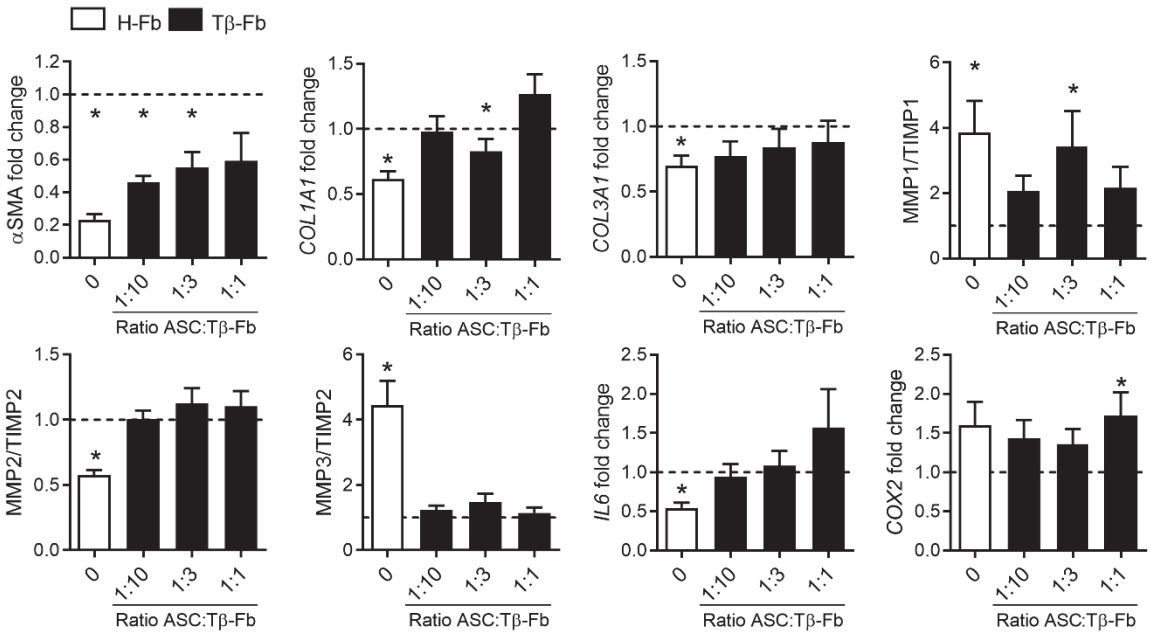

Table 1

Gene	sequence forward	sequence reverse
ACTA2(αSMA)	CATCGGGATGGAGTCTGCTG	AGAAGCATTTGCGGTGGACA
COL1A1	CCTGGATGCCATCAAAGTCT	CGCCATACTCGAACTGGAAT
COL3A1	CGCCCTCCTAATGGTCAAGG	AGGGCCTGAAGGACCAGCTT
COX2	CGGTGAAACTCTGGCTAGACAG	GCAAACCGTAGATGCTCAGGGA
IL1β	TGGCTTATTACAGTGGCAATGAGGAT	TCGGAGATTCGTAGCTGGATGCC
IL6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
MMP1	AGGCCCAGGTATTGGAGGGGA	GCCGATGGGCTGGACAGGATT
MMP2	GCCGTCGCCCATCATCAAGTT	ATAGAAGGTGTTCAGGTATTGCACTG
MMP3	GTACCCACGGAACCTGTCCCTC	TTGCGCCAAAAGTGCCTGTCT
RPS9	GATTACATCCTGGGCCTGAA	ATGAAGGACGGGATGTTCAC
TIMP1	CCGGGGCTTCACCAAGACCT	AGGCAAGGTGACGGGACTGG
TIMP2	AGGGCCAAAGCGGTCAGTGA	AACGTCCAGCGAGACCCCAC

DISCUSSION ET PERSPECTIVES

En résumé, les conclusions de ce travail de thèse se résument en trois points :

- Les EVs sont aussi efficaces que les CSMs pour traiter la ScS murine et miR-29a est responsable, au moins en partie de leur action bénéfique.
- La stimulation de CSMs par l'IFNγ améliore l'efficacité thérapeutique des EVs au niveau pulmonaire chez les souris malades.
- Les CSMs de patients sclérodermiques présentent des propriétés immunosuppressives et anti-fibrotiques équivalentes *in vitro* à celles de CSMs saines.

Nous allons discuter ici des résultats et des perspectives autour de chacun de ces points, puis nous proposerons, dans un 4ème chapitre, différentes perspectives envisagées suite aux travaux que nous avons initiés concernant le rôle physiopathologique des CSMs de patients et de leurs EVs.

I) Effet thérapeutique des EVs et rôle de miR-29a

Bénéfices d'une thérapie acellulaire

Avant mon arrivée au laboratoire, notre groupe avait démontré que les CSMs murines et humaines étaient efficaces pour stopper l'évolution de la maladie dans un modèle murin de ScS très proche de la maladie humaine (621,622). Même si l'implication de certaines molécules dans l'effet thérapeutique des CSMs semble primordiale (467), les mécanismes d'action des CSMs à l'échelle systémique n'avaient pas été démontrés dans la ScS. Alors que le rôle majeur des EVs dans la fonction trophique des CSMs ne fait plus débat, elles ont prouvé leur efficacité thérapeutique à la hauteur de celle de leurs cellules mères pour le traitement de différentes pathologies. Cependant, elles n'avaient pas été évaluées dans le traitement de la ScS. Dans ce travail, nous avons démontré, pour la première fois, que les EVs de CSMs murines et humaines sont un traitement efficace pour stopper l'évolution de la ScS dans un modèle pré-clinique systémique.

Ainsi, les EVs de CSMs pourraient constituer une approche thérapeutique alternative aux CSMs et dénuée des contraintes inhérentes à la thérapie cellulaire.
Tout d'abord, même si les données concernant les CSMs sont rassurantes à ce jour (623), la formation de tissu ectopique et le développement de tumeur sont des risques potentiels liés à l'utilisation des CSMs qui sont des médicaments de thérapie innovante (MTI). Les EVs de

CSMs n'ont pas de noyau cellulaire et ne sont donc pas capables de proliférer ou de se différencier : ainsi, elles ne peuvent être responsables de ces effets secondaires graves. La vigilance reste le maître mot, bien sûr, puisque les EVs restent des outils thérapeutiques complexes pour lesquels tous les mécanismes d'action ainsi que les cibles précises ne sont pas élucidés. D'ailleurs, en restreignant le champ d'action des CSMs à celui de leurs EVs, on restreint les effets indésirables et imprévisibles aux seuls mécanismes d'action attribués aux EVs.

Ensuite, malgré la faible immunogénicité des CSMs, un faible pourcentage de patients développent tout de même des anticorps anti-HLA contre les CSMs de donneurs. En effet, les CSMs expriment des CMH de classe I à l'état basal et des CMH de classe II à l'état activé. Malgré ce, elles échappent au système immunitaire en utilisant notamment leur propriétés immunosuppressives et en évitant ainsi une réaction de rejet (624). Mais ce ne sont pas des cellules immuno-privilégiées et l'allo-immunisation chez certains patients n'autorise pas la réinjection de CSMs, ce qui est une limite importante pour les patients nécessitant des réinjections régulières. Les EVs ont probablement un risque immunologique moindre. En effet, de nombreuses EVs passent dans la circulation sanguine et sont donc collectées lors de dons du sang. Les patients ayant eu recours à de nombreuses transfusions sanguines ne présentent pas, à notre connaissance, de réaction immunitaire contre les EVs présentes dans les produits sanguins même si la compatibilité HLA entre ces EVs et le receveur de transfusion serait un point à explorer pour affirmer cette hypothèse (625,626). Ces données sont importantes, car la thérapie cellulaire ou vésiculaire dans une approche allogénique semble incontournable pour des pathologies aiguës comme l'infarctus du myocarde, l'accident vasculaire cérébral ou encore le SDRA, pathologies qui ne laissent pas le temps d'amplifier des cellules du malade et de produire et isoler leurs EVs.

De plus, l'utilisation de CSMs en clinique nécessite la plupart du temps, des étapes d'amplification des cellules qui sont extrêmement couteuses et chronophages, dont la standardisation est un point complexe du fait de la nature vivante de ce matériel biologique. Les EVs sont considérées par les instances réglementaires comme des produits biologiques et comme pour les MTI, l'utilisation en clinique est très encadrée. Les étapes de développement pré-clinique doivent être rigoureusement établies, afin d'assurer une standardisation des lots d'EVs et une sécurité dans leur utilisation. Lorsque ces conditions seront établies, la production des EVs pourra être réalisée à large échelle et leur conservation pourrait être simplifiée afin de pouvoir les utiliser à un instant voulu sans délai, ce qui est un argument important pour la pratique clinique (627) : je pense notamment à la conservation sous forme lyophilisée qui est en cours de développement. Concernant la standardisation des procédures d'isolement et de caractérisation des EVs, un travail collaboratif international vise à améliorer ce point et plusieurs recommandations ont été émises (628,629).

Les EVs de CSMs sont des vecteurs de médiateurs anti-fibrotiques

Les EVs contiennent différents médiateurs qu'elles protègent des enzymes de dégradation présentes dans les compartiments extracellulaires grâce à leur bicouche lipidique. Les EVs de CSMs sont ainsi un véritable vecteur de molécules thérapeutiques, stables dans les liquides biologiques du corps humain, qui sont leur environnement biologique naturel, et capables de délivrer leur contenu à des cibles précises grâce notamment à leurs marqueurs de surface. Au début de ma thèse, aucune étude ne s'était intéressée à l'effet des EVs de CSMs dans la ScS. Ce travail est le premier à démontrer que la présence de miR-29a au sein des EVs de CSMs est nécessaire à leur effet thérapeutique dans le modèle murin de ScS. Nos résultats sont confortés par une étude qui a montré que l'injection de miR-29b par voie intraveineuse améliore localement la fibrose pulmonaire induite à la bléomycine (630). Notre travail a montré l'intérêt thérapeutique de miR-29a dans la fibrose systémique mais un traitement général par une injection intraveineuse de miR-29a serait limité par la courte durée de vie des microARNs non empaquetés dans la circulation sanguine. Une stratégie de sur-expression de miR-29a au sein des CSMs pourrait être une alternative mais nos travaux n'ont pas permis de voir un bénéfice thérapeutique. Il est possible que l'effet thérapeutique observé était déjà maximal ou que la régulation d'autres cibles de miR29a annihile l'effet bénéfique des CSMs. Une autre approche consisterait à intégrer miR-29a dans les EVs, en utilisant des outils de transfert comme exofect^{TM} par exemple qui est un outil ayant fait ses preuves dans le transfert de microARN et de siRNA au sein des EVs (631,632). D'autres outils de transfert sont également en cours de développement préclinique, comme l'extrusion des EVs qui semblent conserver leur fonction malgré leur modification post-production (633).

Dans une perspective clinique, les EVs s'avèrent de très bons vecteurs de microARN, comme cela a été montré pour d'autres molécules thérapeutiques (614,634,635). En effet, elles sont moins immunogènes et moins toxiques qu'un liposome artificiel, elles ont des capacités intrinsèques pour se diriger vers leurs cibles et elles sont stables dans la circulation et les tissus biologiques. De plus, la transfection des EVs, l'inclusion de molécules thérapeutiques dans les EVs ou encore la modification de leurs molécules de surface pour favoriser le ciblage thérapeutique sont différents outils pour améliorer leur effet thérapeutique. Une autre possibilité est également de modifier leur contenu en changeant l'environnement dans lequel les cellules les produisent. C'est donc une deuxième modalité que nous avons testée dans la deuxième partie de ce travail.

II) La pré-activation des CSMs par l'IFNγ augmente l'effet thérapeutique des EVs

Du fait de l'importance du système immunitaire dans la physiopathologie de la ScS, les propriétés immunosuppressives des CSMs sont particulièrement intéressantes pour la thérapie. Ces propriétés sont stimulées naturellement *in vivo* lorsque les cellules sont soumises à un environnement inflammatoire et *in vitro*, par différentes cytokines dont l'IFNγ (636,637). Ainsi les CSMs injectées chez l'animal modulent leur sécrétome en fonction de l'environnement malade dans lequel elles se trouvent. Nous souhaitions nous approcher de cet environnement, en stimulant les CSMs par l'IFNγ, afin d'obtenir des EVs probablement plus proches de celles qu'elles produisent dans l'organisme malade. Nous avons montré que la pré-activation des CSMs par l'IFNγ en culture améliore l'effet thérapeutique des EVs injectées chez la souris malade, ce qui n'avait pas encore été démontré dans la ScS. Nous avons testé deux doses d'IFNγ pour la pré-activation des CSMs et avons observé que le bénéfice thérapeutique primait sur les marqueurs de fibrose à faible dose et sur les marqueurs d'inflammation à forte dose. Dans tous les cas, l'amélioration de l'effet thérapeutique était surtout observable au niveau des poumons. Pour comprendre cet effet différentiel entre la peau et le poumon, cibles dans notre modèle de ScS, une hypothèse serait que la stimulation à l'IFNγ module certains marqueurs de surface des EVs les adressant préférentiellement au tissu pulmonaire. Une autre hypothèse est que le bénéfice thérapeutique des EVs isolées de CSMs non stimulées étant moins prononcé sur le poumon que sur la peau : il est donc plus facile de mettre en évidence une amélioration d'efficacité au niveau des poumons.

Ce bénéfice thérapeutique semble être médié par l'induction de molécules immunosuppressives et anti-fibrotiques dans les CSMs et leurs EVs. En effet, nous avons montré une augmentation de l'expression de différents transcrits de molécules immunosuppressives dans les CSMs et les EVs, dont l'HGF et iNOS. Des résultats similaires ont été montrés avec des EVs d'ASCs humaines exprimant l'IDO après stimulation à l'IFNγ (638). Ces données sont préliminaires et restent à confirmer par des expérimentations complémentaires, notamment à l'échelle protéique. En effet, l'effet thérapeutique des EVs peut être lié au transfert de certains ARNm qui seront transcrits en protéines effectrices dans les cellules cibles, ou au transfert des protéines contenues dans les EVs. Enfin, nous avons évalué l'effet de la pré-activation des CSMs à l'IFNγ sur l'expression de miR-29a dans les cellules et les EVs sans constater de modulation nette de son expression. Ceci est concordant avec les données récentes de la littérature qui n'ont pas démontré de différences notables dans l'expression des microARNs dans des EVs isolées à partir de CSMs humaines pré-conditionnées par l'IFNγ qu'elles soient isolées de moelle osseuse ou de tissu adipeux (639,640).

Cette amélioration d'efficacité *in vivo* est à démontrer dans un autre modèle pré-clinique avant de pouvoir conclure qu'il apporte un bénéfice suffisamment important pour la clinique. En effet, cette différence d'effet entre les EVs isolées de CSM murines activées ou non activées pourrait ne pas être reproduit en fonction des cellules sources utilisées et du modèle d'étude. Une étude a d'ailleurs montré que la pré-activation d'ASCs humaines n'a pas augmenté l'effet immunosuppresseur de leurs EVs *in vitro* (638). De plus, cette stimulation à l'IFNγ pourrait potentiellement induire des effets indésirables. Par exemple, la stimulation à l'IFNγ des CSMs augmente l'expression de leurs CMH de classe I et II (641–643), ce qui les rend plus immunogènes. Une des possibilités pour surseoir à ce problème serait de travailler dans une approche autologue, ce qui semble intéressant pour des pathologies non aiguës pour lesquelles nous pouvons planifier le traitement, comme au cours de la ScS.

III) Effet thérapeutique des CSMs de patients sclérodermiques

L'effet biologique des EVs dépend de la cellule qui les a produites, elles-mêmes dépendant du tissu source qui peut provenir de donneurs sains ou de malades. En thérapie cellulaire impliquant les CSMs, de plus en plus d'équipes travaillent avec des CSMs provenant de tissu adipeux car celui-ci est plus facilement accessible et permet un rendement en cellules isolées plus important. Cependant, lorsqu'il est prélevé chez des malades pour un traitement autologue, il faut s'assurer qu'il ne présente pas d'altérations qui pourraient être à l'origine d'un défaut fonctionnel des CSMs. Cette approche autologue a été retenue dans les essais cliniques de la ScS malgré que le tissu adipeux soit altéré chez les patients sclérodermiques. Il nous semble donc important de s'assurer que les ASCs isolées à partir de tissu adipeux de patients (et leurs EVs) soient aussi efficaces que des ASCs de donneurs sains.

Les données concernant d'éventuelles altérations des propriétés des CSMs de patients atteints de ScS sont controversées dans la littérature. Au démarrage de ma thèse, la plupart des travaux avait évalué les caractéristiques de CSMs isolées de moelle osseuse et certains avaient mis en évidence des altérations (644). Concernant les ASCs de patients ScS, l'absence d'altérations au niveau phénotypique et de leur capacité de différenciation a été démontrée mais les ASCs étaient prélevées chez des patients atteints de sclérodermie localisée et non systémique (645). Une autre équipe a montré qu'elles prolifèrent de la même façon que des ASCs saines et qu'elles ont un effet pro-angiogénique semblable (646). A l'inverse, une étude a mis en évidence une altération dans la prolifération et les capacités de migration des ASCs de patients sclérodermiques (647). Quoiqu'il en soit, nous n'avions pas de données sur le potentiel anti-fibrotique et immunosuppresseur des ASC de patients sclérodermiques (ASC-ScS).

Notre travail a montré que les ASCs-ScS ont un potentiel anti-fibrotique et immunosuppresseur *in vitro* semblables à celui des ASCs de donneurs sains. Dans ce projet, du fait de la rareté des fibroblastes de patients ScS, nous avons travaillé dans un modèle de coculture de fibroblastes au départ sains, stimulés par le TGFβ1 et avons validé ce modèle en le comparant au comportement de fibroblastes de patients atteints de ScS. De manière complémentaire à des travaux publiés récemment (358), nous avons conclu que les ASC-ScS ne présentent pas d'altération majeure dans leurs fonctions anti-fibrotiques ou immunosuppressives. Toutefois, nous ne pouvons pas utiliser directement des ASCs fraichement isolées, une étape d'amplification est nécessaire. De ce fait, nous ne pouvons pas exclure que les cellules testées ont perdu *in vitro* leurs caractéristiques initiales. De plus, comme tous les modèles *in vitro*, le modèle utilisé dans notre étude ne reproduit pas l'ensemble de la pathologie sclérodermique et n'est donc pas suffisant pour attester de leur efficacité thérapeutique. C'est pourquoi, il nous semble primordial d'évaluer l'effet thérapeutique des ASCs-ScS *in vivo*, ce que nous allons débuter très prochainement. En effet, pour des raisons techniques et de restriction d'accès pour des raisons sanitaires, nous n'avons malheureusement pas pu effectuer cette partie de mon projet de thèse dans le temps imparti. Cette évaluation va contribuer grandement à répondre à cette question essentielle : peut-on utiliser des CSMs de patients dans une approche autologue avec le même espoir thérapeutique que les CSMs de donneurs sains ?

L'évaluation *in vitro* va permettre d'investiguer des pistes pour décortiquer les mécanismes d'action des ASCs. Nos données préliminaires n'ont pas montré d'effet anti-fibrotique des EVs produites par des ASCs saines *in vitro* alors que l'utilisation du surnageant de culture des ASCs a un effet anti-fibrotique puissant. Nous devons bien sûr reproduire cette expérimentation avec différents prélèvements de patients avant de conclure définitivement que l'effet des ASCs est lié à la production de médiateurs solubles non pris en charge dans les EVs. Nous avons donc pour projet d'évaluer l'effet thérapeutique de nouveaux prélèvements d'EVs d'ASCs saines et de patients sclérodermiques. Si nous confirmons ces résultats, nous pourrons comparer le protéome des SN de culture et des EV afin d'évaluer la présence de molécules actives d'intérêt thérapeutique et décrypter le mécanisme d'action des ASCs dans ces effets anti-fibrotiques et anti-inflammatoires.

Finalement, la compréhension des mécanismes d'action responsables de l'effet thérapeutique des CSMs et de leurs EVs sur les fibroblastes ou les PBMCs dans nos modèles nous permettrait de définir un « potency assay » afin de tester différents prélèvements avant de les utiliser en clinique. En effet, si tous les prélèvements ne se comportent pas de la même façon, l'identification du meilleur traitement pour un patient donné serait un outil puissant pour optimiser et/ou personnaliser le traitement.

De plus, l'identification d'une molécule thérapeutique responsable de l'effet des ASCs et de leurs EVs sur ces fibroblastes en culture, pourrait également aider à l'élaboration d'une stratégie thérapeutique plus ciblée. En effet, dans la partie précédente, nous avons identifié miR-29a comme étant nécessaire à l'effet thérapeutique systémique des CSMs et de leurs EVs dans un modèle animal. Mais nous ne savons pas d'une part, si miR-29a agit sur les fibroblastes et/ou sur d'autres cellules cibles et d'autre part, si l'apport de miR-29a permet de restaurer des niveaux physiologiques de miR-29a au sein des tissus. Cette année, une autre équipe a démontré qu'un traitement par des CSMs restaure le niveau tissulaire de miR-29a dans un modèle de fibrose pulmonaire et permet d'améliorer la maladie (648), mais les auteurs n'ont pas apporté la preuve que miR-29a était apporté par les CSMs. De plus, ils démontrent la restauration du niveau de miR-29a au niveau tissulaire et non au niveau cellulaire. L'utilisation d'un modèle *in vitro* apportera ici l'avantage de pouvoir étudier cette question à l'échelle cellulaire et ainsi évaluer si les ASCs sont capables de restaurer les niveaux basaux de miR-29a au sein des fibroblastes sclérodermiques. Si nous démontrons la modulation de l'expression de miR-29a dans les fibroblastes à la suite de leur coculture avec les ASCs, une autre question intéressante serait de comprendre les mécanismes sous-jacents. En effet, les ASCs peuvent apporter directement miR-29a au sein de fibroblastes malades ou bien induire son expression par l'intermédiaire de l'effet lié à d'autres molécules qu'elles libèrent, comme l'HGF. Une étude a en effet démontré que l'HGF induit l'expression de miR-29a au niveau de cellules hépatiques (333). Au total, ces perspectives permettraient de contribuer à la compréhension des mécanismes d'action et élaborer de nouvelles approches thérapeutiques plus ciblées et plus personnalisées.

IV) Perspectives : rôle physiopathologique des CSMs et de leurs EVs dans la ScS

Initialement, une partie de ce projet visait l'étude du rôle des CSMs et de leurs EVs dans la physiopathologie de la ScS. La ScS est une maladie systémique et le rôle potentiel des EVs dans la diffusion de la maladie est possible. Le lien entre les CSMs et les fibroblastes ainsi que l'implication majeure du fibroblaste dans le processus pathologique ont été détaillés dans l'introduction de ce manuscrit. Ainsi, nous émettons l'hypothèse que les EVs diffusent la maladie au niveau systémique et proviennent de CSMs dont le sécrétome est altéré du fait, notamment, d'une niche environnementale pathologique.

Pour étayer notre hypothèse, nous avons initié un travail en collaboration avec la plateforme de protéomique clinique du CHRU de Montpellier, afin de comparer le contenu en protéines des EVs isolées à partir d'ASC saines et celui d'EVs isolées à partir d'ASCs-ScS. J'ai récemment isolé des EVs produites par des ASCs de cinq donneurs sains et de cinq patients atteints de SSc : les échantillons sont en cours d'analyse par la plateforme. J'ai quantifié les EVs produites et je les ai caractérisés en NTA. Nous avons observé que les ASCs-ScS produisent significativement plus d'EVs que des ASC saines, que ce soit en nombre de particules ou en termes de contenu total en protéines. L'analyse en spectrométrie de masse devrait nous apporter des informations au niveau quantitatif et qualitatif sur les protéines contenues dans les EVs d'ASCs saines ou d'ASC-ScS. Par la suite, l'identité des protéines identifiées de manière putative devra être confirmée par des techniques ELISA et/ou de western blot et leur rôle fonctionnel validé dans des modèles *in vitro* dédiés.
Les résultats de l'analyse des protéomes pourront potentiellement apporter des réponses à plusieurs questions. D'une part, un protéome différent entre ASCs saines et ASC-ScS pourrait expliquer qu'après injection, les ASCs de patients puissent être plus ou moins efficaces et permettre d'identifier les molécules thérapeutiques potentielles. D'autre part, l'étude de ces différents protéomes pourrait permettre d'identifier des molécules « pathologiques » à l'origine du transfert de la maladie à l'échelle systémique, même si cela reste une hypothèse et qu'il faudra la démontrer dans un modèle pré-clinique pertinent.

Enfin, nous avons émis une autre hypothèse selon laquelle les EVs pathologiques proviendraient de fibroblastes malades et seraient transmises aux ASCs des patients, provoquant des altérations fonctionnelles. Dans cette perspective, nous nous sommes intéressés à l'effet de fibroblastes malades sur des ASC saines et avons conservé tous les ARNs des ASCs des cocultures réalisées dans la 3ème partie de ce projet. Les résultats obtenus permettront d'évaluer à l'échelle moléculaire l'effet de fibroblastes sains ou malades sur les ASCs saines et seront une première étape dans la compréhension du rôle physiopathologique des EVs dans la ScS.

En conclusion de cette partie, nous souhaitons contribuer à la compréhension des mécanismes physiopathologiques de la ScS avec trois perspectives envisagées. Premièrement, elle permettrait d'améliorer l'approche thérapeutique par les CSMs et leur EVs en privilégiant un traitement autologue ou allogénique. Sur ce point, nos résultats montrent que les CSMs de patients ont les mêmes propriétés que des CSMs saines dans nos modèles *in vitro*, tout en étant conscients que les prélèvements utilisés ont peut-être perdu leurs caractéristiques lors de l'étape d'amplification *in vitro*. Si nos futures études chez l'animal confirment leur efficacité thérapeutique, nous pourrons soutenir l'approche autologue en thérapie cellulaire dans la ScS. Deuxièmement, cette meilleure compréhension de la

physiopathologie de la maladie nous permettrait d'identifier une ou plusieurs voies dérégulées et de nouvelles cibles thérapeutiques à développer. Sur ce point, si nous arrivons à identifier une protéine qui aurait un intérêt fonctionnel dans la pathologie et dont le niveau d'expression dans les EVs de patients serait significativement différent à celui retrouvé dans les EVs de donneurs sains, nous pourrions proposer une nouvelle approche thérapeutique visant à apporter ou au contraire réprimer l'expression de cette protéine nouvellement identifiée. Troisièmement, les EVs circulantes au niveau sanguin sont facilement accessibles par une ponction de sang veineux qui est un acte quotidien en pratique clinique. Ainsi l'identification de particularités sur les EVs de patients pourraient potentiellement constituer un nouveau biomarqueur de la ScS. Il serait évidemment nécessaire de confirmer la présence de ces EVs dans le sang des patients dans une étude clinique à large échelle et de corroborer la présence de ces EVs aux données cliniques des patients, afin de définir plus précisément l'intérêt de ce potentiel nouveau biomarqueur de la maladie au niveau diagnostic ou pronostic. En effet, identifier précocement la maladie ou une forme particulièrement grave de la maladie serait une réelle avancée pour les patients, à qui nous pourrions proposer un traitement précoce avant que la maladie fibrosante soit installée et quasi irréversible.

En conclusion générale, ce projet de thèse a permis de démontrer que les EVs isolées de CSMs sont aussi efficaces que leurs cellules mères dans le traitement de la ScS murine. Nous avons mis en évidence l'importance de miR-29a dans leur effet thérapeutique dans ce modèle pré-clinique et mis en place un modèle *in vitro* afin de clarifier les effets des CSMs et des EVs à l'échelle cellulaire dans des modèles moins complexes. Cette approche de thérapie acellulaire dont les avantages en clinique chez l'homme sont indéniables, nécessite d'élucider au mieux les mécanismes d'action afin de proposer des tests fonctionnels (potency assay) nécessaires à leur validation thérapeutique dans des essais cliniques à large échelle et potentiellement améliorer leurs effets en sélectionnant/générant des EVs de deuxième génération plus efficaces.

ANNEXES

ANNEXE 1

frontiers in Immunology

REVIEW
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Intriguing Relationships Between Cancer and Systemic Sclerosis: Role of the Immune System and Other Contributors

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Systemic sclerosis (SSc) is an autoimmune connective tissue disorder, characterized by multisystem involvement, vasculopathy, and fibrosis. An increased risk of malignancy is observed in SSc (including breast and lung cancers), and in a subgroup of patients with specific autoantibodies (i.e., anti-RNA polymerase III and related autoantibodies), SSc could be a paraneoplastic syndrome and might be directly related to an immune response against cancer. Herein, we reviewed the literature, focusing on the most recent articles, and shed light onto the potential relationship between cancer and scleroderma regarding temporal and immunological dimensions.

Keywords: Systemic sclerosis (scleroderma), cancer, immunosurveillance, paraneopastic syndrome, immunoediting of cancer, autoimmunity

INTRODUCTION

Systemic sclerosis (SSc, or scleroderma) is a rare connective tissue disorder affecting middle-aged women and characterized by tissue fibrosis, vascular dysfunction (microangiopathy) and autoimmunity with the production of specific autoantibodies (1). Skin fibrosis (or scleroderma) is the hallmark of SSc and a leading cause of disability. According to the extent of skin thickening, two main phenotypes are described: limited or diffuse SSc. In diffuse SSc, multi-organ involvement (i.e., pulmonary fibrosis) is associated with poorer prognosis and shorter life expectancy. Unfortunately, there remains an unmet medical need in SSc, a peculiar autoimmune condition where usual immunosuppressant drugs lack efficacy (2).

With population aging, cancer incidence is continuously increasing and represents a major public health problem. In France for instance, in 2017, 400,000 new cases were estimated, including 214,000 men and 186,000 women (3). The most common cancers were prostate cancer followed by lung cancer and colorectal cancer in men. In women, breast cancer, followed by colorectal cancer and lung cancer were the most common cancers, with an increase in lung cancer incidence that tends to become the second cancer in frequency. On the whole, cancer caused about 150,000 deaths that year.

Many studies have shown an increased risk for malignancy in SSc Similar to the general population, an increase in the incidence of malignant tumors has also been noted in the last decades. Together with a better management of scleroderma-related complications due to medical progress in SSc, cancer has thus become a leading cause of mortality in this disease, resulting in about 11% of deaths (third cause) according to the study on death certificates EUSTAR database (4). As a consequence, overall mortality has not improved in SSc in the last four decades, and the management of cancer including early diagnosis and adapted treatment becomes a challenging issue in this disease (2, 5). If no consensual recommendation has been made to date, some authors have suggested screening strategies, based on the most recent data on identified risk factors in this disease (6, 7).

The association of SSc and cancer is not fortuitous and the temporal clustering observed in some patients raises the possibility of SSc as a paraneoplastic syndrome in some patients, as described in other autoimmune conditions such as dermatomyositis (6, 8). However, the connections between the two are more complex than once imagined, and may result from many and diverse mechanisms (9). While immunosuppressants used to treat this autoimmune condition may lead to cancer development (10, 11), cytotoxic anti-cancer therapies have been associated with the development of scleroderma-like features such as Raynaud phenomenon, digital ischemia and fibrosis (12–15). Moreover, common mechanisms and pathways may be involved both in fibrogenesis and oncogenesis, and recent data have suggested that autoimmunity in SSc may be triggered by antigen mutation in tumor cells (6, 16, 17). In that sense, the immune response observed in these patients could be considered as protective, enhancing anti-tumor defenses. More recently, the advent of immune checkpoint inhibitors to treat numerous cancer types, sometimes triggering autoimmune responses, underscores the importance of immunity in cancer emergence and spread. Altogether, a growing body of data points out the intriguing bilateral relationships between SSc and cancer. Herein, we reviewed the most recent literature on this subject, and shed light onto their relationships through epidemiological, biological, and immunological dimensions.

CANCER IN SYSTEMIC SCLEROSIS: EPIDEMIOLOGY

General Aspects

Published studies demonstrated an increased risk of all cancers in SSc, but the associated factors and the increased risk according to cancer subtypes widely differs. This heterogeneity is probably related to the number of patients included, to different follow-up durations, and to the different methods of analysis used in each study. In the study by Wooten and coll. published in 2008, 3.6 to 10.7% of SSc patients had developed at least one cancer. Lung cancer was the most common, followed by breast cancer. The risk factors included female gender, older age, and cutaneous diffuse form of SSc (18).

In a Danish cohort of 2,205 SSc patients, 222 had cancer after the onset of SSc, a higher incidence than in the general population with a standardized incidence ratio (SIR) of 1.5 (95% CI 1.3–1.7) (19). In this cohort, male patients had a higher risk than female and lung cancer and lymphoma exhibited the highest incidence rates. Of note, there was no association between SSc and breast cancer.

Two meta-analyzes performed in 2013 provided additional information. The first one by Bonifazi et al. included 16 studies involving more than 7,000 patients (20). The relative risk of cancer in SSc patients was 1.75 (95% CI 1.41–2.18). There was a strong association with lung cancer with a relative risk (RR) of 4.35 (95% CI 2.08–9.09), and hematological disorders with a RR of 2.24 (95% CI 1.53–3.29). Again, the association with breast cancer was not confirmed.

The second meta-analysis by Onishi et al. involved more than 6,000 patients through six cohort studies and observed a SIR of 1.41 (95% CI 1.18–1.69) and a higher SIR in male than in female patients (1.85 vs. 1.33) (21). An increased SIR was observed for the following cancers: lung cancer (SIR = 3.18, 95% CI 2.09–4.85), malignant hematological disorder (2.57, 1.79–3.68), hepatocellular carcinoma (SIR = 4.36, 2.00–9.51), and bladder cancer (SIR = 2.00, 1.06–3.77). There was no significant difference between cutaneous limited and diffuse SSc.

Recently, Igusa et al. analyzed the cancer risk in subgroups according to SSc phenotype, autoantibodies, and temporal clustering (22). In this study involving 2,383 SSc patients, 205 patients also had cancer. Surprisingly, the overall risk of cancer was not found increased compared to the general population. However, an increased risk for malignancy was found in anti-RNA-Pol-III-positive patients (SIR = 2.84, 1.89–4.10) and sero-negative patients (SIR = 1.83, 1.10–2.86). In contrast, anti-centromere-positive patients had a lower risk of cancer (SIR = 0.59, 0.44–0.76). Interestingly, among anti-RNA-Pol-III-positive patients, diffuse SSc phenotype was associated with breast cancer, while limited SSc phenotype was associated with lung cancer. These data would allow stratification of cancer risk by clinical and serological phenotype and thus allow targeted screening in this population.

DEMONSTRATED ASSOCIATIONS WITH SPECIFIC CANCERS

Lung Cancer

The association of lung cancer and SSc is widely documented in the literature with a SIR ranging from 4.2 to 5.9 (23–25). In a retrospective Italian study of 318 patients, 16 patients had lung cancer (5%) (26). There was an association with the male gender, a longer duration of the disease, a younger age at the diagnosis of SSc. Pulmonary fibrosis was a risk factor for lung cancer with an OR of 6.7 (95% CI 2.2.−20.7). Anti-Scl70 antibodies were also a risk factor (95% CI 1.7–24.1). Over the sixteen SSc patients with lung cancer, thirteen died because of lung cancer. Another study did not identify any increase in lung cancer incidence compared to the general population, but in this single-center study there was a significant number of lung cancer in their general population (27). Notably, both the existence and the duration of interstitial lung disease (pulmonary fibrosis)

in SSc have been confirmed in numerous other studies as an independent risk factor for cancer (5, 28, 29). Various common mechanisms involving chronic inflammation, tissue remodeling, cell cycle, and the sequestration of carcinogens by fibrosis have been suggested as a possible explanation for this association (5, 6).

Breast Cancer

The incidence of breast cancer in scleroderma patients is extremely variable and discordant according to the studies. This variability remains unexplained to date but is potentially related to study methods and inter-country heterogeneity (19, 30). In an Italian study on 318 scleroderma patients, a significant increase in breast cancer incidence compared with the general population was observed. The SIR was evaluated at 2.1 (95% CI 1.13–3.90) (26). An increased risk of breast cancer has been described mainly in other small cohorts of patients but one of the largest cancer studies (Danish cohort of 2,205 patients with systemic scleroderma, 222 with cancer) and two recent meta-analyses did not confirm these results (19–21). However, a close temporal relationship may be observed between SSc and breast cancer in a subgroup of patients. Indeed, there appears to be a short delay between breast cancer and SSc diagnosis, according to several case series or retrospective cohort studies. In a literature review, a majority of patients (61.4%) were diagnosed with cancer within a period from 1 year before to 1 year after SSc diagnosis (31). In the Italian study by Colaci, the median time between these two conditions was 2.5 years (26). These findings might suggest common pathogenesis pathways between these two diseases (6, 8, 15, 29).

Additionally, the group of SSc patients with breast cancer would exhibit some characteristics, such as the presence of lung fibrosis and the absence of anti-nuclear antibodies (32). Patients who developed breast cancer after the diagnosis of SSc had distinct characteristics from others: older age at the time of SSc diagnosis, more frequent interstitial pneumonia with lung fibrosis, and less frequent familial history of breast cancer. However, more recent studies have suggested a link between specific autoantibodies such as RNA Polymerase 3 (RNA-PolIII), PM/Scl or RNA binding Protein Containing 3 (RNPC) antibodies and breast cancer (7, 17, 33–35).

Concerning the association between SSc and breast cancer, two points should be underlined. First, the female susceptibility observed in SSc suggests influence of the same hormonal factors that are found involved in breast cancer. For instance, elevated prolactin levels and decreased levels of DHEA (dehydroepiandrosterone sulfate) were found in patients with SSc and in those breast cancer patients (36, 37). Secondly, calcium channel blockers (CCB), a cornerstone treatment for SSc vasculopathy, have been suspected as a risk factor for breast cancer in the general population in a case-control study (38). The long-term use of CCB (over 10 years) was associated with an OR of 2.3 (95% CI 1.2–4.9) for ductal carcinomas and an OR of 2.6 (95% CI 1.3–5.3) for lobular carcinomas. Another study showed that patients with CCB had an increased risk of aggressive breast tumors with an OR of 1.96 (95% CI 1.09–3.53) (39). Some authors therefore suggested that CCB are potentially a confounding factor that might explain the increased incidence of breast cancer in SSc observed in some studies (40). However, all these results have recently been questioned by an epidemiological study taking into account confounding factors and involving 28,000 patients (41). The possible pathophysiological relationship between breast cancer and CCB remains unclear, but a hypothetical mechanism might be an impaired functionality of intracellular calcium associated with CCB, particularly in the initiation of the pro-apoptotic signal.

Esophageal Cancer

The standardized ratio of esophageal cancer incidence in scleroderma patients is estimated at 15.9 (95% CI 4.2–27.6) (42). This increased risk of esophageal cancer is undoubtedly related to the high frequency of chronic gastro-esophageal reflux disease (GERD), increasing the risk of Barrett's esophagus, and thus the risks of dysplasia and adenocarcinoma of the esophagus. Several studies focused on this risk, which was estimated at 3% per year for patients with Barrett's esophagus over a three-year prospective study from EULAR network centers (43, 44). Among 46 SSc patients who had Barrett's esophagus at baseline and completed the follow-up, four developed esophageal adenocarcinoma. Dysplasia at baseline was a major factor risk of cancer. These data prompt endoscopic follow-up of Barrett's esophagus in SSc.

Hematological Malignancies

The association between autoimmune diseases and lymphoma has been described in several studies (45). An epidemiological study in Denmark and Sweden of 25,000 patients with non-Hodgkin's lymphoma found an association with autoimmune diseases (including SSc) with an OR ranging from 1.6 to 5.4 (46). In another Swedish study, the risk of lymphoma in SSc patients was increased with an SIR of 2.1 (47). In these studies, associated Sjogren's syndrome was not always reported, although it remains an independent risk factor for the emergence of lymphoma.

Bladder Cancers

The use of high cumulative dose of cyclophosphamide may lead to the development of bladder cancer in SSc, as reported in various studies (5, 48).

Suspected Associations With Other Cancers

Concerning gynecological malignancies, a Canadian study found a higher prevalence of cervical dysplasia in the scleroderma population than in the general population (25.4 vs. 13.8%), particularly in patients with diffuse SSc (49). In another study, overall frequency of human papilloma virus (HPV) was not higher in SSc patients than in general population (50). However, the high risk HPV52 was the most frequent genotype and a greater multi-HPV infection rate was observed in SSc, particularly in diffuse SSc. Interestingly, cyclophosphamide was reported as a risk factor for cervical intraepithelial neoplasia with a dose-dependent cumulative risk (plus 13% increased risk of cervical dysplasia per each increase of one gram according to a series of patients with systemic lupus erythematosus)

(51). These data prompt to screen carefully patients for whom immunosuppressive therapy is indicated.

Autoimmune thyroiditis is a condition commonly associated with SSc. The percentage of patients with hypothyroidism varies from 2.4 to 26% and the percentage of anti-TPO antibodies varies from 12 to 52% according to the studies (52). Some cases of scleroderma patients with thyroid cancer have been reported. In a study of 769 patients with SSc, no significant association was found and only 2 cases of thyroid cancer were observed (42). Another recent study suggested an increase in thyroid papillary carcinomas in SSc patients but all those patients with cancer had autoimmune thyroiditis (53). Once again, chronic inflammation might promote cancer development in this condition.

Rare cases of cutaneous squamous cell carcinoma have been reported in scleroderma patients, mostly associated with localized scleroderma (morphea, including pansclerotic morphea), (5, 54). Intriguingly, some patients with melanoma treated with interferon or immune checkpoint inhibitors have developed SSc (55–57).

Interestingly, a few cases of soft tissue malignant tumors (i.e., sarcomas) have been described in association with SSc, although no statistical link can be established, mainly because of the rarity of both conditions (58–64). This association between connective tissue disease and malignancy highlights the possibility of common mechanisms between the loss of connective tissue homeostasis in SSc and oncogenesis in sarcoma, including angiogenesis defects.

We summarized the demonstrated and putative associations between SSc and cancer in a table (see **Table 1**).

CHRONIC INFLAMMATION, TISSUE HOMEOSTASIS, FIBROSIS AND CANCER DEVELOPMENT

SSc cannot be considered as a systemic inflammatory disease such as systemic vasculitides or inflammatory bowel diseases. However, some locations of the disease (in particular, esophagus and lungs) are exposed to chronic tissue inflammation, which is considered nowadays as a major factor leading to the development of cancer. At least 25% of cancers might be related to persistent inflammation or chronic infection (65). In fact, inflammatory mediators, such as pro-inflammatory cytokines and free radicals, could induce genetic and epigenetic modifications, leading to alterations in the cell signaling pathways and thus breaking normal cell homeostasis. For example, free radicals can randomly induce point mutations in tumor suppressor genes and contribute to the progression of cancer (66–68).

According to this concept, some authors proposed that chronic tissue damage with recurrent tissue repair mechanisms may be one of the mechanisms for the development of lung and esophageal cancer in SSc patients with pulmonary fibrosis and GERD, respectively (15). This phenomenon is well documented for SSc patients with chronic GERD, since these patients exhibit an increased risk of Barrett's esophagus, high-grade dysplasia and esophageal cancer. Similarly, chronic inflammation within fibrosis could contribute to cancer development in SSc patients with lung fibrosis (5, 15).

It is noteworthy that since SSc may be associated with other connective tissue disorders or organ-specific autoimmune diseases, patients may be exposed to an increased specific risk of cancer through these additional autoimmune conditions and their related-chronic inflammation. Notably, primary biliary cirrhosis (whose association with SSc represents "Reynolds syndrome") and autoimmune thyroiditis (Hashimoto's) are associated with increased risk of cholangiocarcinoma (69) and papillary thyroid carcinoma respectively (53).

Another point to consider is fibrosis itself, as a condition potentially associated with cancer. Indeed, the association between pulmonary fibrosis and cancer is well known, especially in patients with idiopathic pulmonary fibrosis (IPF), which represents the key condition of lung fibrosis. About one in ten patients with IPF will develop lung cancer during follow-up (70). Apart from the role of chronic inflammation in pulmonary carcinogenesis, carcinogenic molecules may be sequestrated by fibrosis, secondary to an altered lymphatic drainage (71, 72).

Moreover, mesenchymal cells are crucial for the development of cancer (73). This is well documented in lung cancer, in which tumor-associated fibroblasts contribute to a "growing" loop with malignant epithelial cells, through the production of specific growth factors such as epidermal growth factor (EGF), fibroblastic growth factor (FGF), or transforming growth factor (TGF). In this context, epithelial–mesenchymal transition (EMT) contributes to the development and spread of tumor cells, and is also a source of fibroblasts. This complex process including numerous phenotypic transitions toward mesenchymal cells (i.e., fibroblasts) may thus be potentially implicated both in cancer and fibrosis. On the one hand, EMT has been clearly observed during the development of lung cancer, when epithelial cells transform into malignant cells under the activation of oncoproteins such as mutant Kras (Kirsten rat sarcoma viral oncogene) (74, 75). Thus, EMT may participate to epithelial cell plasticity and modify their properties in the context of lung injury. On the other hand, EMT also represents a potential mechanism in SSc, where epidermal cells may acquire mesenchymal and fibroblastic features under the activation of TGF-beta signaling pathway within lesional skin, further increasing the fibrotic burden (76).

SHARED MECHANISMS IN CANCER AND SYSTEMIC SCLEROSIS

Genetics and Epigenetics

Common features may be observed in cancer and scleroderma, with common actors promoting disease development. Strikingly, a recent gene profiling study revealed oncogenic gene patterns in SSc (77). As abovementioned, the implication of EMT in both diseases is particularly interesting and may originate from common genetic and epigenetic alterations, involving telomere shortening, chromosomal instability, senescence, increased

TABLE 1 | Associations between systemic sclerosis and cancer: evidence from epidemiological data and suspected risk factors.

Cancer	Evidence for association	Mean SIR	Scenario	Comments
Lung	YES	4–6	A,B	There is a strong association between lung cancer and SSc in the presence of interstitial lung disease and anti-scl-70 Ab. Chronic inflammation in PF may lead to tumor development.
Breast	YES	2–3	B,C	Breast cancer is the most frequent cancer found in SSc patients. The risk of developing breast cancer in SSc is associated with the presence of anti-RNA-PolIII Ab through a pathophysiological process leading to paraneoplastic autoimmunity. CCB could also contribute to the development of breast cancer.
Esophageal	YES	15	A	GERD, a hallmark of SSc, is responsible for chronic inflammation in esophagus, and can lead to Barrett's esophagus, dysplasia, and adenocarcinoma.
Bladder	YES	2	C	The use of cyclophosphamide in SSc patients can lead to the development of hemorrhagic cystitis and bladder cancer through a cumulative dose-effect.
Hematological	YES	2	A	An increased risk of lymphoma has been described in numerous autoimmune diseases, including SSc, and may be more specifically associated with the presence of secondary SjS.
Cervix	NO	N/A	C	An increased frequency of high-risk HPV infection has been reported in SSc. The use of cyc may lead to the development of cervical neoplasia in these patients.
Thyroid	NO	N/A	A	Thyroiditis, an autoimmune condition frequently associated with SSc may lead to chronic inflammation of the thyroid and the development of papillary carcinoma.
Skin	NO	N/A	A	Cases of squamous cell carcinoma have been reported in association with morphea, (i.e., localized scleroderma), a condition where chronic tissue inflammation may lead to cancer development.
Sarcoma	NO	N/A	A,B	Few cases of soft tissue malignant tumors (i.e., sarcomas) have been described in association with SSc. No statistical link can be established because of the rarity of both conditions.

Scenario A: SSc leads to cancer development (via chronic tissue inflammation); Scenario B: suspected paraneoplastic phenomenon between SSc and cancer onset; Scenario C: SSc treatments favor cancer development.
SIR, standardized incidence ratio; PF, pulmonary fibrosis; Ab, antibody; CCB, calcium channel blockers; GERD, gastro-esophageal reflux disease; cyc, cyclophosphamide; SjS, Sjogren's syndrome; HPV, human papilloma virus.

proliferation rates, immune deregulation, and impaired cell metabolism.

Telomere Shortening

While telomere maintenance is complex as well as essential for cancer progression (78), deficient telomerase activity and telomere shortening have been both reported in SSc (79, 80). In another fibrotic condition, IPF, telomere shortening is also a potential contributor for the pathological process and reduction in telomere length of circulating leukocytes could even have an impact on overall mortality (81).

Epigenetic Alterations

Epigenetic mechanisms could contribute to the pathogenesis of SSc, as a consequence of the exposure to environmental factors such as silica (see below paragraph 2.4.1). This would result in cytokine network modulation toward the development of autoimmunity. Similarly, an important body of data argues for the essential contribution of epigenetics to the development of cancer. Three main mechanisms should be considered with striking similarities between both diseases: changes in DNA methylation, histone modifications, and microRNAs.

First, a reduced expression of several genes regulating the process of methylation (such as DNA methyltransferase-1, methyl-CpG-binding domain 3, and MBD4) could induce DNA hypomethylation in CD4+ T cells in SSc (82, 83). Recently, Lian et al. observed an overexpression of CD40L in CD4+ T cells from female SSc patients (but not from male patients), which could be explained by a demethylation of specific CpG–DNA within regulating regions of CD40L (84). This overexpression of CD40L may be of great importance in SSc, since the interaction CD40/CD40L play key roles in autoimmune diseases, in particular in SSc. The demethylation within CD40L gene could result in the reactivation of the inactive X chromosome, maybe contributing to the female susceptibility of the disease. Other alterations in the methylation patterns have been observed in SSc. Endothelial cells are concerned by such epigenetic changes, since CpG hypermethylation of the bone morphogenetic protein receptor factor type 2 (BMPR2) was recently reported. Hypermethylation resulted in downregulation of the protein, inducing an increased sensitivity of endothelial cells to apoptosis and oxidative stress (85). Another example is represented by the hypomethylation of TNFSF7, the promoter of CD70, observed in SSc, which results in the overexpression of CD70 by CD4+ T cells (86). In fact, CD70 is a B cell co-stimulatory molecule, whose overexpression may contribute to autoimmunity, as observed in other autoimmune diseases like lupus and Sjögren's syndrome. So, CD70 hypomethylation could contribute to autoimmune diseases whereas hypermethylation would be rather associated with the development of malignancies, in particular breast cancers (87). Last but not least, SSc fibroblasts also appear to

be affected by methylation changes. Indeed, hypermethylation of the CpG rich regions in the Fli-1 (Friend leukemia virus-induced erythroleukemia-1) promoter region was demonstrated in fibroblasts and skin from SSc patients (88). Interestingly, the latter also plays an important role in cancer development and appears as a possible link between autoimmunity and malignancy (89). Furthermore, in an experimental animal model of bleomycin-induced lung fibrosis, the regulatory effects of the methyl CpG binding protein 2 (MeCP2) and its implication in tissue homeostasis have been shown. The authors demonstrated that MeCP2 binds to the methylated region of α-SMA gene promoter and activates its expression (90). They then studied the effects of a loss of expression of MeCP2, using siRNA or knockout mice. Resulting in the methylation of α-SMA promoter, they observed less activation of this gene. Hence, MeCP2 deficient mice were less sensitive to bleomycin, highlighting the pivotal role of MeCP2 in myofibroblast transformation and fibrosis development.

Second, impaired transcription of genes and deregulated gene expression induced by histone modifications may also contribute to the development of both cancer (91) and SSc (83). Notably, novel therapies targeting epigenetic contributors such as histone demethylase and histone deacetylase inhibitors have been developed in the field of oncology and may emerge in the field of pulmonary fibrosis (92). In SSc, a recent study highlights the role of the histone acetyltransferase p300 in the development of fibrosis (93). In this study, increased p300 levels within SSc skin under TGF-beta activation resulted in collagen transcription in a Smad-independent manner involving Egr-1 (early growth response 1). Actually, p300-mediated histone H4 hyperacetylation appears to be a pivotal epigenetic modification in SSc, whose deleterious profibrotic effects could be counteracted by the blocking effects of antiaging deacetylase enzyme sirtuin 1 (SIRT-1) on Smad-dependent transcription (94). SIRT-1 belongs to the group of histone deacetylases called sirtuins (SIRTs) that includes 7 proteins (from SIRT1 to SIRT7) implicated in the regulation of aging process, clock control, and cell metabolism. Interestingly, in this study, the expression of SIRT-1 was found reduced in SSc skin and its activation by resveratrol reversed the fibrotic response of fibroblasts (94). Emerging data corroborate this observation in SSc (95) and also suggests the role of SIRTs proteins in the development of cancer (96). Altogether, SIRTs proteins could contribute to SSc and cancer through different mechanisms: TGF-β signaling, mTOR pathway, oxidative stress and cellular senescence.

Third, numerous microRNAs, including miR-21, miR-29 family and let-7d, have been reported to play key roles in the pathogenesis of cancer and fibrosis, and could even represent potential therapeutic targets in both diseases (77). In SSc, their effects concern collagen gene expression in fibroblasts, collagen degradation, thus extracellular matrix remodeling, apoptosis, and epithelial-mesenchymal transition. Firstly, miR-21 may function as an amplifying factor, enhancing TGF-β signaling events in SSc fibrosis (97). Exosomal miR-21 might be used as a cancer biomarker, and its combination with other miRNAs within a specific panel may become a relevant diagnosis tool for cancer (98). Notably, it has been associated with breast cancer. Secondly, the miRNA-29 family, consisting of miR-29a, miR-29b, and miR-29c, could also be involved in cancer development and fibrosis. miR-29b functions either as a tumor suppressor or an oncogene under specific conditions and could mediate cancer chemo-sensitivity or resistance. The miR-29 family members also appear to be antifibrotic mediators, modulating collagen expression and degradation. Indeed, the expression of miR-29a is reduced both in fibroblasts and skin in SSc (99, 100), and its overexpression induced a reduction of fibroblast proliferation and collagen synthesis *in vitro* (101). A similar reduced expression of miR-29a has also been found both in broncho-alveolar cells from IPF and lung cancer patients, suggesting a common link between the two conditions (102). Altogether, miR-21 and miR-29 family members exhibit synergistic functions to modulate fibroblast fate both in healthy and fibrotic conditions. Thus, an imbalance between these two mediators might contribute to fibrosis. Other miRNAs strongly involved both in cancer and fibrosis are miR-16 (77) and let-7d. The latter is considered as a key regulator of cell proliferation and can act as a tumor suppressor (103). It is also involved in the regulation of EMT and prevention of lung fibrosis (104). Its expression is reduced in SSc skin (105). The role of the miRNAs in cancer and scleroderma also underscores the importance of exosomes in both diseases, since miRNAs are contained and conveyed by such vesicles (106). Interestingly, anti-PM/Scl autoantibodies found in SSc and associated with malignancy recognize a complex that is the human homolog of *saccharomyces cerevisiae* exosome (35, 107). This further strengthens a pathological link between autoimmune response in SSc and cancer.

Common Signal Transduction Pathways

Signal transduction pathways may also be shared by cancer and fibrosis, as highlighted by the recent gene profiling study by Dolcino et al. revealing oncogenic signature in SSc patients, involving numerous well-known oncogenic proteins such as Ras, janus kinase (jak), Avian Myelocytomatosis Viral Oncogene Homolog (c-myc), B-cell lymphoma (bcl-2), Myeloid differentiation primary response 88 (myd88), poly(ADP-ribose) polymérase (PARP), and the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (77). Interestingly, the transcription factor Fra (Fos-related antigen) is also involved both in breast cancer (108) and in scleroderma (109).

The PI3K/Akt pathway is implicated in lung fibrosis, and has been documented in experimental *in vitro* and *in vivo* models and may be a target for therapy (110). The antagonist of this pathway, Protein Phosphatase and Tensin homolog (PTEN), is also implicated in SSc: its expression is reduced in skin fibroblasts from patients with cutaneous diffuse SSc. Furthermore, the gene deletion of PTEN in adult mouse fibroblasts induced skin thickening, activation of PI3K/Akt pathway and increased expression of connective tissue growth factor (CTGF/CCN2) (111), the latter mediator representing an essential actor in this model (112). The Wnt/beta-catenin pathway, strongly implicated in cancer development, has also been reported playing a role in lung fibrosis (113). An aberrant activation of this pathway would result in accumulation of beta-catenin within pulmonary

tissue, promoting EMT. In the field of cancer, the dysfunction in Wnt pathway could favor cancer emergence and spread, but also resistance to anti-tumor treatments (including immunotherapy such as immune checkpoint inhibitors). Aberrant Wnt pathway could in fact induce deficient immunosurveillance toward cancer, leading to immune-evasion (114). The activation of the Wnt pathway was also observed in lesional skin from SSc patients. It stimulated a Smad-dependent fibrotic process in mesenchymal cells. It repressed adipogenesis in subcutaneous pre-adipocytes, while inducing myofibroblast differentiation (115, 116).

Some authors hypothesize that dysfunction of regulation of the TGFβ / SMAD pathway by caveolin-1 (Cav-1) is involved in both the development of fibrosis and breast cancer (117). In SSc, there is a decrease in Cav-1 expression in the lung and skin of SSc patients. KO mice for Cav-1 develop pulmonary and cutaneous fibrosis. *In vitro*, when Cav-1 function is restored in cultured SSc fibroblasts, their phenotype was normalized and stimulation of the TGFβ pathway was stopped by the inhibition of SMAD3 phosphorylation. Thus Cav-1 would allow the inhibition of SMAD3 phosphorylation and regulate the fibrosis process (118). In breast cancer, Cav-1 gene is a tumor suppressor gene, and a dominant negative mutation is present in 16% of breast cancers. Using a transgenic mouse model of breast cancer, the authors have demonstrated that cav-/- mice have a significant increase in tumor volume and rapidly develop pulmonary metastases, unlike cav+/+ or cav+/- mice (119).

On the whole, the oncogenic pathways found activated in SSc might contribute to fibrosis development while predisposing to malignancy in this disease.

Environmental Factors and Treatments as Inducers of Both Diseases

Environmental and Occupational Exposures in SSc and Cancer

Crystalline silica is a known carcinogen for bronchopulmonary cancers and also an environmental factor involved in the development of SSc (120). Many other environmental components have been suspected or confirmed as playing a role in the emergence of SSc, such as organic solvents, pollutants, welding fumes, pesticides, etc (121–124). Some of them are recognized as carcinogens.

Immunosuppressant-Induced Cancers

Several immunosuppressive drugs can be used in SSc, as recommended by EULAR, but may contribute to cancer (125). Among them, cyclophosphamide is a well-known agent, which is able to promote bladder cancer with a dose-dependent relation (10, 11). In a retrospective study with control group, there was 4 times more cancer in the SSc population treated with cyclophosphamide, with a consequent higher number of hematological malignancies (126). Recent studies have suggested that mycophenolate mofetil (MMF) is an alternative to cyclophosphamide for the treatment of interstitial lung lesions, including improved tolerance (127, 128). However, few case series have suggested a possible relationship between squamous cell carcinoma and mycophenolate mofetil in scleroderma, with cancer regression after MMF withdrawal (129).

Anti-cancer Therapy-Induced SSc

Several scleroderma-like syndromes have been described after anti-mitotic treatment. Docetaxel, a molecule used in the therapeutic arsenal of many cancers, including breast cancer, is known to have skin toxicity, with possible scleroderma-like lesions. More than a dozen cases of patients with authentic limited or diffuse SSc have also been reported secondary to docetaxel. The mechanisms leading to docetaxel cutaneous fibrosis are not fully understood. Some authors suggest that the deposition of an extracellular matrix glycoprotein (i.e., versican) after docetaxel or paclitaxel treatment may play a role in the pathogenesis of docetaxel-induced scleroderma (130). Recently, the first case of scleroderma secondary to docetaxel with organ involvement (PAH and renal scleroderma crisis) has been published (131). One of the explanations would be the possibility of endothelial cell damage induced by oxidative stress secondary to docetaxel (132).

Other chemotherapies have been associated with tissue fibrosis and/or scleroderma, such as bleomycin and gemcitabine (12, 14).

Ionizing radiation may be responsible for morphea (133) and may exacerbate pre-existing systemic scleroderma, according to some authors (13, 134, 135).

Strikingly, graft versus host disease (GVHD), a common complication of allogeneic hematopoietic stem cell transplantation (HSCT), somehow mimics SSc (136). Indeed, is skin fibrosis is a hallmark of chronic GVHD, resembling scleroderma. Beyond common clinical features, shared pathways are involved as observed in preclinical models for the two conditions (137–139).

More recently, anticancer immunotherapy using immune checkpoint inhibitors has been shown to trigger autoimmunity, and a few cases of scleroderma have been reported in the literature (55–57, 140). In this context, the complexity of the interplay between anti-cancer pre-existing autoimmunity, genuine paraneoplastic syndromes and the effects of immune system stimulation by biologics is striking, and strengthens the relationships between SSc and malignancy.

AUTOANTIBODIES AS BIOMARKERS PREDICTING MALIGNANCY IN SYSTEMIC SCLEROSIS?

Anti-RNA Polymerase III

In a monocentric retrospective study in England, out of 2,177 patients with SSc, 7.1% had a history of cancer (33). The frequency of cancers was significantly increased in patients with anti-RNA-PolIII (14.2%) compared to patients with anti-Scl70 (6.3%) and anti-centromere (6.8%) autoantibodies ($p < 0.0001$ and $p < 0.001$). In patients diagnosed with cancer within 3 years of SSc onset, 55.3% had anti-RNA-PolIII. In addition,

SSc patients with anti-RNA-PolIII had twice as much risk of developing cancer as anti-centromere patients ($p < 0.0001$).

These data were confirmed by a study of EULAR, where anti-RNA-PolIII antibodies were associated with synchronous malignancy (-6 months + 12 months) with an OR estimated at 7.38 (95% CI 1.61–33.8) (7). The association with breast cancers was even stronger, with an OR of 20.2 (95% CI 1.45–355). Epidemiology and the shared role of sex hormones predisposing to both conditions may explain this specific association. In multivariate analysis anti-RNA-PolIII was also associated with older age, acute renal crisis and diffuse skin involvement. These results led to specific cancer-screening recommendations in anti-RNA-PolIII positive patients, over a period of 2 to 5 years, including routine mammography (repeated every year) and non-invasive investigations, such as prostatic specific antigen (PSA) testing, blood stool test, and gynecological examination. Of note, in an Australian prospective study, patients with anti-RNA-PolIII positive and anti-RNA-PolIII negative antibodies had the same percentage of cancer (13%) but the diagnosis of cancer within 5 years after the diagnosis of scleroderma was more frequent in anti-RNA-PolIII positive patients (13 vs. 3.9%) (141). A direct pathophysiological link between RNA-PolIII antigen modification in tumor and autoimmunity is even suggested in this condition, as developed below (paragraph Anti-RNPC-3).

Anti-RNPC-3

Recent studies have identified in "triple negative" scleroderma patients with cancer (patients without anti-centromere, anti-scl70 and anti-RNA-PolIII antibodies), a new autoantibody that targets RNPC-3. RNPC-3 is a protein member of the minor spliceosome, ribonucleoprotein complex that participates in the splicing of pre-messenger RNAs (17). Like the anti-RNA-PolIII positive patients, these patients have 4 times more risk of developing cancer within 2 years after the onset of scleroderma than the anti-centromere positive patients (95% CI 1.1–16.9) (142). The authors also noted an association between anti-RNPC-3 and severe interstitial lung disease, as well as more frequent muscle involvement (142). These anti-RNPC3 autoantibodies would possibly be indicative of cancer-induced autoimmunity in this subgroup of patients.

Anti-PM/Scl

A recent Spanish retrospective study involving 432 patients including 53 cancers (12.2%), found no association between anti-RNA-PolIII antibodiesand cancer (35). In contrast, the anti-PM/Scl autoantibody was associated with cancer with an OR of 3.90 (95% CI 1.31–11.61), while aspirin treatment was protective with an OR of 0.33 (95% CI 0.12–0.90) (35). As above mentioned, the link between miRNAs regulation and PM/Scl, a complex homolog to yeast exosome is intriguing. However, these results have not been confirmed by a Dutch study (143).

Anti-Scl70

Rosen's team has shown that patients with anti-scl70 antibodies were associated with short cancer-scleroderma interval (34). Advanced age was an independent risk factor for cancer in this study. An Italian team has demonstrated that anti-Scl70 antibodies were associated with lung cancer (26).

PARANEOPLASTIC SCLERODERMA: FROM EPIDEMIOLOGICAL OBSERVATION TO PATHOPHYSIOLOGICAL DEMONSTRATION

Overview of the Concept of Paraneoplastic Autoimmune Disease

As said earlier, the observation of an elevated risk of cancer in patients with numerous systemic autoimmune conditions compared with the general population argues for close and bidirectional relationships between malignancy and autoimmunity. Even more striking is the close temporal clustering in some patients, which leads to consider autoimmune disease as a genuine paraneoplastic entity. The exact definition of a paraneoplastic syndrome may vary greatly among authors and remains debatable. First, one prerequisite for paraneoplastic phenomenon lies in this "temporal clustering," viz. a short interval between the onset of cancer and the onset of autoimmune disease. Of note, cancer could precede SSc onset, and vice-versa. This "short" interval is however vague, but 3 to 5 years before and after SSc diagnosis could be acceptable since it corresponds to a "peak" of frequency in cancer diagnosis in previous studies (33). Second, there should be a parallel evolution between cancer and autoimmunity, with autoimmune flares accompanying cancer relapses. Conversely, cancer resection should lead to the remission of the associated autoimmune condition. However, such a theoretical conception of paraneoplastic phenomena may be far away from the reality observed concerning SSc, where no resolution of autoimmunity has been described after cancer healing.

Conceptually, autoimmunity may be associated with oncogenesis and anti-tumor defense. This is also consistent with the observation of cancer developing some time after autoimmunity, since autoimmune response in this context may be triggered in the very early stages of cancer development, even in premalignant disease. For instance, autoantibody production has been shown to appear years before cancer diagnosis in such context (144). Long ago considered as non-specific, the presence of some antibodies could have some clinical relevance for patients developing autoimmune disorders and cancer, and become novel biomarkers for both conditions, in terms of early diagnosis, but also prognosis and response to therapy. Interestingly, specific anti-tumor activity of antinuclear antibody (ANA) *via* antibody-dependent cell-mediated cytotoxicity (ADCC) has been described (145). For some authors, antibody clustering could even correspond to distinct underlying malignancies, beyond autoimmune disease classification (145). For instance, in dermatomyositis, anti-NXP2 and anti-TIF1γ– but not anti-MDA-5 antibodies- are strongly associated with

cancer development (more than 80% of patients presenting with cancer and dermatomyositis) (146).

Interestingly, and consistent with what is usually reported with paraneoplastic syndromes, autoimmunity in the context of cancer is most often associated with a better outcome. For instance, the presence of a paraneoplastic syndrome is associated with smaller tumors and less metastatic disease. Moreover, tumor-infiltrating lymphocytes, the presence of ANA

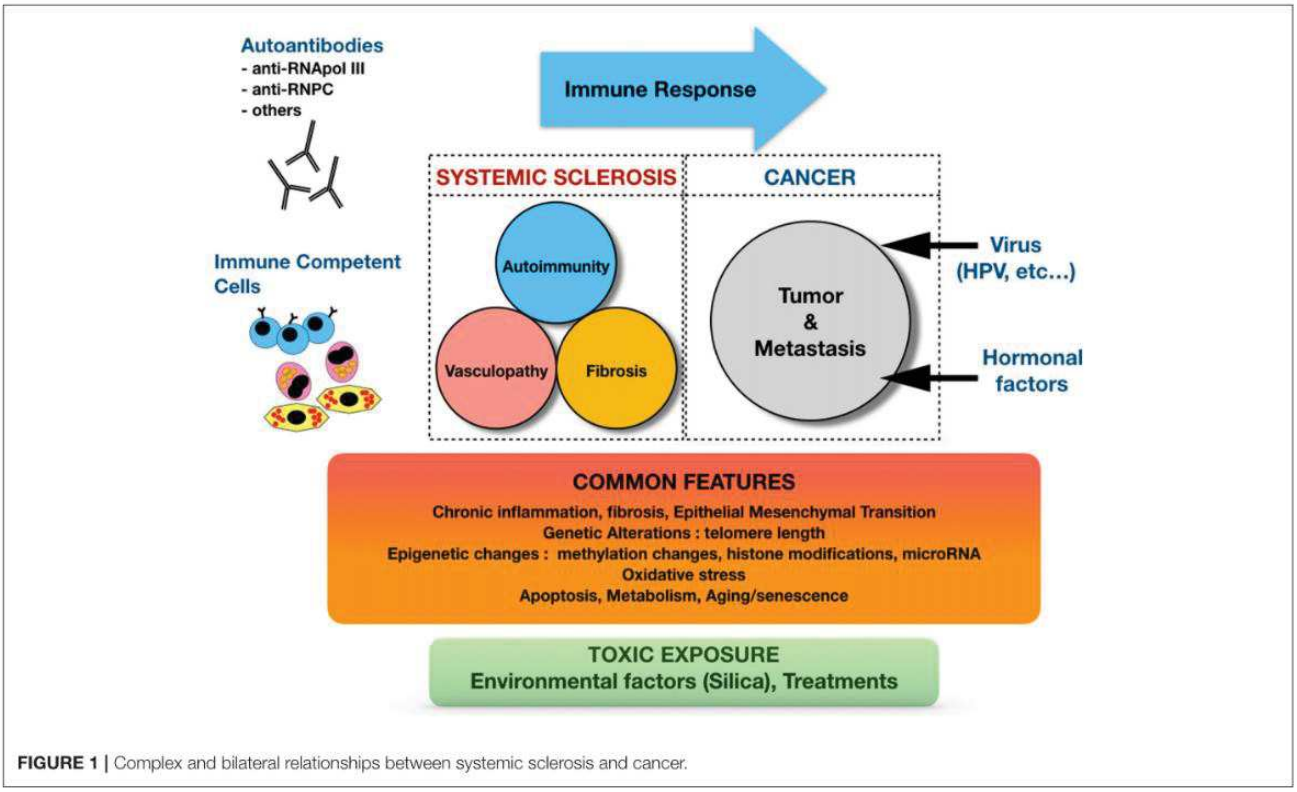

FIGURE 1 | Complex and bilateral relationships between systemic sclerosis and cancer.

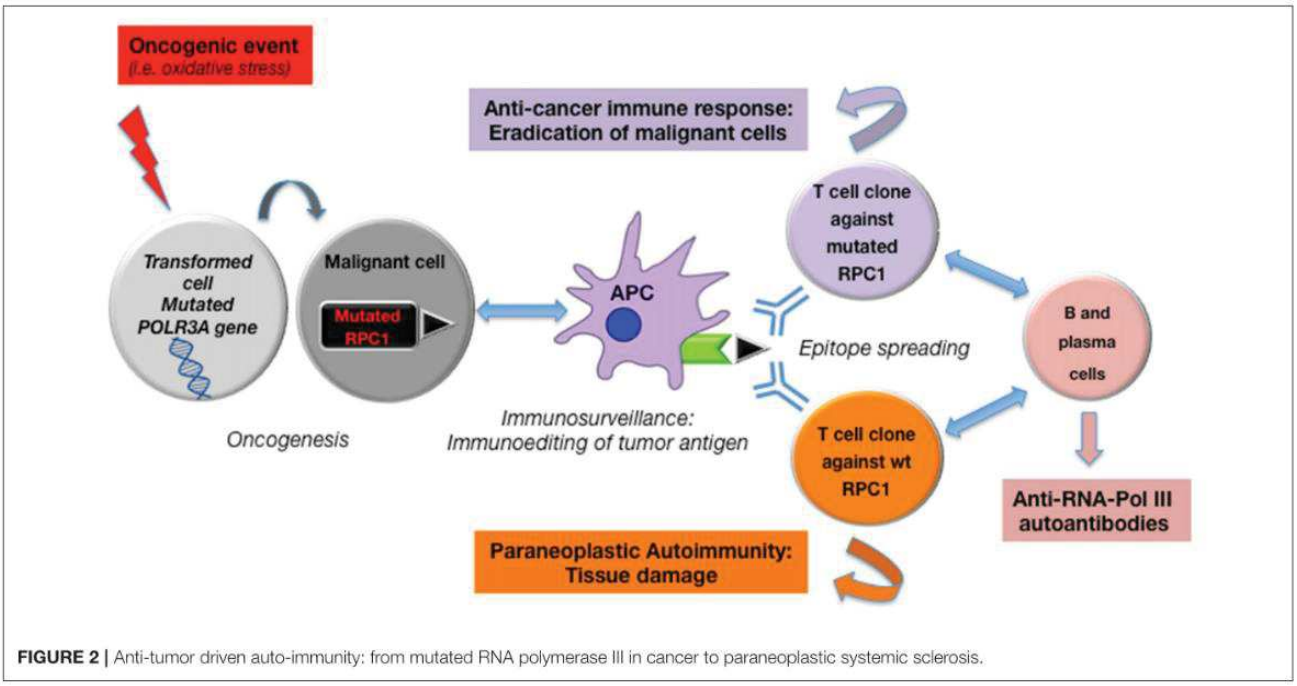

FIGURE 2 | Anti-tumor driven auto-immunity: from mutated RNA polymerase III in cancer to paraneoplastic systemic sclerosis.

and vitiligo are clearly demonstrated as positive prognostic factors (8, 145). In addition, in the context of cancer immunotherapy using immune checkpoint inhibitors (i.e., anti-PD1, -PDL1 and CTLA-4 agents), the presence of tumor infiltrating lymphocytes before treatment and the observation of autoimmunity (i.e., immune related adverse events) under treatment are usually predictive of a better response to therapy (147).

All these observations support the hypothesis of a common primary event in oncogenesis that could make a self-antigen become immunogenic, and next trigger an autoimmune response against tumor cells. This specific autoimmune response would contribute to anti-tumor defense, but in some extent, in the case of a shared antigen, this response could spread to non-mutated antigens and be responsible for healthy tissue damage in relation with autoimmunity. In SSc, this concept has recently been thoroughly demonstrated in patients with anti-RNA-PolIII antibodies and contributes to the possibility of scleroderma being a paraneoplastic disease.

Cancer-Induced Scleroderma: The Role of Mutated RNA-PolIII in Autoimmunity

Several recent studies conducted by the team of Rosen, Casciola-Rosen and Shah (Johns Hopkins University School of Medicine, Baltimore, USA) among others, have shown that the anti-RNA-PolIII antibodies were associated with cancer in SSc, with a temporal clustering between the two conditions suggestive of a paraneoplastic disease and a common underlying mechanism (7, 16, 33, 141). Patients with anti-RNA-PolIII antibodies are indeed 5.08 (95% CI 1.60–16.1) times more likely to develop cancer within 2 years of SSc onset (34). Furthermore, a specific nucleolar expression of RNA-PolIII was observed in the malignant cells from these patients, suggestive of a link between cancer-related auto-antigen and autoimmune response (16).

In order to decipher the potential common pathophysiological process underlying this association, Rosen et al. comparatively studied eight tumors from anti-RPC1 positive patients (RNA polymerase III subunit) and eight tumors from anti-Scl70 or anti-cm positive patients. Genetic alterations (somatic mutation or loss of heterozygosity) of the POLR3A gene coding for RNA polymerase III were identified in the tumors of six out of eight anti-RCP1 positive patients, while no mutation of this gene was found in the tumors of anti-cm or anti-Scl70 positive patients. Hence, the presence of this mutated auto-antigen in malignant cells could be the *primum movens* triggering autoimmunity in these patients, inducing cellular and humoral responses, with the production of anti-RNA-PolIII autoantibodies (148). Of particular interest, anti-RNA-PolIII antibodies in these patients were found to recognize both mutated and non-mutated (wild-type, wt) RNA-PolIII. This important finding, observed in other situations (149), is related to an "epitope-spreading" mechanism, that would be responsible for healthy tissue damage in SSc. In other terms, their demonstration argues for the role of a shared antigen (i.e., RNA polymerase III) that could undergo genetic alterations (for instance under DNA oxidative damage), and lead to the emergence of a mutated and immunogenic antigen in transformed cells. These alterations would next trigger a mutant-specific clonal immune response that could subsequently spread to wt antigens in healthy cells, contributing to autoimmune-mediated tissue damage in SSc (**Figure 2**). According to this hypothesis, the appearance of scleroderma in the context of malignancy would be the "price to pay" for eliminating the cancer (6). This could also explain why 80% of RNA-PolIII patients never develop cancer: the latter ones may have benefited from anti-tumor immunosurveillance with efficient eradication of malignant cells. However, as summarized by Schreiber et al., immunosurveillance in cancer is a dynamic process, better-called immunoediting, that evolves in three stages, from tumor elimination, equilibrium, and finally to escape under tumor high mutational rate with loss of expression of immunogenic antigens(150).

CONCLUSION AND PERSPECTIVES

Beyond simple epidemiological observations, intriguing and complex bilateral relationships exist between SSc and malignancy, supported by a growing body of data involving the immune system and other contributors such as genetic and epigenetic changes, environmental factors, including oxidative stress. These relationships are summarized in **Figure 1**.

On the whole, based on these observations, a novel concept of autoimmunity as a response to underlying malignancy is coming up in scleroderma, and may contribute to new strategies in patients' care. Indeed, autoantibodies could be useful biomarkers for screening strategies, as proposed by some authors who postulate that early diagnosis of malignancy may ameliorate cancer but also SSc outcome (6, 7, 35). A detailed screening algorithm has been proposed by Shah et al., based on gender, specific risk factors and "red flags," leading to specific non-invasive and invasive investigative procedures (6). Even if the beneficial effects in terms of overall survival in the scleroderma population remain to be demonstrated, a strategy based on repeated and more aggressive screening in patients with specific autoantibodies subsets (i.e., anti-RNA-PolIII, Pm/Scl or RNPC3) or in seronegative patients may be tantalizing.

Deciphering the mechanisms of autoimmunity through the prism of cancer immunosurveillance is even more fascinating in the era of anticancer immunotherapy, and will undoubtedly lead to new breakthroughs both in the field of autoimmunity and cancer. One could hope this may 1 day contribute to a better prognosis of scleroderma.

AUTHOR CONTRIBUTIONS

AM, LP, RG, and PG participated in the review of literature, and in the manuscript redaction. SR, PR, CB, AL, JM, and DN participated in the manuscript redaction and final approval.

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ANNEXE 2

frontiers in Immunology

ORIGINAL RESEARCH
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Fibrosis Development in HOCl-Induced Systemic Sclerosis: A Multistage Process Hampered by Mesenchymal Stem Cells

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Objectives: Skin fibrosis is the hallmark of systemic sclerosis (SSc) a rare intractable disease with unmet medical need. We previously reported the anti-fibrotic potential of mesenchymal stem cells (MSCs) in a murine model of SSc. This model, based on daily intra-dermal injections of hypochlorite (HOCl) during 6 weeks, is an inducible model of the disease. Herein, we aimed at characterizing the development of skin fibrosis in HOCl-induced SSc (HOCl-SSc), and evaluating the impact of MSC infusion during the fibrogenesis process.

Methods: After HOCl-SSc induction in BALB/c mice, clinical, histological and biological parameters were measured after 3 weeks (d21) and 6 weeks (d42) of HOCl challenge, and 3 weeks after HOCl discontinuation (d63). Treated-mice received infusions of 2.5×10^5 MSCs 3 weeks before sacrifice (d0, d21, d42).

Results: HOCl injections induced a two-step process of fibrosis development: first, an 'early inflammatory phase', characterized at d21 by highly proliferative infiltrates of myofibroblasts, T-lymphocytes and macrophages. Second, a phase of 'established matrix fibrosis', characterized at d42 by less inflammation, but strong collagen deposition and followed by a third phase of 'spontaneous tissue remodeling' after HOCl discontinuation. This phase was characterized by partial fibrosis receding, due to enhanced MMP1/TIMP1 balance. MSC treatment reduced skin thickness in the three phases of fibrogenesis, exerting more specialized mechanisms: immunosuppression, abrogation of myofibroblast activation, or further enhancing tissue remodeling, depending on the injection time-point.

Conclusion: HOCl-SSc mimics three fibrotic phenotypes of scleroderma, all positively impacted by MSC therapy, demonstrating the great plasticity of MSC, a promising cure for SSc.

Keywords: mesenchymal stem cells, systemic sclerosis, fibrosis, hypochlorite, oxidative stress, scleroderma, cell therapy, autoimmunity

INTRODUCTION

Skin fibrosis is the hallmark of systemic sclerosis (scleroderma, SSc), a rare intractable autoimmune disorder with high morbidity and mortality due to multi-organ involvement (1). Although precise etiology for SSc remains unknown, fibrosis development in the disease may originate from a complex interplay between environmental and intrinsic triggers–including oxidative stress-leading to tissue damage, immune response, endothelial cell and myofibroblast activation. The latter results in fibroblast proliferation and extracellular matrix (ECM) production (2–4). However, fibrogenesis is a dynamic process, with counteracting mechanisms involved to dampen immune activation or up-regulate tissue remodeling. So, different stages of tissue fibrosis may be observed, with variable participation of immune cell activation, angiogenesis or ECM synthesis by fibroblasts. Thus, targeting fibrosis in SSc may be challenged by the complexity of this multistage process, as well as the unpredictability of clinical evolution. Studies are still on going to help defining more precisely the clinical and biological status of SSc patients, hence refining therapy (5, 6).

Among new potential approaches for treating SSc, mesenchymal stem cells (MSCs)-based therapy stands as a promising lead (7). Indeed, these mesodermal multipotent progenitors can be easily isolated and expanded from virtually all tissues, and display both immunomodulatory and anti-fibrotic properties when infused (8). We recently demonstrated the feasibility and the therapeutic benefits of an approach based on MSCs in a preclinical murine model for SSc (9, 10). In this model, fibrosis is induced by repeated exposure to hypochlorite (HOCl), an oxidative agent administered by daily intra-dermal injections during 6 weeks. This model, mimicking early diffuse cutaneous SSc, is particularly useful to evaluate new therapeutic approaches. However, we still lack data on the development and the spontaneous evolution of fibrosis in HOCl-induced SSc (HOCl-SSc), as well as the impact of MSCs-based therapy along the process.

In this study, we first precisely characterized the fibrotic process induced by HOCl injections, as well as the evolution of fibrosis after discontinuation of HOCl challenge. Doing so, we further investigated the impact of MSC infusion, at each stage of the process, on skin fibrosis, inflammation and remodeling.

MATERIALS AND METHODS

Isolation and Culture of Mesenchymal Stem Cells

MSCs from BALB/c mice were isolated from bone marrow (BM). BM was flushed out from long bones and the cell suspension was plated in DMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza, France). Cells were passaged till obtaining homogeneity for mesenchymal marker expression and lack of hematopoietic markers as analyzed by flow cytometry. They were used between passages 10 and 15.

Flow Cytometry Analysis

Cells were harvested by treatment with 0.05% trypsin and 0.53 mM EDTA, and resuspended in PBS containing 0.1% BSA and 0.01% sodium azide. Cells were incubated on ice with conjugated antibodies against CD11b, CD44, CD45, CD73, and Sca1 (BD Pharmingen, France) or conjugated isotypic controls. Samples were analyzed on the FACS Canto II and analysis performed using the BD FACSdiva software (BD Pharmingen).

Differentiation of MSCs

Differentiation of MSCs was induced as reported elsewhere (11). In brief, for adipogenesis, MSCs were plated at 10^4 cells/cm^2 in inductive medium and adipocytes characterized by presence of lipid droplets as visualized by Oil red O staining and expression of specific markers by RT-qPCR. Chondrogenic differentiation was induced by culture in micropellet and chondrogenesis was assessed by RT-qPCR. Osteogenesis was induced by culture at low density in osteogenic medium. Differentiation was assessed by RT-qPCR quantification of osteoblast markers and ECM mineralization visualized after staining with a 2% Alizarin Red S solution.

T-Cell Proliferation Assay

For T-cell proliferative experiments, 10^5 splenocytes were stimulated with 1 µg/ml concanavalin A (conA; Sigma-Aldrich, France) in presence of different ratios of MSCs as already reported (11). After 3 days, cell proliferation was measured using the CellTiter-Glo™ luminescent cell viability assay (Promega, France).

HOCl Preparation

HOCl was generated extemporaneously by adding NaClO (9.6% as active chlorine) to KH_2PO_4 solution (100 mM, pH: 6.2), usually using a 1:100 ratio. The right amount of NaClO was adjusted to obtain the desired HOCl concentration, defined by the absorbance of the mixture at 292 nm (optical density between 0.7 and 0.9 read on a Nanodrop spectrophotometer, Thermoscientific). Stock solutions were stored at 4°C in the dark and NaClO was replaced every 3 weeks.

Experimental Design and Animals

Six-week-old female BALB/c mice purchased from Janvier were housed and cared for according to the Laboratory Animal Care guidelines. Approval was obtained from the Regional Ethics Committee on Animal Experimentation before initiation of the study (approval APAFIS#5351-2016050919079187). All experiments were performed after final approval given by the French Ministry for Education, Higher Education and Research. Mice had their backs shaved the day before disease induction. Skin thickness was assessed with a caliper before disease induction and every week during the whole experiment by a blinded experimenter. As previously described, a total amount of 300 µl of freshly prepared HOCl was injected in two sites into the backs of the mice with a 29 G needle, 5 days a week for 6–9 weeks [d0 to d42 or day 63; (12)]. Control mice received PBS in the same conditions. MSCs-treated SSc-HOCl mice received an infusion of MSCs (2.5×10^5 cells in 100

μl PBS), in the tail vein of the mice at indicated time points (d0, d21, d42). Groups of 7 to 10 mice were made for each condition (PBS-, HOCl- and MSCs-treated HOCl-mice). Three weeks after MSCs infusion, and after a 2-day recovery time without HOCl injections, animals were sacrificed, at indicated time points (d21, d42, or d63). Skin biopsies (6 mm punches) were taken on the backs of mice. Samples were stored at −80°C for RT-qPCR, ELISA and collagen content determination or fixed in 4% formaldehyde for histopathological analysis. Overall experimental scheme is shown in **Figure 1**, and representative pictures of mice during experimental procedure are shown in **Supplementary Figure 1**.

Histopathology

Samples were embedded in paraffin and 5 μm thick sections were stained with Masson trichrome or immunostained with antibodies for α-sma (ab5694, Abcam, 1/100) TGFβ (ab66043, Abcam, 1 /100), Ki67 (SP6, VP-RM04, Vector laboratories, 1/200), CD3-epsilon (M-20, sc−1127, Santa Cruz Biotechnology, 1/250), F4/80 (MF4800, Invitrogen, 1/50), Pax5 (C-20, sc−1974, Santa Cruz Biotechnology, 1/250). Histological slides were scanned using Nanozoomer (Hamamatsu) or Pannoramic 250 Flash II (3DHistech) for immunofluorescence. Quantification of immunostaining was made using Definiens Tissue Studio software.

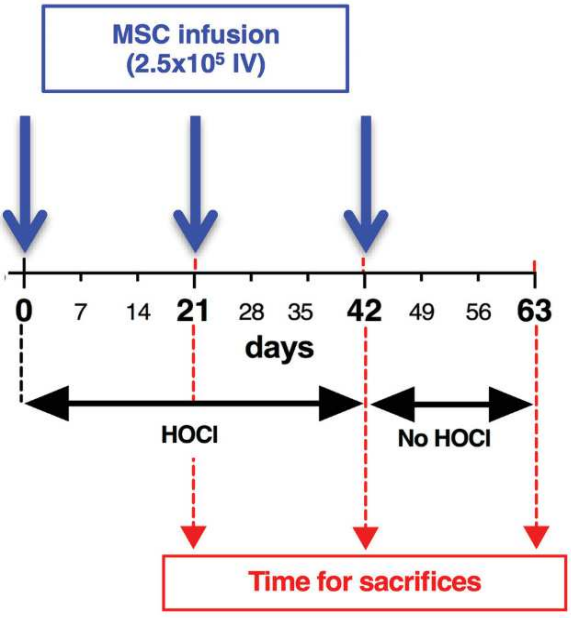

FIGURE 1 | Development of skin fibrosis in HOCl-mice: experimental scheme. To induce systemic sclerosis, BALB/c mice underwent 6 weeks of daily HOCl intradermal injections, while control mice received PBS injections. Skin thickness was measured weekly during the experiment. After d42 (6 weeks), HOCl injections were abrogated, and groups of mice were kept in order to investigate skin thickness evolution till d63. Groups of mice were sacrificed at indicated time points (d21, d42, d63).

RT-qPCR Analysis

Samples (cells or skin biopsies) were crushed in RLT-buffer and total RNA was extracted using the RNeasy mini kit and Qiacube robotic workstation (Qiagen, France). 1 μg RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, France). qPCR was performed on 20 ng cDNA using LightCycler 480 SYBRGreen I Master mix and real-time PCR instrument (Roche Applied Science, France). The following conditions were used: 95○C for 5 min; 40 cycles at 95○C for 15 s; 64○C for 10 s and 72○C for 20 s in a LightCycler 480 system (Roche diagnostics, France) and analyzed with the dedicated software. Primers were designed using the web-based applications Primer3 and BLAST (**Table 1**). Samples were normalized to mRNA expression of TATA binding protein *(Tbp)* gene for tissue samples or GAPDH for MSCs, and results were provided either as relative expression to the housekeeping gene using the formula $2^{-\Delta Ct}$ or as fold change using the formula $2^{-\Delta\Delta Ct}$.

Collagen Content in Skin

Collagen content assay was based on the quantitative dye-binding Sircol method (Biocolor, Ireland). Skin biopsies were suspended in 2 ml of a 0.5 M acetic acid—pepsin (2.5 mg/ml) solution and dissociated using UltraTurrax (vWR, France). Collagen extraction was performed overnight at 4°C under stirring. Suspension was then centrifuged at 12,000 g for 10 min and 20 μl of each sample were added to 1 ml of Syrius red reagent. Tubes were rocked at room temperature for 30 min and centrifuged at 12,000 g for 10 min. The supernatants were discarded and tubes washed with 750 μl of ice-cold salt acid. After another 12,000 g centrifugation for 10 min, the collagen-dye pellets were suspended in 1 ml of 0.5 M NaOH. Optical Density (OD) was read at 555 nm on a microplate reader (Varioskan Flash, Thermo scientific) vs. a standard range of bovine collagen type I concentrations (supplied as a sterile solution in 0.5 M acetic acid). Results were expressed as collagen content in $\mu g/mm^2$ of skin.

Anti-topoisomerase 1 Antibody Elisa

Anti-topoisomerase 1 or anti-scl-70 antibodies were detected using scl-70 Ig ELISA kit (Abnova, Taiwan). In brief, 200 μl of 1:5 diluted sera were dispensed into scl-70 pre-coated wells and incubated at room temperature for 90 min. Goat total anti mouse IgG antibody, HRP conjugate (BD Biosciences, France), diluted 1:1,000 was then incubated for 60 min at room temperature. TMB substrate was incubated for 5 min and stopped with an equivalent amount of sulfuric acid. Absorbance was read at 450 nm under Varioskan Flash and results expressed as arbitrary unit (AU) for optical density (OD).

Statistical Analysis

All quantitative data were expressed as mean +/- SEM. Gaussian distribution of values was tested using the Shapiro-Wilk normality test. Data were then compared using Mann-Whitney's test for nonparametric values or Student's *t*-test for parametric values. When analysis included more than two groups, one-way ANOVA was used. All statistical analyses

TABLE 1 | List of primers designed and used in RT-qPCR experiments.

Gene abbreviation	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Ap	GTGGTGGACGGTGAACGGGA	TCCACCGTGGGTCTCATGGC
αSma	AAGGCCAACCGGGAGAAAAT	AGCCAAGTCCAGACGCATGA
Col1	TGTTCAGCTTTGTGGACCTC	TCAAGCATACCTCGGGTTTC
Col2B	CTGGTGCTGCTGACGCT	GCCCTAATTTTCGGGCAT
Col3	CGGTGAACGGGGCGAAGCTGGTT	GACCCCTTTCTCCTGCGGCTCCT
Col10	TGCTGCCTCAAATACCCTTT	CAGGAATGCCTTGTTCTCCT
Fabp4	CGTAAATGGGGATTTGGTCA	TCGACTTTCCATCCCACTTC
Gapdh	GGTGCTGAGTATGTCGTGGA	GTGGTTCACACCCATCACAA
Il1b	TTTGACAGTGATGAGAATGACCTGTTC	TCATCAGGACAGCCCAGGTCAAAG
Il6	TGGGACTGATGCTGGTGACA	TTCCACGATTTCCCAGAGAACA
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Lpl	TTTGGCTCCAGAGTTTGACC	GTCTTGCTGCTGTGGTTGAA
Mmp1	TTCAAAGGCAGCAAAGTATGGGCT	CCAGTCTCTTCTTCACAAACAGCAGCA
Oc	GCGCTCTGTCTCTCTGACCT	GCCGGAGTCTGTTCACTACC
Pparγ	AAGAGCTGACCCAATGGTTG	GGATCCGGCAGTTAAGATCA
Runx2	ACAGTCCCAACTTCCTGTGC	ACGGTAACCACAGTCCCATC
Sox9	AGGAAGCTGGCAGACCAGTA	CTCCTCCACGAAGGGTCTCT
Tbp	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT
Tgfβ1	TGCGCTTGCAGAGATTAAAA	CTGCCGTACAACTCCAGTGA
Timp1	CTCCGCCCTTCGCATGGACATT	GGGGGCCATCATGGTATCTGCTCT
Tnfα	AGCCCACGTCGTAGCAAACCA	TGTCTTTGAGATCCATGCCGTTGGC

Ap, alkaline phosphatase; aSma, alpha, smooth muscular actin; Col, collagen; Fabp, fatty acid binding protein; Gapdh, glyceraldehyde-3-phosphate deshydrogenase; Hmox, heme oxygenase; Il, interleukin; Lpl, lipoprotein lipase; Mmp, metalloprotease; Oc, osteocalcin; Ppar, peroxisome proliferator-activated receptor; Runx, Runt related transcription factor; Sox, SRY (sex determining region Y)-related HMG (high mobility group)-box gene; Tbp, TATA binding protein; Timp, tissue inhibitor of metalloproteinase; Tnf, tumor necrosis factor.

were performed using Prism 6 GraphPad software for Mac OS (California, United States). A $P < 0.05$ was considered significant.

RESULTS

Induction of Skin Fibrosis Under HOCl Challenge Is a Two-Step Process With Early Proliferative and Inflammatory Stage and Late Constitution of ECM Deposition

As previously shown, the induction of skin fibrosis by daily HOCl injections was clinically characterized by progressive thickening of skin from d7 to d42 when compared with control PBS-mice (**Figure 2A** and **Supplementary Figure 1**). At d42, histology disclosed strong collagen deposition in skin from HOCl-mice compared with PBS mice, as shown by sirius red coloration (**Figure 2B**). Histological examination from sequential sacrifices revealed a discontinued process with two main stages. First, in the third week of experiment (d14–d21), we observed transparietal polymorphous cellular infiltrates; second, in the last week of experiment (d35–d42), these cellular infiltrates were gradually replaced by ECM deposition, resulting in disorganization of dermis and complete loss of hypodermic adipose tissue (**Figure 2C**). During the process, we noted a strong and steady expression of myofibroblastic markers *αSma* and *Tgfβ1* in dermis from SSc mice, with no obvious difference between d21 and d42 (**Figure 2D**).

While comparing these two main time points (i.e., d21 and d42), we observed a higher number of proliferative cells in skin tissues at d21 compared with d42, as shown by Ki67 staining (**Figures 3A,B**). Looking at cytokine mRNA expression within the tissue, we noted a stronger expression of pro-inflammatory cytokines *Il1β* and *Tnfα* at d21 compared with d42, together with a lower expression of the anti-inflammatory cytokine *Il10* (**Figure 3C**). We therefore focused on d21, and further characterized these cellular infiltrates by immunostaining. We observed high number of CD3+ T-lymphocytes, of F4/80+ macrophages, but no Pax5+ B cells (**Figure 4**). Of note, we also noticed some cells co-expressing CD3 and Ki67 (data not shown).

Altogether, the analysis of skin fibrosis kinetics during the induction of HOCl-SSc revealed a two-step process, with an early cellular phase made of highly proliferative T-lymphocytes, macrophages and myofibroblasts, culminating at d21, and a late evolution toward paucicellular matricial fibrosis mainly characterized by ECM deposition at d42. Conversely, as previously reported, lung fibrosis development in this model is a continuous and progressive process, leading to tissue fibrosis at the end of the 6-wk experiment [(9) and **Supplementary Figure 2**]. For these reasons and because skin fibrosis is the hallmark of SSc, we kept focused on skin fibrosis development in the present study.

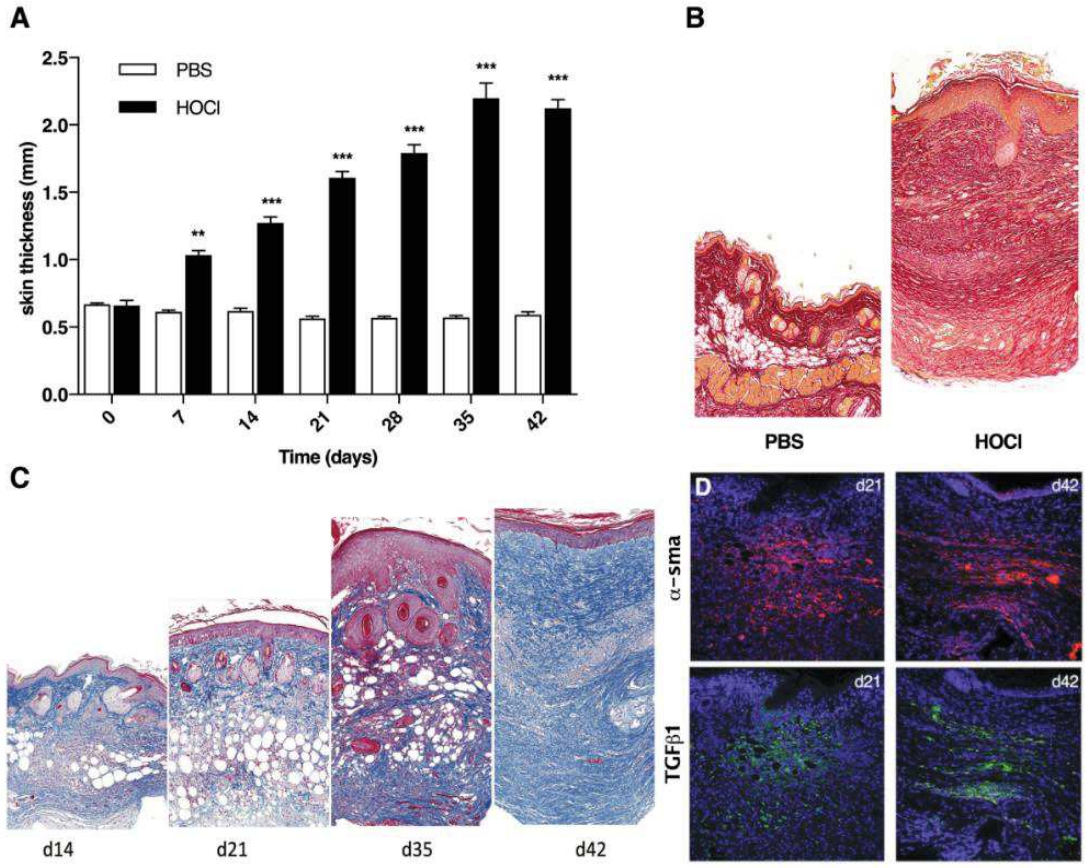

FIGURE 2 | Development of skin fibrosis in HOCl-mice. **(A)** Skin thickness from PBS or HOCl-injected mice at different time-points during the induction of HOCl-SSc (d0 to d42) ($N = 8$ per group) $^{**}P < 0.001$, $^{***}P < 0.001$. **(B)** Representative skin sections of PBS and HOCl mice at d42 (original magnification 10x; Sirius Red staining). **(C)** Representative skin sections at different time-points during the induction of HOCl-SSc (original magnification 10x; Masson Trichrome staining). **(D)** Immunostaining for αSma (in red) and Tgfβ1 (in green) in representative skin sections at d21 and d42.

MSCs-Based Treatment Efficiently Reduces Inflammation During the First Stage of Fibrosis Constitution in HOCl-SSc

In a first experiment, we aimed at evaluating the anti-inflammatory impact of MSCs-based treatment administered during the first stage of fibrosis induction in the model (d0–d21). Therefore, we used MSCs isolated from BALB/c mice in a syngeneic approach. These cells were characterized by the expression of CD44, CD73, CD29, CD105, CD106, and stem cell antigen 1 (Sca1), and the absence of expression of the haematopoietic markers CD45, CD3, CD19, CD31, CD11b, and HLA-DR by cytometry analysis (**Figures 5A,B**). MSCs exerted immunosuppressive properties on the mitogen-induced proliferation of T-lymphocytes (**Figure 5C**) and were able to differentiate into adipocytes expressing lipoprotein lipase *(Lpl)*, peroxisome proliferator-activated receptor *(Pparγ)*, fatty acid binding protein *(Fabp4)* (**Figure 5D**), chondrocytes expressing SRY (sex determining region Y)-related HMG (high mobility group)-box gene *(Sox9), Col2B, Col10* (**Figure 5E**) and osteoblasts expressing osteocalcin *(Oc)*, alkaline phosphatase *(Ap), Col1*, Runt related transcription factor (*Runx2*; **Figure 5F**).

A unique dose of 2.5×10^5 MSCs was administered intravenously before HOCl induction at d0, and mice were sacrificed 3 weeks later. A significant reduction of skin thickness was obtained at d21 in MSCs-treated SSc mice compared with untreated SSc mice (**Figure 6A**). Because inflammation was the highest at d21, we analyzed the effect of MSCs on inflammatory mediators in skin. MSCs treatment was associated with a significant decrease of *Il1β*, *Tnfα*, *Il6*, and *Il10* expression compared with untreated SSc mice (**Figure 6B**). Histological analysis revealed overall less cellular infiltrates in MSCs-treated mice, together with less Ki67 staining, strong decrease in CD3 staining and almost abolition of F4/80 staining (**Figures 6C,D**). MSC infusion also prevented specific anti-scl70 autoantibody production as detected in the serum (**Supplementary Figure 3**). On the whole, MSCs treatment during the first phase of HOCl-induced fibrosis prevented skin inflammation

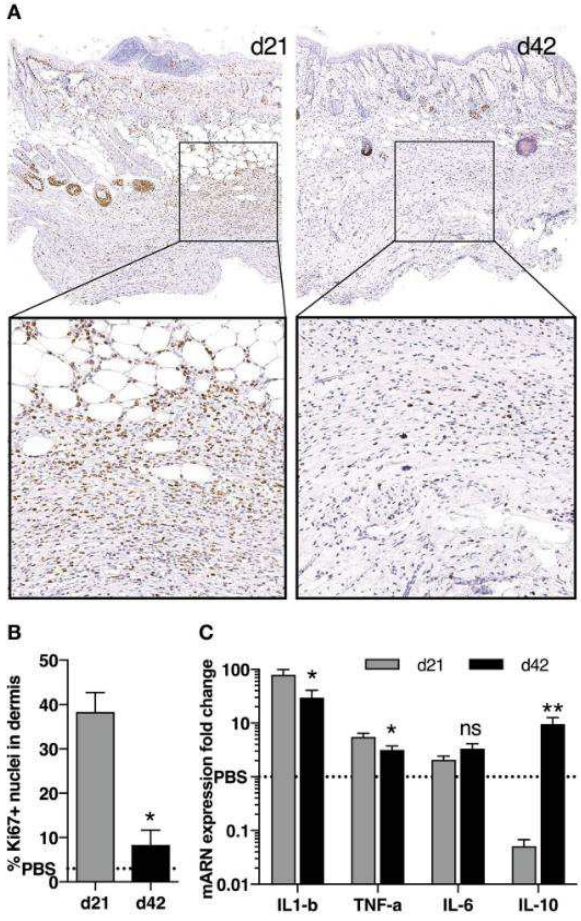

FIGURE 3 | Cell proliferation and cytokine production in skin during fibrosis induction in HOCl-mice. **(A)** Immunostaining for nuclear Ki67 in representative skin sections at d21 and d42 (top panels: magnification 10x, bottom panels: top image box, 30x). **(B)** Percentage of Ki67$^+$ proliferative cells in dermis as quantified on immunostaining in **A** using Definiens Tissue Studio software ($n = 3$ per group, $^*P < 0.05$, $^{**}P < 0.01$). **(C)** mRNA expression of *Il1b, Tnfa, Il6* and *Il10* in skin sections from HOCl-SSc mice at d21 and d42. Results are given as fold-change *vs.* control PBS-mice normalized at 1. $N = 8$ per group.

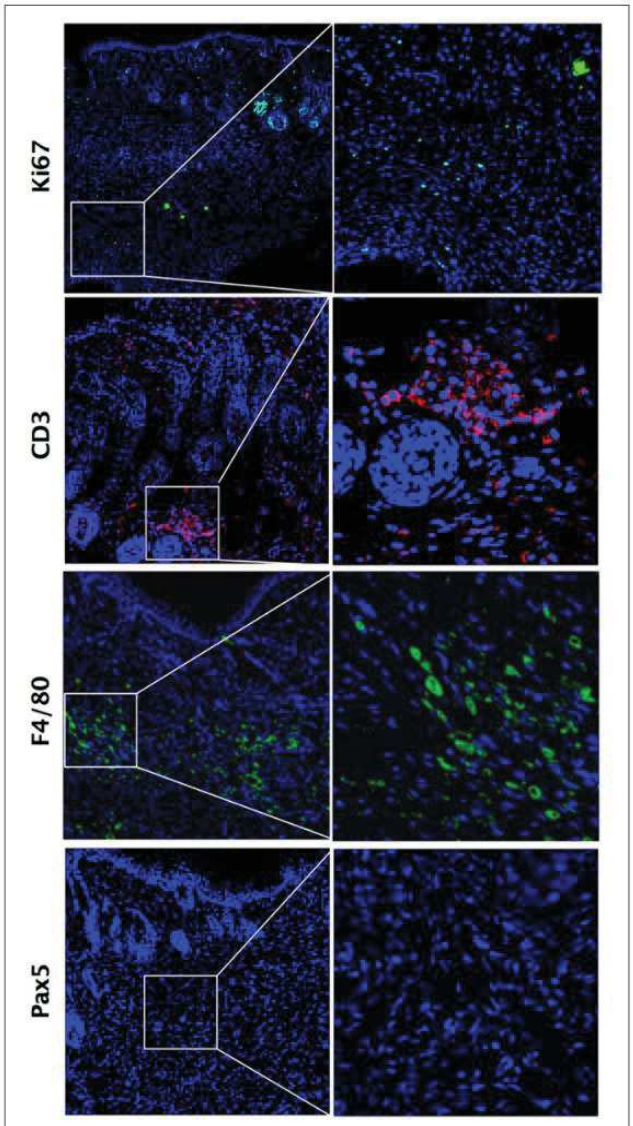

FIGURE 4 | Inflammatory cell infiltrates within dermis during fibrosis induction in HOCl-mice. Immunostaining for Ki67$^+$ proliferative cells, CD3$^+$ T-lymphocytes, F4/80$^+$ macrophages and Pax5$^+$ B lymphocytes in representative skin sections at d21 from HOCl mice (original magnification 10x; enlargement 60x).

by down-regulating T-cell and macrophage immune response resulting in reduced cytokine and autoantibody production.

MSCs Treatment Reduces Myofibroblastic Activation and Collagen Deposition During the Second Stage of Fibrosis Constitution in SSc Mice

We then investigated the effect of a single infusion of 2.5×10^5 MSCs administered at d21 on the second phase of HOCl-SSc induction (d21–d42). MSCs treatment completely inhibited skin thickening during the whole period, resulting in significantly reduced skin thickness at d42 (**Figure 7A** and **Supplementary Figure 1**). At the end of the experiment, MSCs-treated mice exhibited reduced skin fibrosis characterized by less ECM deposition in Masson Trichrome staining (**Figure 7B**) and reduced total collagen content in skin (**Figure 7C**), almost reaching the level of control PBS mice. Immunostaining for α-Sma and Tgfβ confirmed less myofibroblastic activation in MSCs-treated mice compared with untreated mice (**Figures 7D,F**). Of note, fewer F4/80+ macrophages were also noted in skin from MSCs-treated mice compared with SSc mice, whereas no difference could be detected concerning CD3+ lymphocyte infiltrates, since there was almost none in SSc mice at d42 (**Figures 7E,F**). However, in this setting, we did

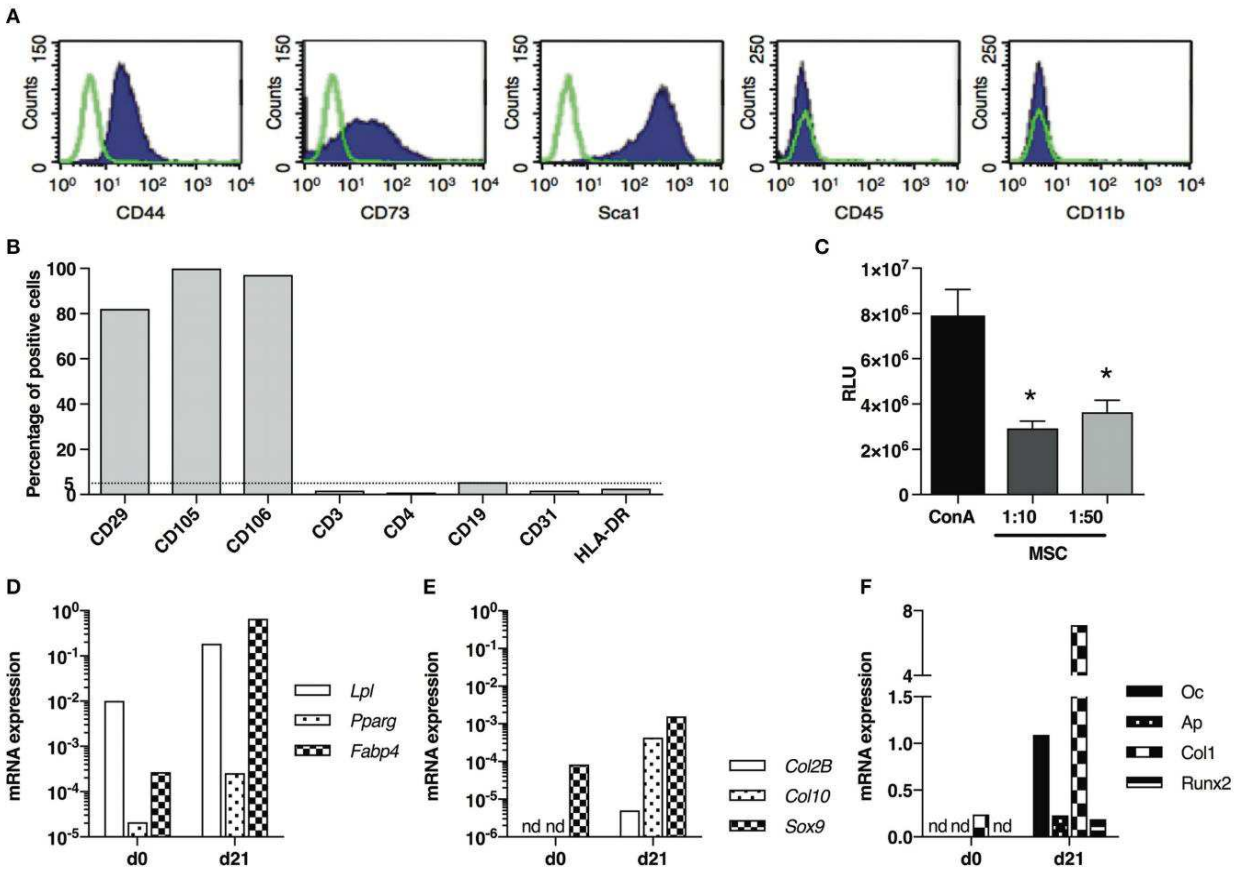

FIGURE 5 | Phenotypical and functional characterization of BM-derived BALB/c MSCs. **(A)** Representative graph of flow-cytometry analysis of MSCs stained with antibodies against CD44, CD73, Sca1, CD45 and CD11b. **(B)** Percentage of MSCs stained with antibodies against CD29, CD105, CD106, CD3e, CD4, CD19, CD31 and HLA-DR in flow-cytometry analysis. **(C)** T cell proliferation assay expressed as relative luminescent units (RLU) for splenocytes stimulated with concanavalin A (ConA) and cultured in presence of MSCs (ratio 1:10 or 1:50; $n = 3$ per group) $^*P < 0.05$. **(D)** mRNA expression of *Lpl, Pparg* and *Fabp4* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) adipogenesis induction. **(E)** mRNA expression of *Col2B, Col10* and *Sox9* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) chondrogenesis induction. **(F)** mRNA expression of *Oc, Ap*, *Col1* and *Runx2* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) osteogenesis induction. Data are presented as average ± SEM. nd: not detected. *Ap,* alkaline phosphatase; *Col,* collagen; *Fabp,* fatty acid binding protein; *Lpl,* lipoprotein lipase; *Oc,* osteocalcin; *Ppar*, peroxisome proliferator-activated receptor; *Runx,* Runt related transcription factor; *Sox,* SRY (sex determining region Y)-related HMG (high mobility group)-box gene.

not observe a reduction of anti-scl70 antibody production (**Supplementary Figure 3**).

MSCs Treatment After HOCl Discontinuation Activates Tissue Remodeling Toward Fibrosis Clearance

In a last experiment, we aimed at investigating both the evolution of HOCl-SSc after HOCl discontinuation and the effect of MSCs treatment in this condition. Therefore, we administered a single infusion of 2.5×10^5 MSCs at d42, stopped HOCl injections and sacrificed the mice 3 weeks later (d63). We first observed a spontaneous decrease in skin thickness in untreated HOCl-induced mice, significant in the last week of experiment (**Figure 8A**). MSCs-treated mice exhibited an earlier and stronger decrease in skin thickness compared with untreated HOCl-induced mice, significant in the last 2 weeks (**Figure 8A**). Histological analysis confirmed the strong anti-fibrotic effect of MSCs treatment, with an almost normal aspect of skin compared with untreated HOCl-induced mice (**Figure 8B**). Immunostaining for α-SMA and TGF-β corroborated these observations (**Figures 8C,E**), while mRNA expression for the main fibrotic markers confirmed a significantly reduced expression of *Col1, Col3, Tgfβ1*, and α-*Sma* in MSCs-treated mice compared with untreated HOCl-induced mice (**Figure 8F**). Notably, levels of these four markers were found below the levels of control PBS-mice. Interestingly, untreated HOCl-induced mice also exhibited lower levels of α-*Sma* in comparison with control PBS mice. Concerning tissue inflammation, we noted residual F4/80 macrophage infiltrates in SSc mice, which was reduced in MSCs-treated mice (**Figures 8D,E**). In MSCs-treated mice we also observed a reduced expression of *Il1β*, and *Tnfα*, while *Il6* was increased

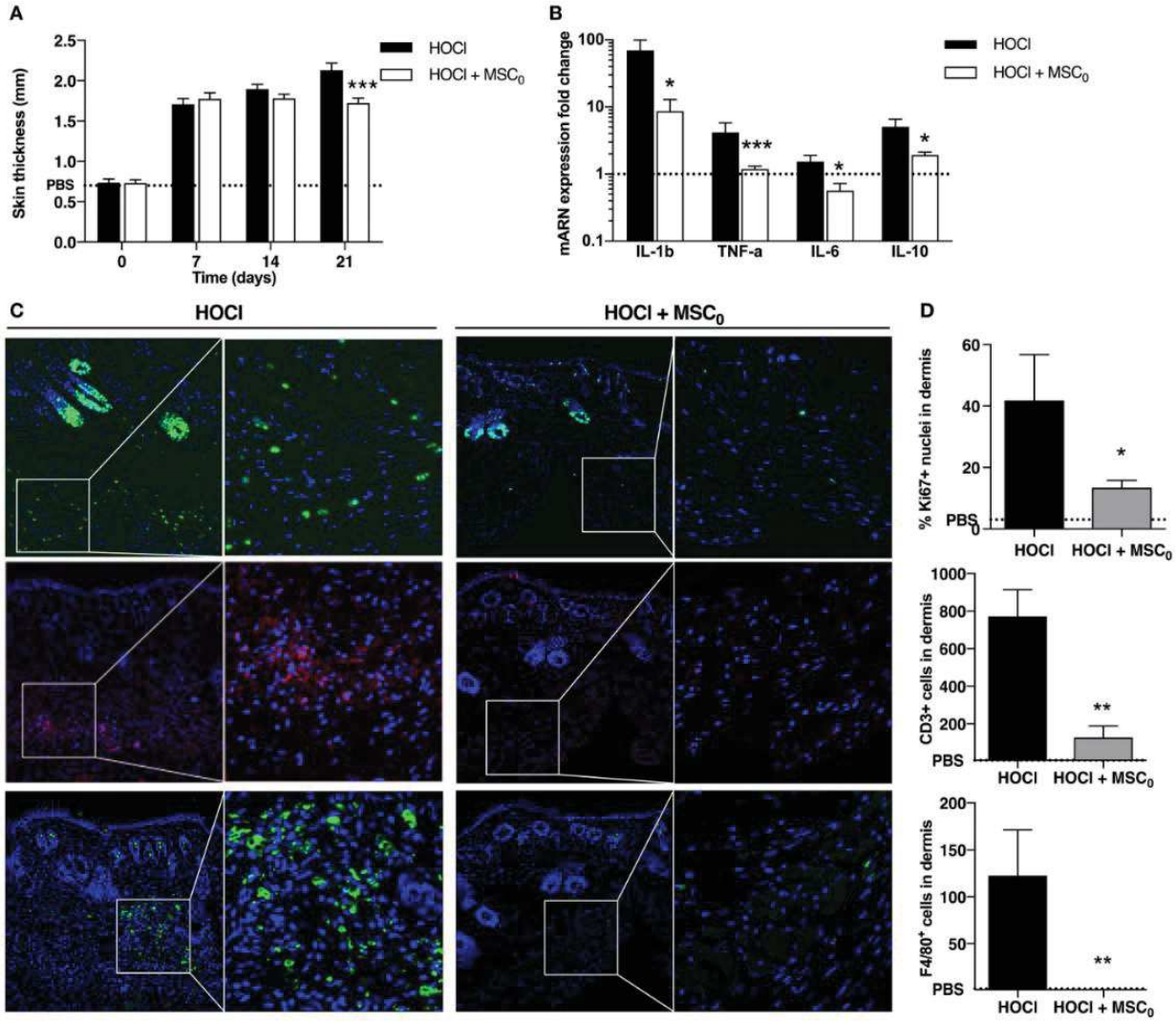

FIGURE 6 | Anti-inflammatory effects of MSC treatment during fibrosis induction in SSc mice at day 21. **(A)** Skin thickness measured from d0 to d21 in control PBS mice, non-treated SSc mice and SSc mice treated with 2.5×10^5 MSCs at d0 (MSC_0). **(B)** mRNA expression of *IL1b, TNF-a, IL-6* and *IL-10* in skin at d21. Results are given as fold-change vs. control PBS-mice normalized to 1. **(C)** Nuclear immunostaining for proliferative cells using Ki67 (top panels, in green), CD3+ T-lymphocytes (middle panels, in red), and F4/80+ macrophages (bottom panels, in green), in representative skin sections at d21 from HOCl-SSc mice and MSCs-treated HOCl-SSc mice. Left: original magnification 10x; middle: part of left image 40x. **(D)** Quantification of immunostainings in **C** using Definiens Tissue Studio IF software (top: percentage of Ki67+ nuclei among all nuclei observed; middle and bottom: absolute number of CD3+ or F4/80+ cells; $n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, data are presented as mean ± SEM. $N = 8$ for HOCl-mice, $N = 7$ for MSCs-treated mice.

and *Il10* remained unaffected (**Figure 8F**). Looking for changes in tissue remodeling, we noted a positive effect of MSCs treatment, associated with significant increased expression of metalloproteinase 1 (MMP1) and decrease of its main inhibitor tissue inhibitor of metalloproteinase 1 (TIMP1) vs. untreated HOCl-induced mice, resulting in a favorable MMP1/TIMP1 ratio in tissue (**Figure 8F**). Of note, untreated mice also exhibited a significantly higher ratio in comparison with PBS mice, or SSc mice sacrificed at d42 (data not shown). On the whole, HOCl discontinuation was responsible for tissue remodeling activation leading to progressive fibrosis clearance, a phenomenon significantly improved by MSCs therapy.

DISCUSSION

This study provides original data regarding skin fibrosis development in a preclinical inducible model for diffuse SSc. In this murine model, we demonstrated a multi-stage process leading to skin fibrosis under repeated exposure to HOCl.

First, during the first 3 weeks of the experiment, daily injections of HOCl were shown to trigger inflammation and led to transparietal cellular polymorphous infiltrates, with high proliferative rate, culminating at d21. These cellular infiltrates were mostly made of T-lymphocytes and macrophages,

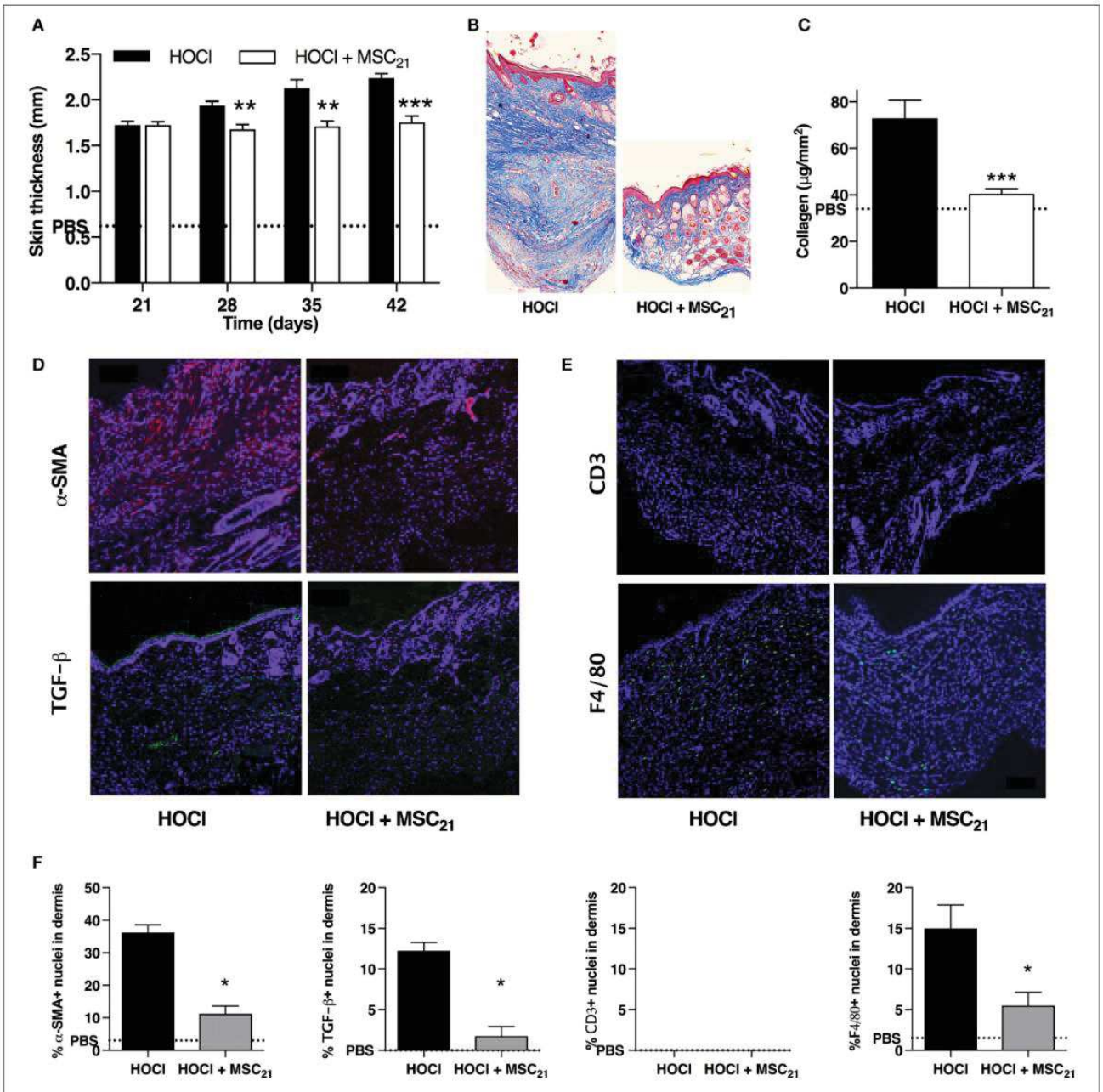

FIGURE 7 | Anti-fibrotic and anti-inflammatory effects of MSCs treatment on skin fibrosis at day 42. **(A)** Skin thickness from d21 to d42 in HOCl-SSc mice, HOCl-SSc mice treated with 2.5×10^5 MSCs at d21 (MSC_{21}), or control PBS mice. $N = 8$ per group. **(B)** Representative skin sections at d42 from HOCl-SSc mice and MSCs-treated HOCl-SSc mice (original magnification 10x; Masson trichrome staining). **(C)** Collagen content in skin from HOCl-SSc mice and MSCs-treated HOCl-mice (MSC_{21}). Mean level for control PBS mice are represented by a discontinued line. $N = 8$ per group. **(D)** Immunostaining with a-SMA (in red) and TGF-b1 (in green) in skin sections from HOCl-SSc mice (left) and MSCs-treated HOCl-SSc mice (MSC_{21}, right) at d42. **(E)** Immunostaining with CD3 (in red) and F4/80 (in green) in skin sections from HOCl-mice (left) and MSC-treated HOCl-mice (MSC_{21}, right) at d42. **(F)** Quantification of immunostainings in D and E (percentage of positive cells among all cells observed; $n = 4$ per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HOCl-mice; data are presented as mean ± SEM.

but also myofibroblasts. These observations are consistent with those made in SSc patients, notably during oedematous scleroderma, an inflammatory phenotype associated with early rapidly progressive diffuse SSc, where incisive treatments may be required (13, 14). In this condition, concordantly with what we observed, immune cells in skin tissue mainly include CD4+ T-lymphocytes and macrophages, with sparse B lymphocytes (15, 16). Macrophages are a well-known source of TGFβ secretion, this soluble mediator being the most critical trigger of fibroblast activation leading to ECM synthesis (3). Regarding cytokine

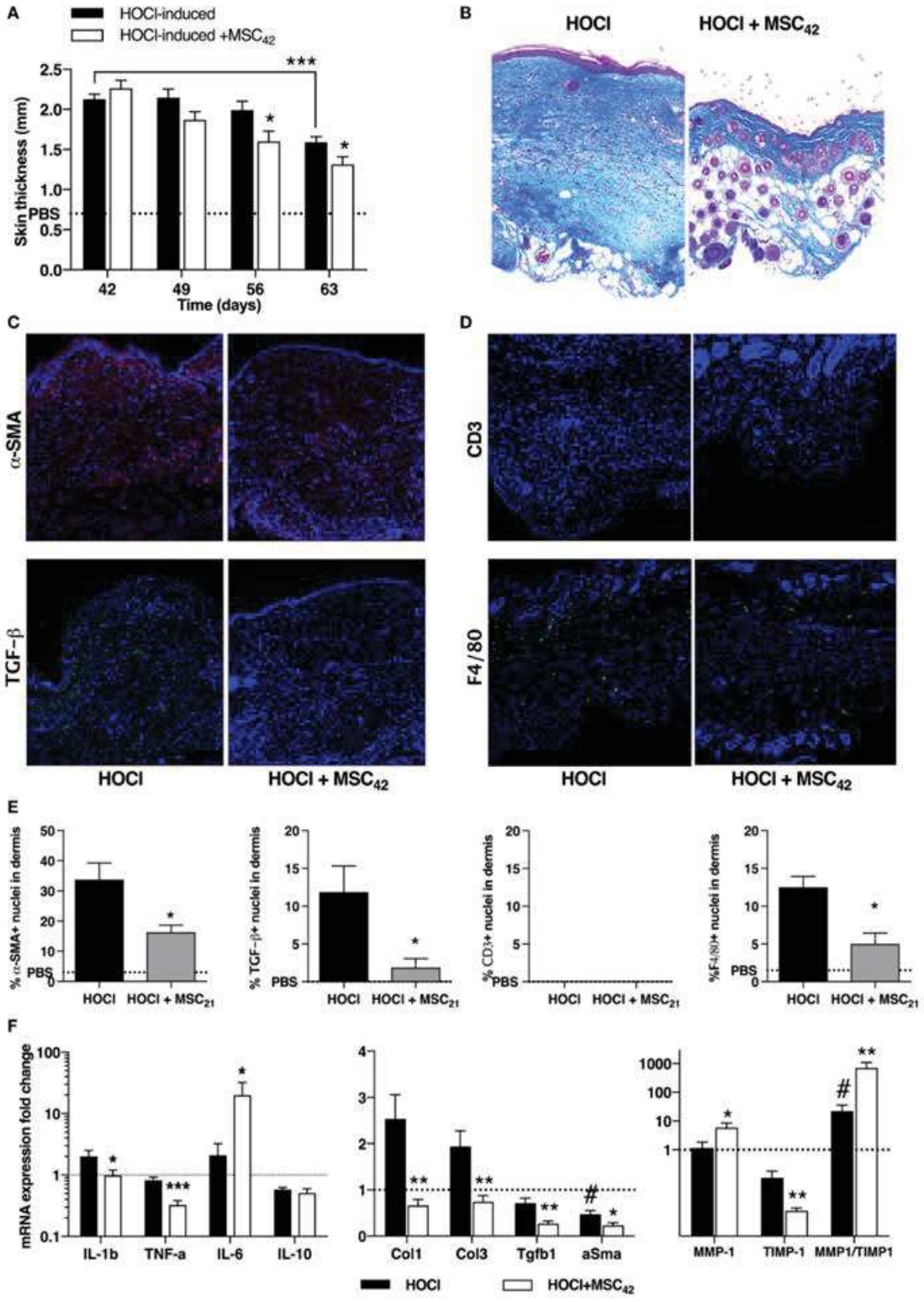

FIGURE 8 | Tissue remodeling after fibrosis induction in HOCl-mice and effects of MSCs treatment at day 63. **(A)** Evolution of skin thickness after discontinuation of HOCl challenge in HOCl-induced mice, HOCl-SSc mice treated with 2.5 × 10^5 at d42 (MSC_{42}), or control PBS mice. **(B)** Representative skin sections from HOCl-SSc mice and MSCs-treated HOCl-mice (MSC_{42}) (original magnification 10x, Masson trichrome staining). **(C)** Immunostaining with a-SMA (in red) and

(Continued)

FIGURE 8 | TGF-b1 (in green) in skin sections from HOCl-induced mice (left) and MSCs-treated HOCl-induced mice (right). **(D)** Immunostaining with CD3 (in red) and F4/80 (in green) in skin sections from HOCl-induced mice (left) and MSCs-treated HOCl-induced mice (right). **(E)** Quantification of immunostainings in C and D (percentage of positive cells among all cells observed; $n = 4$ per group). **(F)** mRNA expression of cytokines (*IL-1b, TNF-a, IL-6, IL-10,* left panel) fibrotic markers (*Col-1, Col-3, TGF-b1, a-SMA,* middle panel), and remodeling parameters (*MMP-1* and *TIMP-1,* right panel) in skin sections from HOCl-induced mice compared with HOCl-mice treated with 2.5×10^5 MSCs. Mean levels for control PBS-mice are represented by a discontinued line. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HOCl-mice; $\#P < 0.05$ vs. PBS-mice; data are presented as mean ± SEM. $N = 8$ per group.

expression, while IL1β and TNFα are associated with cell-mediated immunity, IL6 is also known to promote differentiation of fibroblasts into myofibroblasts (17) and to trigger collagen production (18). Moreover, at d21, we previously showed in this model upregulation of pro-inflammatory metalloproteases (MMP2 and MMP9, also known as gelatinases) and vascular endothelial growth factor [VEGF; (9)], which may activate angiogenesis and epithelial/endothelial-mesenchymal transition, contributing to myofibroblast activation and proliferation (19, 20). On the whole, the *primum movens* of fibrogenesis in HOCl-SSc is greatly related to inflammatory activation of skin tissue, leading to fibroblast proliferation, and preceding ECM synthesis.

In a second phase of HOCl-SSc induction, lasting from day 21 to day 42, we observed the progressive development of "matrix fibrosis," characterized at d42 by paucicellular skin tissue, with panparietal ECM deposition made of disorganized collagen fibers. At the end of the 6-wk experiment, skin fibrosis in SSc mice was characterized by strong ECM deposition made of disorganized collagen fibers, along with strong thickening of epidermis and dermis, destructuring all skin layers. This state of "established fibrosis," previously described in this model (9, 12) and responsible for skin thickening and stiffness, is close to what is usually observed in human SSc. Notably, as previously reported, tissue remodeling is low at d42, as indicated by decreased MMP1/TIMP1 ratio in tissue, contributing to impaired degradation of ECM components (9). Looking at the immune response, when comparing d42 to d21, we previously showed less inflammation and proliferation of cells, lower levels of inflammatory cytokines–except for IL-10 whose level increases during the 6-wk experiment. Of particular interest, dermal and hypodermal adipose tissue progressively disappeared during the whole process, as reported in the bleomycin model (21) and in human SSc (22), where it has been speculated that adipocyte attrition could even contribute to fibrogenesis through a putative adipocyte-to-fibroblast switch under PPAR-γ deregulation (23).

A spontaneous remission of SSc was observed once HOCl injections ceased, consistent with tissue remodeling activation and up-regulated MMP1/TIMP1 ratio. This third phase of fibrogenesis in HOCl-SSc had not been described before, and represents an original and interesting study model for SSc. As a matter of fact, clinical evolution in human scleroderma may be unpredictable, with patients first presenting with early and rapidly progressive diffuse SSc, but who may secondarily evolve with spontaneous improvement of skin fibrosis due to natural remodeling. Hence, this can somehow be disconcerting when considering therapeutic aspects, notably in the design of clinical trials (24, 25). Therefore, preclinical models reflecting disease heterogeneity and mimicking the different stages of fibrosis development are needed. In that perspective, HOCl-SSc, which reproduces three distinct phenotypes of scleroderma (i.e., early inflammatory phase, established matrix fibrosis, and spontaneous remodeling of tissue), seems particularly helpful to study new therapeutic approaches.

We previously reported the therapeutic benefits of a single IV infusion of 2.5×10^5 MSCs, capable of preventing fibrosis development in a preventive approach, or hampering fibrosis extension in a curative approach. In the present study, considering the three steps of fibrosis development and receding in the model, we focused on the effects obtained 3 weeks after MSCs infusion (i.e., d21, d42, d63). We demonstrated beneficial effects of MSCs treatment for each therapeutic strategy, in terms of skin thickness and histological lesions improvement. However, benefits were associated with more specific mechanisms. In the first setting, when infused at d0, MSCs exhibited a pre-eminence of immunomodulatory properties and were able to efficiently prevent immune response, resulting in an extinction of cellular inflammatory infiltrates together with a strong decrease in cytokine production at d21. These immunomodulatory capacities of MSCs principally involve paracrine mechanisms with the secretion of various soluble factors (i.e., IDO, iNOS, PGE2) down-regulating the immune system (8). In the second setting, when infused at d21, MSCs treatment was associated with less myofibroblast activation, and less ECM deposition at d42. These effects were previously shown to depend primarily on TGFβ signaling abrogation by MSCs, resulting in lower expression of TGFβ, TGFβRII and phospho-SMAD in the tissue (9). In the last setting, when infused at d42, after discontinuation of HOCl injections, MSCs were shown to enhance tissue remodeling, leading to enhanced MMP1/TIMP1 ratio and a more rapid clearance of fibrotic lesions. Interestingly, lost adipose tissue seemed to be partially restored during the process, suggesting regenerative potential of MSCs. Of note, MSCs were also able to prevent autoantibody production when infused at d0, indicating a specific inhibition of B-cell activation toward plasma cells. However, we could not observe any effect when mice were treated at d21, maybe because of the *in vivo* half-life of IgG (about 3–4 weeks).

Altogether, we observed benefits of MSCs treatment whatever the setting of infusion, in three distinct conditions of fibrosis development, through immunosuppressive, trophic or regenerative properties. This indicated that not only do MSCs possess wide and pleiotropic capabilities, but they also show adaptability and versatility depending on the surrounding pathological environment. Actually, it has been shown that according to the signals in the vicinity (hypoxia, ischemia, cytokine secretion…), MSCs would be primed differently, and polarized into a specific phenotype [i.e., immunosuppressive, trophic…; (8, 26)]. This plasticity in response to specific injury

makes MSCs-based therapy particularly interesting in treating SSc, considering the heterogeneity of the disease and the unpredictability of its evolution.

AUTHOR CONTRIBUTIONS

AM participated in the design of the study, acquisition, analysis and interpretation of data, manuscript redaction and final approval. KT, MM, PR, and M-CV participated in acquisition and analysis of data, manuscript proofreading and final approval. AL and CJ participated in the design of the study, interpretation of data, manuscript preparation and final approval. DN and PG carried out the conception and design of the study, participated in analysis and interpretation of data, manuscript redaction and final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02571/full#supplementary-material

Supplementary Figure 1 | (A) Skin thickness measurement during experiment (PBS mouse). **(B)** Skin at injection site on the mouse back during experiment (d34), in PBS injected-mice, HOCl-injected mice, HOCl-injected mice receiving MSC infusion at d21 (from left to right).

Supplementary Figure 2 | (A) Representative lung sections at different time points during the induction of HOCl-SSc (Masson trichrome staining). **(B)** Lung sections from PBS mice and HOCl-SSc mice at d42 (red sirius staining).

Supplementary Figure 3 | Levels of anti-scl70 antibodies in sera from PBS-healthy mice, HOCl-SSc mice and HOCl-SSc mice treated with MSC at d0 **(A)** or at d21 **(B)**. $^{**}P < 0.01$, $^{***}P < 0.001$; data are presented as mean ± SEM. $N = 8$ per group.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ANNEXE 3

frontiers in Immunology

ORIGINAL RESEARCH
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iNOS Activity Is Required for the Therapeutic Effect of Mesenchymal Stem Cells in Experimental Systemic Sclerosis

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Objectives: Fibrosis is a hallmark of systemic sclerosis (SSc), an intractable disease where innovative strategies are still being sought. Among novel anti-fibrotic approaches, mesenchymal stromal/stem cell (MSC)-based therapy appears promising. Previously, we reported anti-fibrotic effects of MSC in an experimental model of SSc, through various mechanisms (tissue remodeling, immunomodulation, anti-oxidant defense). Since immunomodulation is a pivotal mechanism for MSC therapeutic effects, we investigated the specific role of critical molecules associated with MSC immunosuppressive properties and hypothesized that MSC defective for these molecules would be less effective in reducing fibrosis in SSc.

Methods: SSc was induced by 6-week daily intradermal injections of hypochlorite (HOCl) in mice. MSC were isolated from the bone marrow of wild type mice (WT) or mice knockout for IL1RA, IL6, or iNOS (IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC, respectively). Treated-mice received 2.5 × 10^5 MSC intravenous infusion at d21. Skin thickness, histological and biological parameters were evaluated in skin and blood at d42.

Results: IL1RA$^{-/-}$ and IL6$^{-/-}$ MSC exerted similar anti-fibrotic properties as WT MSC, with a reduction of skin thickness together with less collagen deposition. Conversely, iNOS$^{-/-}$ MSC did not exert anti-fibrotic functions as shown by a similar skin thickness progression as non-treated HOCl-SSc mice. Compared with WT MSC, iNOS$^{-/-}$ MSC kept some immunosuppressive and tissue remodeling properties, but lost their capacity to reduce oxidative stress in HOCl-SSc mice.

Conclusion: Our study highlights the crucial role of iNOS, whose activity is required for the anti-fibrotic properties of MSC in experimental SSc, with a special emphasis on NO-related anti-oxidant functions.

Keywords: systemic sclerosis, HOCl, mesenchymal stem cells, inducible NO synthase, oxidative stress

INTRODUCTION

Skin fibrosis is the hallmark of systemic sclerosis (SSc), a rare and intractable autoimmune disease characterized by multi-organ fibrosis where innovative therapeutic strategies are still being sought. Among novel anti-fibrotic approaches in development, mesenchymal stromal/stem cell (MSC)-based therapy appears promising (1). Our group previously reported dramatic anti-fibrotic and anti-inflammatory effects of MSC in an experimental mouse model of SSc (2, 3). This inducible model, based on daily exposure to hypochlorite (HOCl-SSc), mimics the main features of human SSc in its diffuse and rapidly progressive form. MSC immunosuppressive properties are pivotal for their therapeutic effects, and mainly rely on paracrine mechanisms depending on soluble factors secretion (4–6).

We previously demonstrated that MSC efficacy in HOCl-SSc was associated with huge decrease in tissue inflammation characterized by less T-lymphocytes and macrophages infiltrates, and lower levels of inflammatory cytokines (7). In the present study, we further investigated the specific role of some critical molecules associated with MSC immunosuppressive properties and hypothesized that MSC defective for interleukine-1 receptor-antagonist (IL1RA), interleukine-6 (IL6) or inducible nitric-oxide (NO)-synthase (iNOS) would be less effective in reducing fibrosis in SSc. Herein, through a concise report, we present preliminary results giving evidence of the crucial role of iNOS for the anti-fibrotic properties of MSC.

MATERIALS AND METHODS

Isolation and Culture of MSC

MSC from C57BL/6 wild-type-(WT)-mice or from IL6-, iNOS-knock-out C57BL/6J-mice, or IL1RA-knock-out BALB/c-mice ($IL6^{-/-}$, $IL1RA^{-/-}$, $iNOS^{-/-}$-MSC, respectively) were isolated from bone-marrow (BM), as reported earlier (4, 5). BM was flushed out from long bones and the cell suspension was plated in DMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria), 2 mM glutamine, 100 U/ml penicillin, 100 mg/mL streptomycin (Lonza, France), and 2 ng/ml human bFGF (R&D Systems, France). Cells were passaged till obtaining homogeneity for mesenchymal marker expression and lack of hematopoietic markers as analyzed by flow cytometry. They were used between passages 10 and 15.

HOCl Preparation

HOCl was generated extemporaneously by adding NaClO (9.6% as active chlorine) to KH_2PO_4 solution (100 mM, pH: 6.2), usually using a 1:100 ratio. The right amount of NaClO was adjusted so as to obtain the desired HOCl concentration, defined by the absorbance of the mixture at 292 nm (optical density between 0.7 and 0.9 read on a Nanodrop spectrophotometer, Thermoscientific). Stock solutions were stored at 4°C in the dark and NaClO was replaced every 3 weeks.

Experimental Design and Animals

Six-week-old female BALB/c mice purchased from Janvier were housed and cared for according to the Laboratory Animal Care guidelines. Approval was obtained from the Regional Ethics Committee on Animal Experimentation (approval APAFIS#5351-2016050919079187) and the French Ministry for Education, Higher Education and Research. The mice had their backs shaved the day before the disease induction. Skin thickness was assessed with a caliper before disease induction and every week during the whole experiment by a blinded experimenter. As previously described, a total amount of 300 µL of freshly prepared HOCl was injected in two sites into the backs of the mice with a 29 G needle, 5 days a week for 6 weeks (8). Control mice received PBS in the same conditions. Treated-mice received an infusion of MSC ($2.5x10^5$ cells conditioned in 100 µL PBS), in the tail vein at day 21. Groups of 7 to 10 mice were made for each condition (PBS-, HOCl-, and MSC-treated HOCl-mice). After 6 weeks and a 2-day recovery time without HOCl injections, all animals were sacrificed. Blood samples were collected and serum was recovered after centrifugation (1,500 g, 10 min) and stored at −20°C for ELISA. Skin biopsies (6 mm punches) were performed on the backs of the mice and lungs were removed and washed in PBS. Samples were stored at −80°C for RT-qPCR and collagen content determination or fixed in 4% formaldehyde for histopathological analysis.

Histopathology

Skin samples were embedded in paraffin and 5µm thick sections were stained with Masson-trichrome. Histological slides were scanned using Nanozoomer (Hamamatsu).

RT-qPCR Analysis

Skin samples were crushed in RLT-buffer and total RNA was extracted using the RNeasy mini-kit and Qiacube robotic workstation (Qiagen, France). One microgram of RNA was reverse-transcribed (M-MLV RT, Invitrogen, France). qPCR was performed on 20 ng cDNA using LightCycler480 SYBRGreenI Master-mix and real-time PCR instrument (Roche, France). Primers were designed using the applications Primer3 and BLAST as already described (2). Samples were normalized to mRNA expression of TATA binding protein *(Tbp)* housekeeping gene, and results provided either as relative expression to *tbp* using the formula $2^{-\Delta Ct}$ or as fold-change vs. PBS-mice using the formula $2^{-\Delta\Delta Ct}$.

Collagen Content in Skin

Collagen content assay was based on the quantitative dye-binding Sircol method (Biocolor, Ireland). Skin biopsies taken from the site of injection were suspended in 2 mL of a 0.5 M acetic acid—pepsin (2.5 mg/mL) solution and dissociated using UltraTurrax (vWR, France). Collagen extraction was performed overnight at 4°C under stirring. The solution was then centrifuged at 12,000 g for 10 min and 20 µL of each sample were added to 1 mL of Syrius red reagent. Tubes were rocked at room temperature for 30 min and centrifuged at 12,000 g for 10 min. The supernatants were discarded and the tubes washed with 750 µL of ice-cold salt acid wash. After another 12,000 g centrifugation of 10 min, the

collagen-dye pellets were resuspended in 1 ml of 0.5 M NaOH Alkali solution. Optical density (OD) was then read at 555 nm on a microplate reader (Varioskan Flash, Thermo scientific) vs. a standard range of bovine collagen type I concentrations (supplied as a sterile solution in 0.5 M acetic acid). Results were expressed as the collagen content in $\mu g/mm^2$ of skin.

Determination of Advanced Oxidation Protein Product (AOPP) Concentrations in Sera

AOPP concentration was measured by spectrophotometry as previously described (3). Twenty microliters of acetic acid was added to 200 µL of serum diluted 1:20 in PBS. In standard wells, 20 µL of acetic acid was added to 200 µL of chloramine-T solution (range from 0 to 1,000 µM) followed by 10 µL of 1.16 M potassium iodide. Absorbance was read at 340 nm on a microplate reader (Varioskan Flash) before and immediately after adding acetic acid and potassium iodide. AOPP concentration was expressed as chloramine-T equivalents (µM).

Total Anti-oxidant Capacity of Serum

The total antioxidant capacity was determined on sera diluted 1:10, measuring the formation of the radical cation 2,29-azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) using the Antioxidant Assay Kit (Cayman Chemical, Interchim, France). The absorbance was read at 750 nm on a microplate reader (Varioskan Flash) vs. a standard range of Trolox, and was expressed as mM Trolox equivalents.

Determination of Glutathione (GSH) and Glutathione Disulfide (GSSG) Concentrations in Serum

At sacrifice, 100 µL of blood was collected with heparinized syringe, immediately mixed with 100-µL trichloro-acetic acid (10% in EDTA) and centrifuged at 10,000 g, at 4°C for 10 min allowing plasma recovery. Concentrations of GSH and its oxidized form GSSG were determined using ultraperformance liquid-chromatography–tandem-mass spectrometry (UPLC, Waters Acquity, Milford, USA).

Statistical Analysis

Quantitative data were expressed as mean ± SEM. Data were compared using Mann–Whitney's test for non-parametric values or Student's *t*-test for parametric values as evaluated using the Shapiro–Wilk normality test. When analysis included more than two groups, one-way ANOVA was used. All statistical analyses were performed using Prism 6 GraphPad software for Mac OS (California, USA). A $P < 0.05$ was considered significant.

RESULTS

iNOS Activity Is Required for MSC-Based Therapy of SSc

In a first series of experiments, we compared the effects of MSC defective for IL1RA, IL6, and iNOS production with those of WT-MSC when injected during the course (d21) of HOCl-SSc. In this setting, we observed that disease progression was hampered in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC, indicating that neither IL1RA nor IL6 were involved in the therapeutic effect of MSC in this model (**Figure 1A**). Conversely, iNOS$^{-/-}$-MSC did not affect the course of skin thickness, which followed the progression of non-treated HOCl-SSc mice. Concordantly, at d42, skin thickness was significantly lower in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC, compared with non-treated mice or iNOS$^{-/-}$-MSC-treated mice; and no difference in skin thickness was found between the two latter groups (**Figure 1B**).

These clinical data were corroborated by the measurement of collagen content in skin, significantly lower in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC compared with HOCl-SSc mice, while no significant effect was noted for iNOS$^{-/-}$-MSC-treated mice (**Figure 1C**). On histology, treatment with WT-MSC and to the same extent with IL1RA$^{-/-}$ or IL6$^{-/-}$-MSC reduced dermal collagen infiltration, while no reduction in collagen deposition was observed in iNOS$^{-/-}$-MSC-treated mice (**Figure 1D**).

iNOS$^{-/-}$ MSC Show Preserved Anti-inflammatory and Remodeling Capacities

Since IL1RA$^{-/-}$ or IL6$^{-/-}$-MSC exerted similar anti-fibrotic effects as WT_MSC, we next focused on iNOS$^{-/-}$-MSC in a second series of experiments. We confirmed that iNOS$^{-/-}$-MSC were unable to reduce skin thickening (**Figure 2A**) or collagen deposition (**Figure 2B**) during the induction of HOCl-SSc, compared with WT-MSC.

In order to decipher the underlying mechanisms, we then investigated the effect of MSC treatment on skin inflammation and tissue remodeling. We first noted that iNOS$^{-/-}$-MSC were almost as efficient as WT-MSC in reducing the expression of IL1β and IL6, two main inflammatory cytokines that are found at high levels within the skin of HOCl-SSc mice (**Figure 2C**). Concerning their ability to improve ECM remodeling, we noted that mice treated with iNOS$^{-/-}$-MSC disclosed reduced expression of tissue inhibitor of metalloprotease-1 (TIMP-1), and higher matrix metalloproteinase (MMP)1/TIMP1 ratio compared with HOCl-SSc mice, indicating enhanced remodeling capacity, similar to what is observed using WT-MSC (**Figure 2D**). Of note, concerning systemic involvement in HOCl-SSc, iNOS$^{-/-}$-MSC were not able to reduce fibrotic markers such as collagen 1 and α-SMA and inflammatory cytokines (IL-1β and IL-6) in lung tissue, while WT-MSC had a positive impact on pulmonary fibrosis in this model (data not shown).

iNOS$^{-/-}$ MSCs Fail to Dampen HOCl-Induced Oxidative Stress

Since immunomodulatory and remodeling capacities of iNOS$^{-/-}$-MSC seemed relatively preserved, we next looked at their effects on oxidative parameters in HOCl-SSc mice. Interestingly, we observed that iNOS$^{-/-}$-MSC failed to reduce the levels of AOPP in serum compared with WT-MSC,

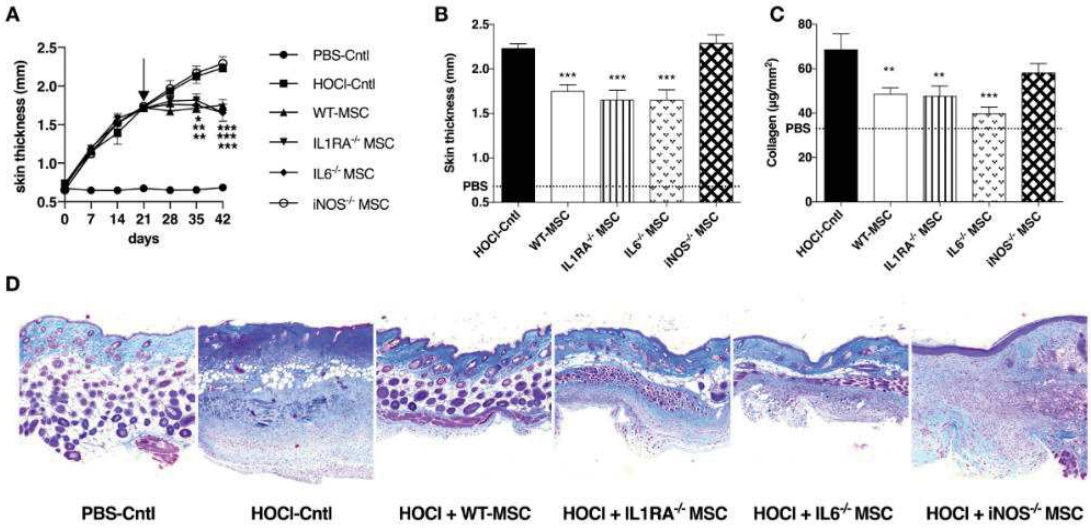

FIGURE 1 | Comparative effects of WT MSC, IL1RA$^{-/-}$, IL6$^{-/-}$, and iNOS$^{-/-}$ MSC in HOCl-SSc. **(A)** Skin thickness evolution (d0 to d42) from control PBS-mice, HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC at d21. **(B)** Skin thickness at d42 in previously mentioned groups of mice (control PBS-mice are represented by a discontinued line). **(C)** Collagen content in skin samples from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC (mean level for control PBS-mice is represented by a discontinued line). **(D)** Representative skin sections at d42 (original magnification 10x; Masson Trichrome staining). $N = 8$ for PBS-mice, HOCl-mice, and IL6$^{-/-}$ MSC-treated mice, $n = 7$ for IL1RA$^{-/-}$ and iNOS$^{-/-}$ MSC-treated mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, data are presented as mean ± SEM.

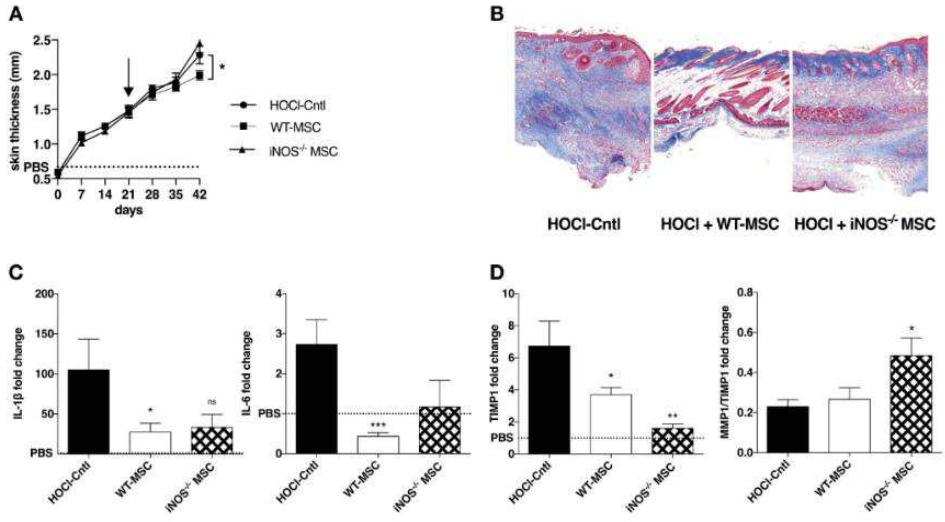

FIGURE 2 | Effects of iNOS$^{-/-}$ MSC on fibrosis, inflammation, and tissue remodeling in HOCl-SSc. **(A)** Skin thickness evolution (d0 to d42) from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSCs at d21 (mean levels for control PBS-mice are represented by a discontinued line). **(B)** Representative skin sections at d42 (original magnification 10x; Masson Trichrome staining). **(C,D)** mRNA expression of *IL1β*, *IL6*, *MMP1*, and *MMP/TIMP1* at d42 in skin sections from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. Results are given as fold-change vs. control PBS-mice normalized at 1.

but induced higher levels of gluthatione and enhanced anti-oxidant capacity (AOC) (**Figures 3A–C**). In the end, the overall oxidative balance represented by AOPP/AOC ratio remained high under iNOS$^{-/-}$-MSC treatment whereas it significantly decreased under WT-MSC treatment (**Figure 3D**).

DISCUSSION

We previously demonstrated pleiotropic mechanism of MSC, acting through the abrogation of myofibroblastic activation and the reduction of tissue inflammation through potent immunosuppression, but also inducing tissue remodeling via

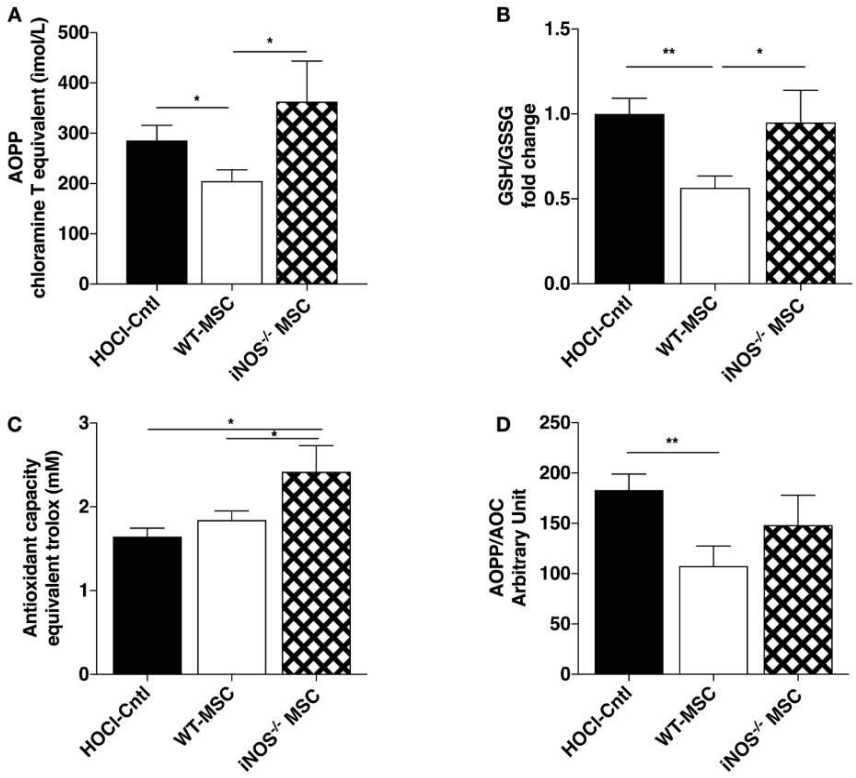

FIGURE 3 | Effects of iNOS$^{-/-}$ MSC on oxidative balance in HOCl-SSc. **(A)** Advanced Oxidation Protein Product (AOPP) concentrations in sera from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. **(B)** Glutathione levels in sera from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC (GSH/GSSG ratio). **(C)** AOC and **(D)** AOPP/AOC serum levels in HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSCs. $N = 7$ HOCl-mice, $n = 6$ for HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. $^*P < 0.05$, $^{**}P < 0.01$, data are presented as mean ± SEM.

metalloproteinase activation, and improving oxidative imbalance (2, 6, 7). These different mechanisms may depend on the pathological environment at the time of MSC infusion and argue for MSC adaptive capacities. This seems even more relevant in the setting of SSc, a heterogeneous and multifaceted disease.

In the present study, we aimed at deciphering possible mechanisms of MSC efficacy in HOCl-SSc and focused on their paracrine functions through molecules that were known to support their immunosuppressive capacities. Using MSC defective for these molecules, we observed that only iNOS seemed to be mandatory for the anti-fibrotic effects of MSC in HOCl-SSc. By contrast, IL1RA and IL6 were not involved in this function while they were required in previous *in vitro* and *in vivo* studies on other inflammatory models, such as collagen-induced-arthritis, a preclinical model for rheumatoid arthritis, another pathological condition where inflammatory cytokines such as IL6 play a critical role (4, 5).

Focusing on inflammation in mice treated with iNOS$^{-/-}$-MSC, we noticed that there were slightly less capable of reducing cytokine production than WT-MSC. This seems consistent with the importance played by iNOS for the immunosuppressive function of MSC in literature (9). Another explanation for these results may lie in the fact that inflammation in HOCl-SSc mainly pertains to the onset of the fibrogenic process (before d21), a period of time where the role of MSC might be more devoted to immunosuppression (Frontiers Immunology, in revision).

Since we did not demonstrate a dramatic reduction of anti-inflammatory function using iNOS$^{-/-}$-MSC, we looked for other mechanisms involved in the lack of therapeutic effect in murine SSc. We first turned to tissue remodeling, because this process seems of particular importance in the last 3 weeks of HOCl-SSc model (from d21 to d42), a phase characterized by less inflammation but strong collagen deposition in tissue. Interestingly, iNOS$^{-/-}$-MSC improved some remodeling parameters, with higher levels of the MMP1/TIMP1 ratio in skin, as compared with those found in WT MSC treated-mice. The strong decrease of TIMP-1, one main inhibitor of metalloproteinases, under iNOS$^{-/-}$ MSC treatment, suggested that iNOS is poorly involved in tissue remodeling.

Still, iNOS$^{-/-}$ MSC failed to prevent collagen deposition in tissue, which led us to consider the impact on oxidative balance. Indeed, in HOCl-SSc as well as in human disease, the role of oxidative stress seems pivotal (10, 11). Notably, AOPP were reported to play a critical role in fibrosis and autoantibody formation both in human and mice (8, 12). Herein, we showed that iNOS$^{-/-}$-MSC, unlike WT-MSC, were not able

to reduce the levels of AOPP in serum. Even though anti-oxidant defenses (i.e., serum AOC and glutathione levels) were upregulated in iNOS$^{-/-}$-MSC-treated mice, to even higher levels than in WT MSC-treated mice, the overall oxidative balance seemed unfavorable in these mice. In that sense, the strong upregulation in some parameters (i.e., glutathione or tissue remodeling enzymes MMP1/TIMP1), even overpassing what is observed using WT-MSC, might be a compensatory mechanism to counteract the persistent oxidative stress in these mice treated with defective iNOS$^{-/-}$-MSC.

On the whole, through this concise report, we demonstrate the crucial role of iNOS in the therapeutic effects of MSC in the murine HOCl-SSc model resulting in a global anti-fibrotic impact. This is supported by another study where iNOS$^{-/-}$-MSC failed to prevent tissue fibrosis in a model of liver cirrhosis (13). Conversely, another study reported that NO increased the anti-fibrotic properties of MSC in the same disease model (14).

Actually, NO plays a complex role in tissue remodeling and in oxidative stress regulation. On the one hand, the short-term production of NO by iNOS induces reactive oxygen species (ROS) formation such as peroxynitrites (15). On the other hand, NO is required for wound healing (16–18) and is also considered as an antioxidant (19, 20). Moreover, while prolonged iNOS blockade induces renal, heart or liver fibrosis in rodents (21–23), NO has shown proper antifibrotic roles, through the inhibition of myofibroblast activation, the abrogation of TGFβ pathway and the activation of MMP and hepatocyte growth factor (HGF), leading to less collagen deposition in other models (13, 20, 24). Interestingly, in the specific context of SSc, molecules up-regulating the NO pathway have been developed to treat pulmonary arterial hypertension in the clinics (25), and demonstrated anti-fibrotic effects in various preclinical models (26). This strengthens the interest of these preliminary results. Even if iNOS might not be the only mediator of importance in MSC therapeutic effects, this work underlines the role played by oxidative stress in SSc, and brings the perspective of enhancing MSC anti-oxidant activity to ameliorate their anti-fibrotic properties for future applications.

AUTHOR CONTRIBUTIONS

AM participated in the design of the study, acquisition, analysis and interpretation of data, manuscript redaction and final approval. PR, GF, TS, MM, and KT participated in acquisition and analysis of data, manuscript proofreading and final approval. J-PC and CJ participated in the design of the study, interpretation of data, manuscript preparation and final approval. DN and PG carried out the conception and design of the study, participated in analysis and interpretation of data, manuscript redaction and final approval.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ANNEXE 4

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TGFBI secreted by mesenchymal stromal cells ameliorates osteoarthritis and is detected in extracellular vesicles

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ABSTRACT

Mesenchymal stem/stromal cells (MSCs) are of interest in the context of osteoarthritis (OA) therapy. We previously demonstrated that TGFβ-induced gene product-h3 (TGFBI/BIGH3) is downregulated in human MSCs (hMSCs) from patients with OA, suggesting a possible link with their impaired regenerative potential. In this study, we investigated TGFBI contribution to MSC-based therapy in OA models. First, we showed that co-culture with murine MSCs (mMSCs) partly restored the expression of anabolic markers and decreased expression of catabolic markers in OA-like chondrocytes only upon priming by TGFβ3. Moreover, TGFβ3-primed hMSCs not only modulated the expression of anabolic and catabolic markers, but also decreased inflammatory factors. Then, we found that upon TGFBI silencing, mMSCs partly lost their inductive effect on chondrocyte anabolic markers. Injection of hMSCs in which TGFBI was silenced did not protect mice from OA development. Finally, we showed that MSC chondroprotection was attributed to the presence of TGFBI mRNA and protein in extracellular vesicles. Our findings suggest that TGFBI is a chondroprotective factor released by MSCs and an anabolic regulator of cartilage homeostasis.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) are multipotent progenitor cells that are primarily isolated from bone marrow and adipose tissue, but that can also be found in many other tissues, including umbilical cord and deciduous teeth [1]. These fibroblastic-like adherent cells are characterized by a panel of positive and negative markers and the potential to differentiate into the three mesenchymal lineages [2]. MSCs also secrete many factors that act in a paracrine fashion and play important roles in their therapeutic effect [3]. Recent findings indicate that most of these factors are conveyed by extracellular vesicles (EVs) that take part in intercellular communication by acting as vehicles for the transfer of mediators between cells [4]. Previous studies on factors that mediate *in vivo* the beneficial function of MSCs showed that interleukin 6 (IL6), IL1 receptor antagonist, and glucocorticoid induced leucine zipper are anti-inflammatory mediators in experimental arthritis, whereas thrombospondin-1 is a chondroprotective factor in osteoarthritis (OA) [5–8]. Nevertheless, the identification of factors responsible for MSC clinical benefit in rheumatic diseases is still incomplete.

OA is the most common rheumatic disease. Its prevalence increases with age, and is higher in patients with metabolic syndromes or obesity [9]. OA is characterized by progressive cartilage destruction, but it also affects all other joint tissues, leading to sub-chondral bone sclerosis, synovium inflammation and fat pad fibrosis [10,11]. In patients with severe forms, it results in the loss of joint function, pain and functional disability. Currently, there is no curative treatment and the available pharmaceutical options only alleviate symptoms, such as pain and inflammation. In patients with advanced OA, joint replacement surgery offers pain relief and restores function and mobility. Recent studies have evaluated the interest of delivering MSCs in the pathological joint, as a possible innovative therapeutic solution. MSC chondroprotective effect has been demonstrated in preclinical models of OA [12–14], and the safety and efficacy of this approach are now evaluated in the clinic (for review, see Ref. [15]). Both allogeneic and autologous MSCs are used; however, questions have been raised on the efficacy of MSCs isolated from aged donors or with age-associated diseases [16]. Indeed, MSCs from aged donors show reduced proliferative capacity, differentiation potential, and migration as well as increased senescence [17]. As MSCs adapt and respond to their microenvironment, a pathological

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state might perturb the stem cell niche where MSCs reside. Therefore, the interactions between MSCs and their niche will result in modifications of their secretome. For instance, several factors secreted in the joint environment, particularly members of the transforming growth factor-β (TGFβ) family [18], are deregulated in OA and contribute to the breakdown of cartilage homeostasis. At basal levels, TGFβ signalling contributes to cartilage homeostasis. On the other hand, high concentrations of TGFβ are found in the synovial fluids of patients with OA [19]. Interestingly, deregulation of TGFβ signalling in MSCs is involved in OA onset or progression, and knock out of TGFβ type II receptor in MSCs attenuates cartilage erosion and subchondral bone sclerosis [20]. We recently reported that TGFβ-induced gene product-h3 (TGFBI/BIGH3/RGD-CAP) is upregulated in cartilage and bone from patients with OA, whereas it is downregulated in bone marrow-derived human MSCs (hMSCs) [21]. We also found that TGFBI downregulation in hMSCs partly compromises their chondrogenic potential. Therefore, we hypothesized that TGFBI downregulation in hMSCs from patients with OA may affect their functional properties and impair their regenerative/repair potential. Here, we investigated whether TGFBI deregulation in OA affects MSC chondroprotective role.

2. Materials and methods

Mesenchymal stromal cell culture. Human specimens were recovered from patients with OA undergoing knee replacement surgery after written informed consent, in accordance with the Declaration of Helsinki. The study was carried out following the recommendations by the Languedoc-Roussillon Committee for the Protection of Persons and was approved by the French Ministry of Higher Education and Research (DC-2010-1185). After isolation from bone marrow, hMSCs were characterized by phenotyping and tri-lineage differentiation, as described [22]. They were cultured in proliferative medium [αMEM, 2 mmol/mL glutamine, 100 µg/mL penicillin/streptomycin, 10% foetal calf serum (FCS), and 1 ng/mL basic fibroblast growth factor (bFGF) (R &D Systems, Lille)], and used between passage 2 and 5. Murine MSCs (mMSCs) were isolated from bone marrow of C57BL/6 mice, expanded in proliferative medium, and characterized as previously reported [5]. They were used between passage 12 and 20.

EV isolation and characterization. EVs were isolated from conditioned supernatants of mMSCs (mMSC-EVs) by differential ultracentrifugation to recover large-size EVs and small-size EVs at 18 000 and 100 000 g, respectively. EVs were characterized following the guidelines provided by the International Society of Extracellular Vesicles (ISEV), as already described in Ref. [23]. Similar protocols were used for the production and isolation of hMSC-EVs. Briefly, hMSCs (10^6/dish) were cultured in proliferative medium with 3% EV-free FCS for 60 h for production of EV-rich supernatant. Total EVs, which contain small-size and large-size EVs, were isolated according to the described protocol [23]. EV characterization included the determination of size and concentration by nanoparticle tracking analysis (NanoSight LM10-12 Malvern Instruments, Orsay), protein quantification with the Micro BCA Protein Assay Kit (Pierce, ThermoFisher Scientific, Illkirsch), and surface marker detection (anti-CD81 from Miltenyi Biotech, Paris; and anti-CD44, -CD63, CD73, -CD90, -CD105 antibodies from BD Biosciences, Le Pont de Claix) on EV-coated beads, as described [23].

Cartilage explant and chondrocyte culture. Femoral heads were dissected from 2-week-old C57BL/6 mice as described [24]. After 72 h of stabilization in proliferative medium, OA-like changes were induced in cartilage explants by culture in serum-free medium with 10 ng/mL IL-1β (R&D Systems) for another 72 h. Thereafter, OA-like explants were co-cultured with 2×10^5 mMSCs seeded in polyethylene terephthalate culture inserts with 0.4 µm pore size (BD, Corning, Boulogne-Billancourt) for 24 h.

Murine articular chondrocytes were isolated from the knees and femoral heads of 3-day-old C57BL/6 mice, as described [25]. Chondrocytes were plated (25 000 cells/cm^2) in 12-well TPP culture plates (TPP Techno Plastic Products, Switzerland) with 1 mL of proliferative medium for 5 days. Thereafter, OA-like chondrocytes were induced by incubation with 1 ng/mL IL-1β (R&D Systems) for 24 h (day 0). In parallel, mMSCs were cultured in 1 mL of proliferative medium in 12-well culture plates. At 80% confluence, they were primed (or not) with 10 ng/mL TGFβ3 (R&D Systems) for 24 h, then rinsed twice with PBS, and cultured for 24 h to obtain conditioned medium (mMSC-CM). mMSC-CM (1 mL/well) was then added to OA-like chondrocytes for 24 h (day 1) before recovering chondrocytes for RT-qPCR analysis.

For co-culture with mMSCs or hMSCs, 2×10^5 OA-like chondrocytes were seeded in 12-well culture inserts with 1 mL of proliferative medium and primed (or not) with 10 ng/mL TGFβ3 for 24 h. After two washes with PBS, mMSC- or hMSC-containing inserts were added to OA-like chondrocytes on day 0 with fresh medium and co-cultured for 24 h (day 1). Chondrocytes were recovered and processed for RT-qPCR.

Glycosaminoglycan (GAG) content measurement. GAG content was measured using the Blyscan Glycosaminoglycan Assay according to the supplier's recommendations (Biocolor Ltd, UK). Supernatants from OA-like explants were collected and cultured OA-like explants were digested overnight with 125 µg/mL papain (Sigma) in sodium acetate buffer (0.1 M; pH = 5.5) containing 5 mM EDTA and 5 mM L-cysteine HCl. Supernatants and digestion products were diluted to fit the calibration curve. Colorimetric values were obtained using a Varioskan LUX microplate reader.

Cell transfections. At 60% confluence, mMSCs and hMSCs were transfected with 50 nM of siRNA-control (siCT), siRNA-mTGFBI or siRNA-hTGFBI (siTGFBI) (Ambion, ThermoFisher Scientific) using the Oligofectamine reagent and according to the supplier's recommendations (Life Technologies, Courtaboeuf). Chondrocytes were transfected with 400 nM of each siRNA using the Lipofectamine reagent, according to the supplier's recommendations (Life Technologies, Courtaboeuf). Cells were used 48 h after transfection.

Proliferation and adhesion assays. For both assays, 96-well TPP plates were coated with 10 µg/mL recombinant human TGFBI (rhTGFBI; R&D Systems) and incubated at 4 °C overnight. Then, wells were washed twice with PBS and blocked with 2% BSA at room temperature for 1 h. For proliferation assays, 2×10^4 murine chondrocytes were seeded on rhTGFBI-coated plates and incubated for 72 h. For adhesion assays, 5×10^4 murine chondrocytes were seeded on rhTGFBI-coated plates and after 2 h, 4 h, and 6 h, wells were washed twice with PBS to remove non-adherent cells before quantification of the adhering cells. Cell number was quantified by measuring cell viability using the CellTiter-Glo luminescent assay (Promega, Charbonnières-les-Bains), according to the manufacturer's protocol. Cell numbers were estimated relative to a standard curve generated using 10-fold serial dilutions of chondrocytes and the cell number in non-treated wells was set to 1.

Splenocyte proliferative assay. Splenocytes were isolated from C57BL/6 mice and cultured with hMSCs transfected with siCT or siTGFBI, as described [8]. After 3 days of incubation, splenocyte proliferation was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Charbonnières-les-Bains) following the manufacturer's instructions. Splenocyte proliferation was quantified by subtracting the signal of unstimulated splenocytes, and calculated as the percentage of the value obtained in concanavalin A-stimulated splenocytes (100%).

RNA extraction and RT-qPCR. Total RNA was isolated from h/mMSCs or chondrocytes using the RNeasy kit according to the supplier's protocol (Qiagen, Courtaboeuf); from cartilage and bone with 0.1 mL TRIzol reagent (ThermoFisher Scientific)/g tissue, followed by chloroform and phenol acid extraction; and from EVs using the miRNeasy Micro Kit (Qiagen). Total RNA (0.5 µg) was reverse transcribed using 100 U of M-MLV reverse transcriptase (ThermoFisher Scientific), and PCR reactions were performed as described [26]. Primer sequences (SYBR Green Technologies) are described in Table 1. All values were normalized to the *RPS9* housekeeping gene, and expressed as relative

Table 1
List of primer sequences for RT-qPCR analysis.

Gene	Sequence forward	Sequence reverse
mADAMTS5	CTGCCTTCAAGGCAAATGTGTGG	CAATGGCGGTAGGCAAACTGC
mAGG	GCGAGTCCAACTCTTCAAGC	GAAGTAGCAGGGGATGGTGA
mCOL2B	CTGGTGCTGCTGACGCT	GCCCTAATTTTCGGGCAT
mCOX2	GCATTCTTTGCCCAGCACTT	AGACCAGGCACCAGACCAAAGA
mIL6	TGGGACTGATGCTGGTGACA	TTCCACGATTTCCCAGAGAACA
mINOS	CCTTGTTCAGCTACGCCTTC	GCTTGTCACCACCAGCAGTA
mMCP1	TGCAGGTCCCTGTCATGCTT	TCCTTCTTGGGGTCAGCACA
mMMP13	TCTGGATCACTCCAAGGACC	ATCAGGAAGCATGAAATGGC
mTGFBI	ACCATCAACGGGAAGGCTGTCA	AGCCAGCTCAAGCAGTGTCTTG
mTNFalpha	AGCCCACGTCGTAGCAAACCA	TGTCTTTGAGATCCATGCCGTTGGC
hTGFBI	GGACATGCTCACTATCAACGGG	CTGTGGACACATCAGACTCTGC

expression or fold change using the respective formulae: $2^{-\Delta CT}$ and $2^{-\Delta\Delta Ct}$. For EVs, gene expression was quantified in 10 ng of cDNA and normalized to the Ct values.

Collagenase-induced osteoarthritis model. The collagenase-induced OA (CIOA) model was generated in accordance with the guidelines and regulations of the Ethical Committee for animal experimentation of the Languedoc-Roussillon region (Approval APAFIS#5349–2016050918198875). Experiments were performed after the final approval by the French Ministry for Education, Higher Education and Research. OA was induced by two injections (day 0 and 2) of 1U type VII collagenase in 5 µL saline in the intra-articular (IA) space of one hind knee joint in 10-week-old C57BL/6 mice. Then, groups of 23 mice received or not IA injections of siCT- or siTGFBI-transfected hMSCs (2.5×10^5 cells/5 µL saline) at day 7. Mice were euthanatized at day 42 and hind paws were fixed in 4% formaldehyde for further analysis.

Bone parameter analysis. Hind paws were scanned in a SkyScan 1176 micro-CT scanner (Bruker, Belgium) using the following parameters: 0.5 mm aluminium filter, 45 kV, 500 µA, resolution of 18 µm, 0.5° rotation angle. Scans were reconstructed using the NRecon software (Bruker). Misalignment compensation, ring artefacts and beam-hardening were adjusted to obtain the correct reconstruction of each paw. Bone degradation was quantified in subchondral bone of the medial plateau for each tibia (CTAn software, Bruker). Calcification of the lateral and medial meniscal/external ligaments and osteophyte formation on joint edges were also quantified. 3D images of joints were reconstructed using the Avizo software (Avizo Lite 9.3.0, FEI Visualization Sciences Group, Lyon, France).

Confocal laser scanning microscopy. A confocal laser scanning microscope (CLSM; TCS SP5-II, Leica Microsystems, Nanterre) was used to acquire images of the medial tibial plateau articular cartilage. Articular cartilage was scanned in depth (XYZ-mode) using the following parameters: voxel size 6 µm, 5× dry objective, and UV laser light source (405 nm). Image stacks were used to reconstruct a 3D image of the medial tibial plateau cartilage and then to quantify cartilage morphometric parameters using the Avizo software.

Histological analysis. Hind paws were decalcified in 5% formic acid solution for 2 weeks, and then processed for paraffin embedding. Coronal sections of tibias were cut (3 slices of 7 µm each 100 µm; first section at 50 µm below the cartilage surface) and stained with Safranin O/Fast Green. Cartilage degradation was quantified using the modified Pritzker OARSI score, as described [27]. Osteophyte size at the edges of the tibia cartilage was scored using an arbitrary score from 0 to 3, as described [14].

Statistical analysis. Statistical analysis was performed with the GraphPad Prism software. Each sample/cell was independent and represented an experimental unit providing a single outcome. The data normal distribution and variance homogeneity were determined with the Shapiro-Wilk and Fisher's exact tests (2 groups) or the Bartlett's test (> 2 groups), followed by the appropriate tests, as detailed in each figure legend. Data are presented as the mean ± SEM with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

TGFBI is deregulated in OA-like femoral head explants and chondrocytes. We previously showed by immunohistochemistry that TGFBI is upregulated in the cartilage from patients with OA and in CIOA mice compared with healthy controls [28]. To better understand the effect of TGFBI deregulation, we first evaluated its expression by RT-qPCR in mice at different ages. We found that *TGFBI* mRNA expression was higher in cartilage than in cortical bone or bone marrow in healthy 3-day-old mice (Fig. 1A). *TGFBI* expression was also higher in the femoral heads of adult mice compared with cortical bone or total bone marrow, and in tibial epiphyses compared with bone marrow (Fig. 1B). This suggests higher expression in cartilage-containing tissues. Importantly, *TGFBI* mRNA levels tended to be higher in tibial epiphyses from CIOA mice than in healthy controls, confirming TGFBI upregulation in OA joint tissues (Fig. 1C). We next determined whether OA-related TGFBI deregulation can be reproduced in *in vitro* models. First, we demonstrated that mouse femoral head explants (Fig. 1D) cultured with IL1β reproduced the OA-like cartilage phenotype, as indicated by the downregulation of the anabolic markers type IIB collagen (*Col2a1*) and aggrecan (*Acan*) and the upregulation of the catabolic markers matrix metalloproteinase 13 (*Mmp13*) and A Disintegrin And Metalloproteinase with Thrombospondin Motifs 5 (*Adamts5*) (Fig. 1E). In addition, IL1β significantly increased GAG release in the culture supernatant, and decreased GAG content in cartilage explants. Moreover, *Tgfbi* level was higher in OA-like explants compared with untreated controls (Fig. 1E).

Similarly, immature articular chondrocytes isolated from neonatal mice displayed an OA-like phenotype after incubation with IL1β (Fig. 1F). Specifically, expression of *Col2a1* and *Acan* was decreased, whereas that of *Mmp13* and *Adamts5* was increased after 24 h of incubation with IL1β (Fig. 1G). Moreover, the genes encoding several inflammatory mediators, such as IL6, inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP)1 and cyclooxygenase (COX)2, were upregulated, whereas tumour necrosis factor (TNF)α remained unchanged. In this model, *Tgfbi* was downregulated (Fig. 1G), indicating a deregulation of TGFBI in both *in vitro* models of OA.

Naive murine and human MSCs cannot normalize the phenotype of OA-like chondrocytes. We then investigated the effect of mMSCs on OA-like femoral head explants (experimental strategy in Fig. 1D). GAG release in the supernatant was decreased and the expression of *Acan* and *Col2a1* was increased in OA-like explants co-cultured with mMSCs compared with explants alone, indicating a chondroinductive effect of mMSCs (Fig. 2A). As femoral head explants are made of several tissues (cartilage, bone and bone marrow), we evaluated the effect of mMSCs specifically on OA-like chondrocytes (experimental strategy in Fig. 1F). Co-culture with mMSCs did not significantly change the expression of anabolic, catabolic and inflammatory factors in OA-like chondrocytes. Conversely, addition of

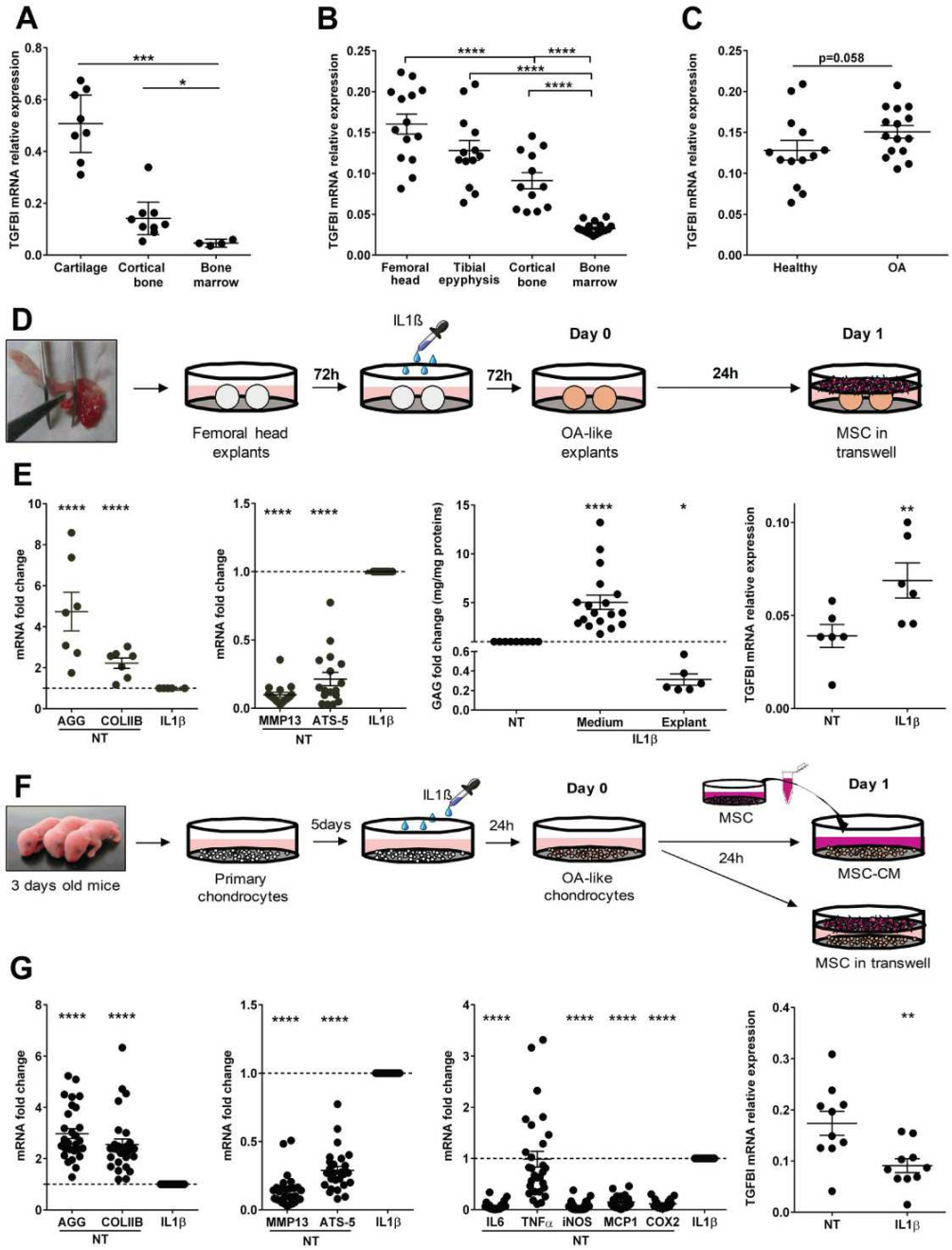

Fig. 1. Modulation of TGFBI expression in mouse tissues and in *in vitro* OA-like models. A) *Tgfbi* mRNA expression in the indicated tissues from 3-day-old healthy mice. B) *Tgfbi* mRNA expression in the indicated tissues from adult mice. C) *Tgfbi* mRNA expression in epiphyses from adult healthy mice and after OA induction. D) Scheme showing the OA-like model based on incubation of adult femoral head explants with IL1β. E) RT-qPCR analysis of different chondrocyte markers and TGFBI in control (NT, not treated with IL1β) and OA-like femoral head explants (IL1β). Glycosaminoglycan (GAG) quantification in femoral head explants, and GAG release in the culture medium from NT and IL1β-treated samples after 3 days. F) Scheme explaining the generation of OA-like chondrocytes by incubation with IL1β. G) RT-qPCR analysis of different chondrocyte and inflammatory markers and of TGFBI in control (NT, not treated with IL1β) and OA-like chondrocytes (IL1β). Each dot represents one biological replicate, and results are expressed as the mean ± SEM. Groups were compared with the Kruskal-Wallis test followed by the Dunn's multiple comparisons test in (A–B), the Mann-Whitney test in (C, E, G), or the Wilcoxon signed-rank test to compare NT and IL1β-treated samples (dotted line) (E, G). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, and ****: $p < 0.0001$.

mMSC-CM increased *Acan* expression, compared with OA-like chondrocytes alone, but without reaching the levels of chondrocytes not exposed to IL1β. The expression of type IIB collagen and of catabolic and inflammatory mediators was not modified by addition of mMSC-CM (Fig. 2B–C). Finally, we evaluated whether co-culture with hMSCs influenced the phenotype of murine OA-like chondrocytes and found no significant change, except for a decrease of iNOS expression (Fig. 2D).

TGFβ3-primed murine and human MSCs reverse the deregulation of cartilage markers in OA-like chondrocytes. As the TGFβ pathway has regulatory functions in cartilage homeostasis (REF [19]) and TGFBI downregulation in OA MSCs impairs their functional properties [21], we tested whether TGFBI upregulation in MSCs modified

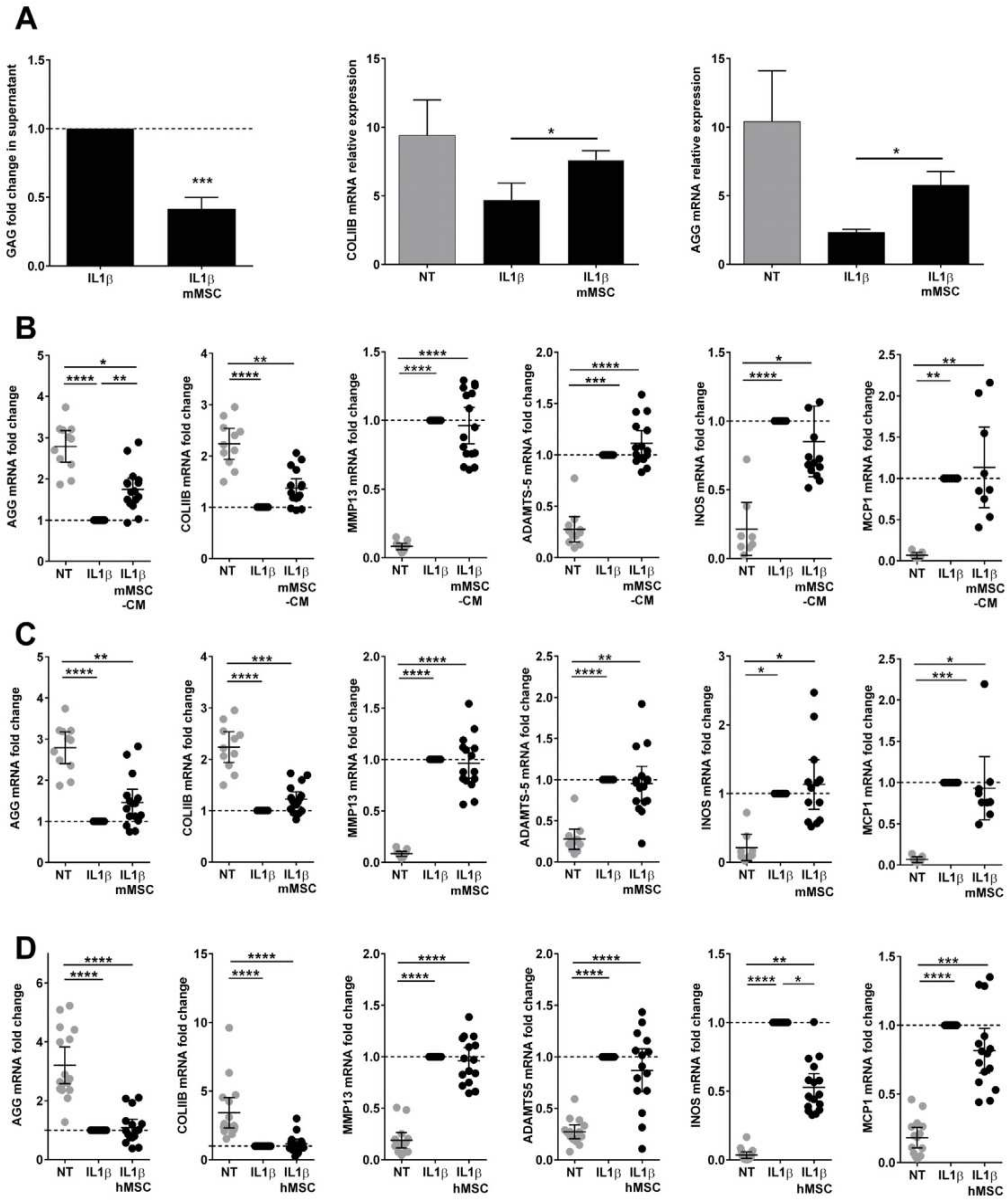

Fig. 2. Effect of mMSCs and hMSCs on chondrocyte gene expression in the *in vitro* OA-like models. A) Glycosaminoglycan (GAG) quantification in the supernatant of OA-like femoral head explants (IL1β) after 3 days of co-culture or not with mMSCs (left). RT-qPCR analysis of the chondrocyte markers *Col2a1* (middle) and *Acan* (right) in control (NT, not treated with IL1β) and OA-like femoral head explants (IL1β) after 3 days of co-culture or not with mMSCs (n = 5–10 biological replicates). B) RT-qPCR analysis of different chondrocyte and inflammatory markers in control (NT, not treated with IL1β) and OA-like chondrocytes (IL1β) incubated or not with mMSC conditioned medium (mMSC-CM). C) RT-qPCR analysis of different chondrocyte and inflammatory markers in control (NT) and OA-like chondrocytes (IL1β) co-cultured or not with mMSCs. D) RT-qPCR analysis of different chondrocyte and inflammatory markers in control (NT) and OA-like chondrocytes (IL1β) co-cultured or not with hMSCs. Each dot represents one biological replicate, and results are expressed as the mean ± SEM. Two groups were compared with the Mann-Whitney test (A), and samples were compared to OA-like chondrocytes (IL1β; set to 1) with the Wilcoxon signed-rank test (dotted line) (B–D). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, and ****: $p < 0.0001$.

their effect on OA-like chondrocytes. We first showed that priming with TGFβ3 upregulated *TGFBI* transcription in mMSCs and hMSCs by 2- and 3-fold, respectively (Fig. 3A–B) and increased TGFBI secretion by 3.5-fold in hMSCs (Fig. 3B, right panel). Moreover, incubation of OA-like mouse chondrocytes with TGFβ3-primed mMSC-CM significantly upregulated the expression of chondrocyte anabolic markers, but did not change the expression of catabolic and inflammatory factors (Fig. 3C). Co-culture of OA-like chondrocytes with TGFβ3-primed mMSCs led to upregulation of anabolic markers and downregulation of catabolic markers, but did not significantly modulate inflammation-associated markers (Fig. 3D). Conversely, co-culture with TGFβ3-primed hMSCs significantly reversed the deregulated expression of all tested markers, including inflammatory mediators (Fig. 3E). Altogether, these results indicated that mMSC pro-anabolic function is regulated by TGFβ3-priming. However, their anti-catabolic role requires both TGFβ3-priming and co-culture, indicating the importance of the crosstalk with OA-like chondrocytes.

TGFBI silencing in murine MSCs partly impairs their chondroinductive effect on OA-like chondrocytes. We then investigated the effect of TGFBI downregulation in mMSCs after transfection with a siTGFBI that reduced by 33% *Tgfbi* mRNA level compared with siCT (Fig. 4A). This decrease was sufficient to inhibit mMSC

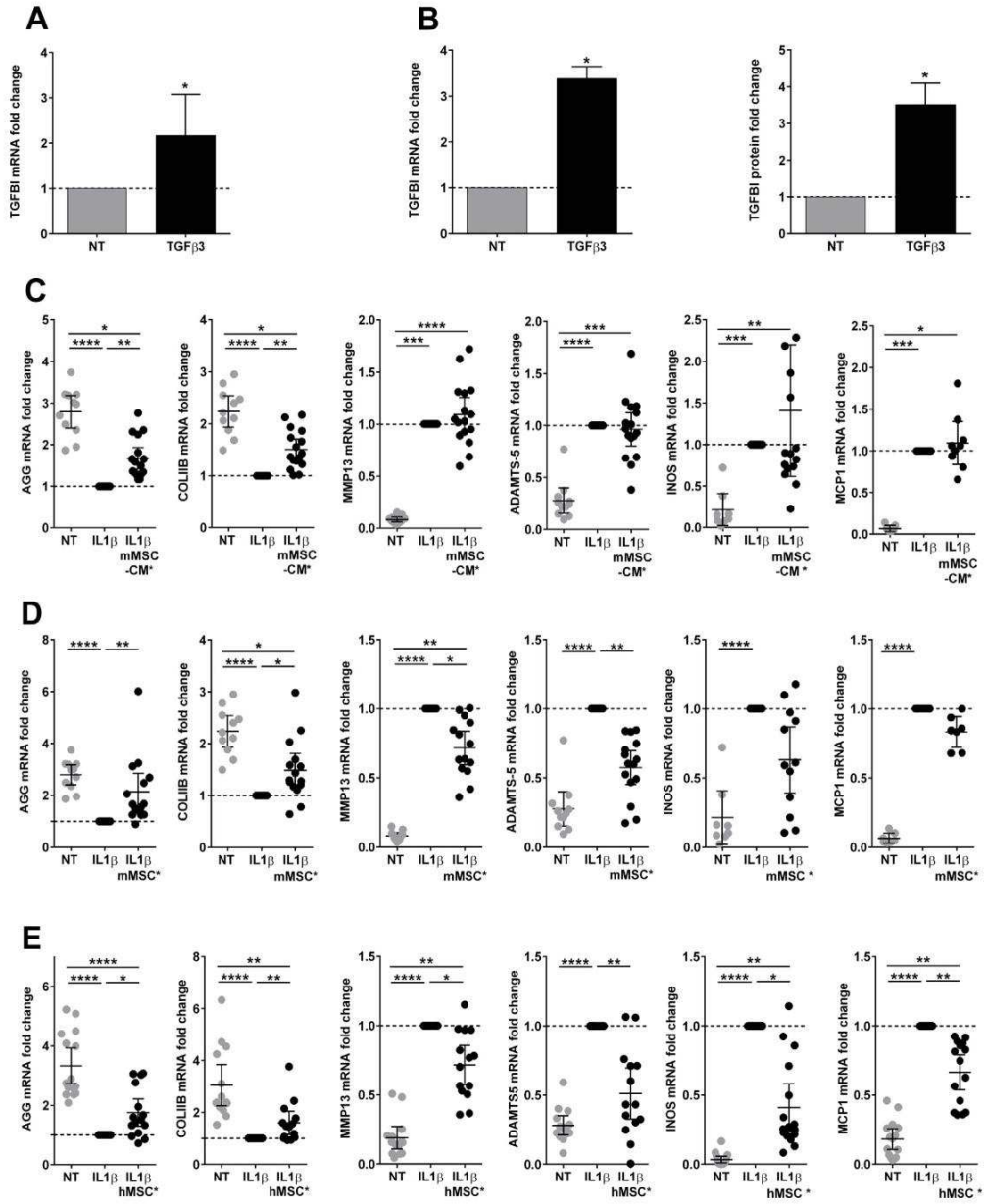

Fig. 3. Effect of TGFβ3-primed mMSCs and hMSCs on chondrocyte gene expression in *in vitro* OA-like models. A) RT-qPCR analysis of *Tgfbi* in mMSCs primed (TGFβ3) or not (NT) with TGFβ3 (n = 17/group). B) RT-qPCR analysis of *TGFBI* (left panel; n = 11 biological replicates) and quantification of TGFBI in the supernatant (right panel, n = 4 biological replicates) of control (NT) and TGFβ3-primed hMSCs. C-D) RT-qPCR analysis of chondrocyte and inflammatory markers in control (NT, not treated with IL1β) and OA-like chondrocytes (IL1β) incubated or not with conditioned medium from TGFβ3-primed mMSCs (mMSC-CM*) (C), or co-cultured or not with TGFβ3-primed mMSCs (mMSC*) (D). E) RT-qPCR analysis of chondrocyte and inflammatory markers in control (NT) and OA-like chondrocytes (IL1β) co-cultured or not with TGFβ3-primed hMSCs (hMSC*). Each dot represents one biological replicate, and results are expressed as the mean ± SEM. The Mann-Whitney test was used to compare two groups (A), and the Wilcoxon signed-rank test to compare samples to IL1β-treated chondrocytes (set to 1; dotted line) (C–E). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ or ****: $p < 0.0001$.

chondroinductive effect, as shown by the absence of *Acan* and *Col2a1* upregulation in OA-like chondrocytes co-cultured with siTGFBI-transfected mMSCs compared with siCT-mMSCs (Fig. 4A). Similarly, *Acan* upregulation was slightly but significantly lower in OA-like chondrocytes co-cultured with TGFβ3-primed siTGFBI-mMSCs than with TGFβ3-primed siCT-mMSCs (Fig. 4B). Finally, siTGFBI transfection in hMSCs reduced *TGFBI* expression by 48% (Fig. 4C). However, TGFBI downregulation did not modify the expression of *Acan* and *Col2a1* in OA-like chondrocytes co-cultured with TGFβ3-primed siTGFBI hMSCs.

TGFBI silencing impairs the therapeutic function of human MSCs in CIOA mice. Then, we evaluated *in vivo* the effect of IA injection of siCT- or siTGFBI-transfected hMSCs (TGFBI expression reduced by 70%) in CIOA mice [29]. Histological analysis showed that the OA score was lower, although not significantly, in CIOA mice treated with siCT-hMSCs compared with untreated mice (Fig. 5A–B). Conversely, the score was significantly higher in mice treated with siTGFBI-hMSCs compared with mice treated with siCT-hMSCs and untreated controls. Histomorphometric analysis of cartilage by CLSM confirmed that cartilage degradation was more important, and cartilage thickness was lower in CIOA mice treated with siTGFBI-hMSCs

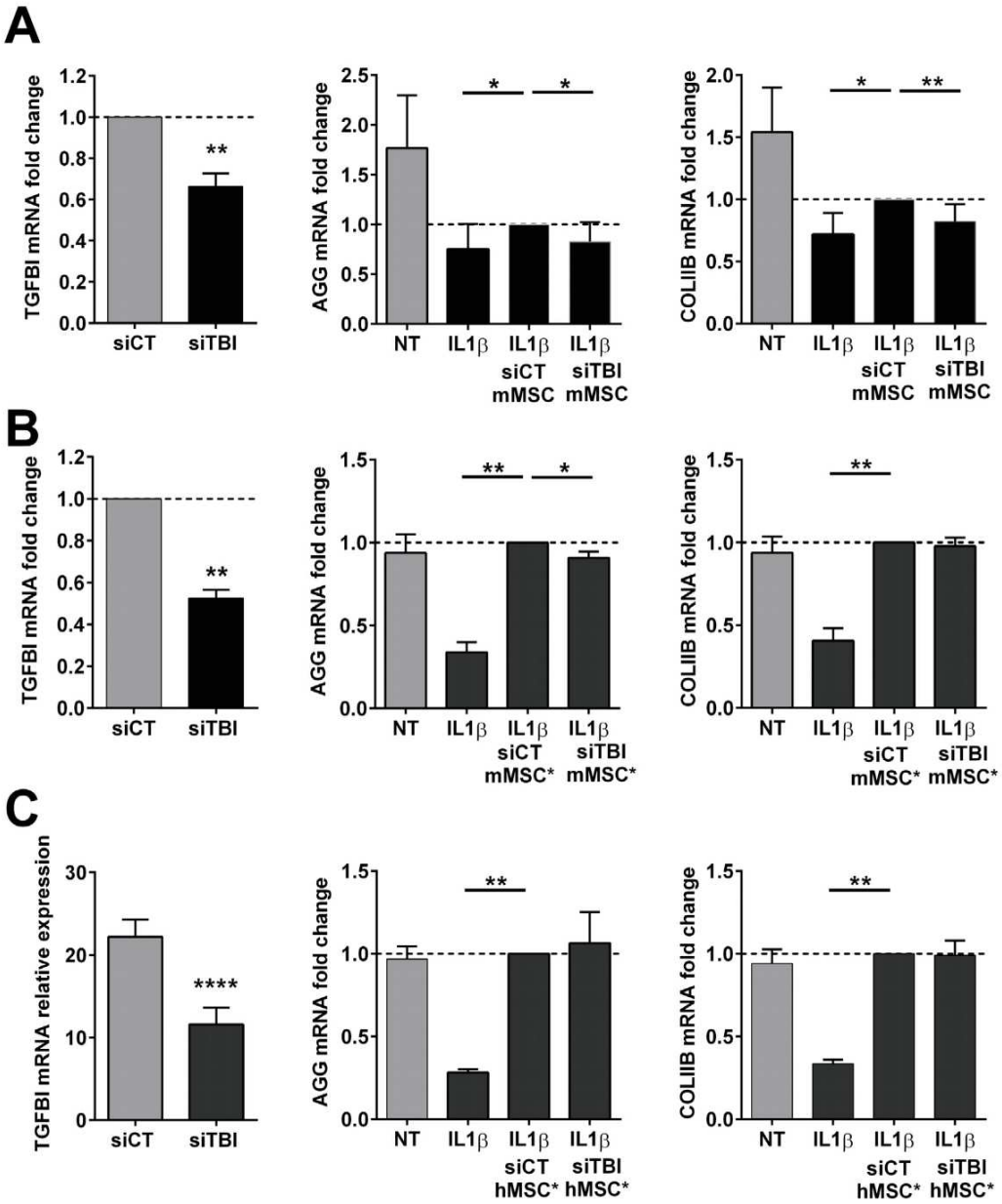

Fig. 4. Effect of TGFBI silencing in mMSCs and hMSCs on co-cultured OA-like chondrocytes. A) RT-qPCR analysis of *Tgfbi* in mMSCs transfected with control (siCT) or *anti*-TGFBI siRNAs (siTBI) and of chondrocyte markers in control (NT, not treated with IL1β) and OA-like chondrocytes (IL1β) co-cultured or not with siCT- or siTBI-transfected mMSCs (n = 8 biological replicates). B) RT-qPCR analysis of *Tgfbi* in siCT- and siTBI transfected mMSCs and of chondrocyte genes in control (NT) and OA-like chondrocytes (IL1β) co-cultured or not with siCT- or siTBI-transfected TGFβ3-primed mMSCs (mMSCs*; n = 12 biological replicates). C) RT-qPCR analysis of *TGFBI* in siCT- and siTBI-transfected hMSCs and of chondrocyte genes in control (NT) and OA-like chondrocytes (IL1β) co-cultured or not with siCT- or siTBI-transfected TGFβ3-primed hMSCs (hMSCs*; n = 8 biological replicates). Results are expressed as the mean ± SEM. The Wilcoxon signed-rank test was used to compare OA-like chondrocytes (IL1β) in different conditions to OA-like chondrocytes co-cultured with siCT-mMSCs or hMSCs (set to 1; dotted line). *: $p < 0.05$; **: $p < 0.01$.

compared with siCT-hMSCs (Fig. 5C–D). Accordingly, micro-CT analysis showed that sub-chondral bone parameters in CIOA mice treated with siCT-hMSCs were similar to those of healthy mice. Conversely, CIOA mice that received siTGFBI-hMSCs were not protected from bone degradation (Fig. 5E–F). Finally, injection of siCT-hMSCs, but not of siTGFBI-hMSCs partly inhibited calcification of the lateral and medial menisci and ligaments in CIOA mice (Fig. 5G–H). Overall, these findings indicated that TGFBI produced by hMSCs contributes to their therapeutic effect in CIOA mice.

TGFBI is conveyed within MSC-derived extracellular vesicles. To understand TGFBI mechanism of action, we assessed its effect on chondrocyte functions. Addition of rhTGFBI enhanced the proliferation of murine chondrocytes, while culture in rhTGFBI-coated dishes reduced chondrocyte adhesion (Fig. 6A–B). Moreover, splenocyte proliferation was slightly, but significantly reduced by siTGFBI-transfected hMSCs, suggesting decreased immunosuppressive properties upon TGFBI silencing (Fig. 6C). Then, we asked whether TGFBI could be conveyed by MSC-EVs that have been shown to have immunomodulatory effects in an arthritis model [23] and to transfer MSC-secreted factors between cells [4]. Using an already validated protocol

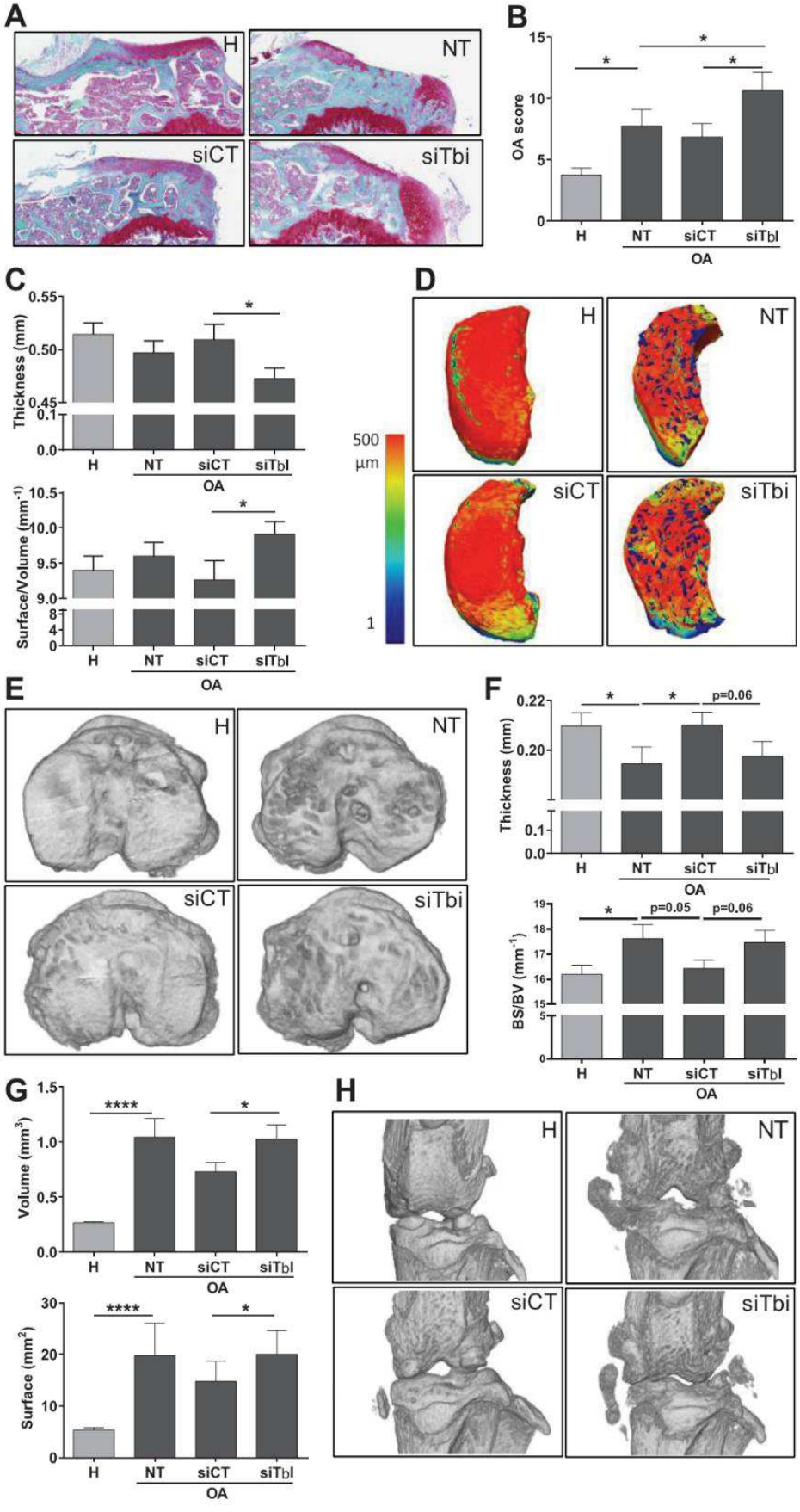

Fig. 5. Effect of siTGFBI-hMSCs in the collagenase-induced osteoarthritic (CIOA) murine model. A) Histological images of healthy (H) mice and CIOA mice not treated (NT) or treated with hMSCs transfected with control (siCT) or *anti*-TGFBI (siTBI) mRNAs. B) OA score of histological sections of knee joints of the mice described in A. C) Histomorphometric analysis of 3D images of cartilage by CLSM. D) Representative 3D reconstructed images of medial tibial cartilage after CLSM analysis; on the left, colour code for cartilage thickness. E) Representative 3D reconstructed images of the sub-chondral bone surface in tibias after micro-CT analysis. F) Histomorphometric analysis of 3D images of sub-chondral bone: thickness and bone surface/bone volume (BS/BV) parameters (n = 15/group). G) Histomorphometric analysis (volume and surface) of mineralized tissues in joints. H) Representative 3D reconstructed images of mouse knee joints after micro-CT analysis showing mineralized menisci and external ligaments. Results are expressed as the mean ± SEM; *: $p < 0.05$; ****: $p < 0.0001$ (Mann-Whitney test; n = 23 mice/group from 2 independent experiments).

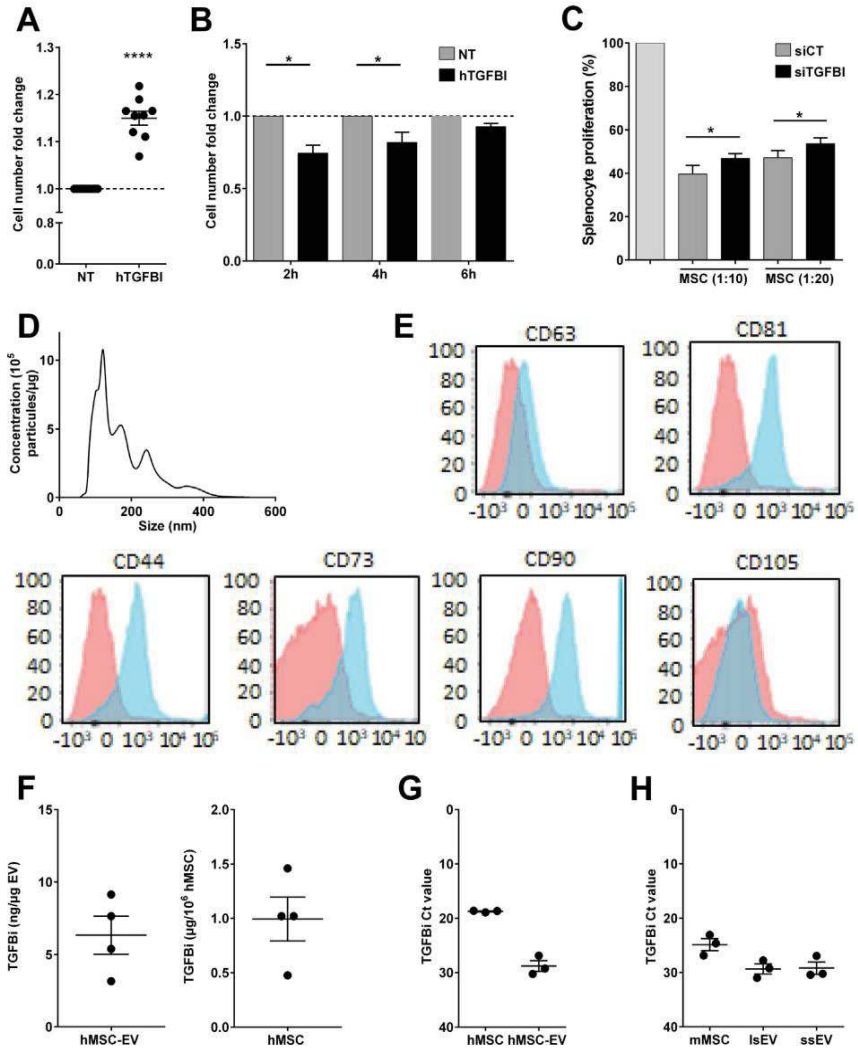

Fig. 6. Functional effect of TGFBI. A) Proliferation of chondrocytes on plates coated with recombinant hTGFBI, expressed as fold change compared with cells cultured on plates coated with PBS (NT). B) Adhesion of chondrocytes on plates coated with PBS (NT) or recombinant hTGFBI, expressed as the cell number fold-change relative to NT at the indicated time points after seeding normalized to PBS-coated plates (n = 9 biological replicates). C) Proliferation of concanavalin A-activated mouse splenocytes co-cultured or not with hMSCs transfected with control (siCT) or *anti*-TGFBI siRNAs at two ratios (1:10 or 1:20, hMSCs:splenocytes) (n = 7 biological replicates). D) Number and median size of extracellular vesicles from hMSCs (hMSC-EVs) by nano-particle tracking analysis. E) Representative histograms of hMSC-EV surface markers by flow cytometry. F) TGFBI protein expression in hMSC-EVs normalized to the total protein amount and expression of TGFBI protein in hMSCs. G) Expression of *TGFBI* mRNA in hMSCs and hMSC-EVs as expressed as Ct value. H) Expression of*Tgfbi* mRNA in mMSCs, mMSC-ssEVs (small-size EVs) and mMSC-lsEVs (large-size EVs), expressed as Ct value. Each dot represents a biological replicate and results are expressed as the mean ± SEM. *: $p < 0.05$; ****: $p < 0.0001$ (Wilcoxon signed-rank test in (A) and Mann-Whitney test in (B–C).

for mMSC-EVs, we isolated and characterized total EVs from hMSCs [23]. The quantity of hMSC-EVs was 8×10^8 particles/µg total proteins/10^6 hMSCs, and their size ranged from 80 to 400 nm (Fig. 6D). They expressed surface markers of hMSCs (CD44, CD73, and CD90) and of exosomes (CD63 and CD81) (Fig. 6E). We detected easily quantifiable amounts of TGFBI protein in hMSC-EVs, although the amounts were lower than in hMSCs (Fig. 6F). We also detected *TGFBI* mRNA in hMSC-EVs and in small-size and large-size mMSC-EVs (Fig. 6G–H).

4. Discussion

This is the first evidence that TGFBI produced by hMSCs exerts a pro-anabolic function on chondrocytes and a therapeutic role in OA by preventing cartilage and bone degradation, while inhibiting soft tissue calcification.

Using two *in vitro* models that mimic the cartilage degradation and chondrocyte deregulation observed in OA, we revealed TGFBI expression deregulation in OA-like cartilage. Specifically, TGFBI was upregulated in OA-like femoral head explants, while it was downregulated in OA-like chondrocytes. A possible explanation for this discrepancy might be related to the developmental stage of the analysed tissues. In femoral head explants from 2-3- week-old mice, TGFBI expression is typical of the adult age, and increases in OA-like conditions as observed in epiphyses of CIOA-induced adult mice. This upregulation might be related to a regulatory loop to inhibit cartilage mineralization that occurs during OA. Indeed, it was previously shown that TGFBI inhibits osteogenesis and mineralization of cultured chondrocytes [30–32]. On the other hand, in immature chondrocytes isolated from femoral and tibial epiphyses of 3-day-old mice, TGFBI was downregulated in OA-like conditions. In mouse embryos (E16.5 to E18.5), TGFBI is expressed in proliferating chondrocytes and in primary endochondral ossification centres during joint cartilage formation, where it may interact with cells and extracellular matrix molecules, thus playing a role in tissue morphogenesis [33–35]. In 3-day-old mice, cartilage is predominantly pre-hypertrophic and hypertrophic, consistent with high TGFBI expression, as observed during embryogenesis. We previously reported that TGFBI is required at the early stages of chondrogenic differentiation of MSCs, and is then downregulated in mineralized hypertrophic chondrocytes [21]. The present study brings evidence that besides promoting endochondral ossification and inhibiting mineralization during *in vitro* differentiation of mature osteoblasts or chondrocytes, TGFBI deregulation in joint tissues might contribute to OA.

TGFBI is a paralogue of periostin (*POSTN*), the only other member of the TGFBI family. The two genes have a similar domain structure, although *TGFBI* is shorter and lacks the C-terminal domain that is subjected to alternative splicing in *POSTN* [36,37]. Both genes have important overlapping functions in cell adhesion, migration, proliferation, and apoptosis. In cancer, they display dual roles, acting as tumour suppressors or promoters, depending on the tumour environment. POSTN levels in serum and synovial fluid are associated with OA

incidence and progression [38,39], and its expression is higher in mouse and human bone and cartilage [40,41]. Importantly, the higher chondroprotective effect in OA mice of FRA-1-overexpressing adipose-derived stromal cells compared with wild type cells has been associated with increased POSTN expression [42]. We recently reported TGFBI upregulation in bone and cartilage from patients and mice with OA [21]. Based on the similarity of functions and expression in OA, all these data suggest a possible common regulation of POSTN and TGFBI in OA and identify these two molecules as important players in joint homeostasis. In agreement, the present study demonstrated the lower *in vivo* therapeutic efficiency of siTGFBI-hMSCs that mimic the lower TGFBI expression of hMSCs from patients with OA. Interestingly, siTGFBI-hMSCs could not reduce osteophyte calcification in CIOA mice (data not shown), further supporting a probable inhibitory role of TGFBI on mineralization *in vivo*. TGFβ pathway deregulation in MSCs was previously associated with OA onset and/or maintenance [20]. Here, we present further evidence that TGFBI deregulation in MSCs impairs their therapeutic chondroprotective function and might affect their physiologic role in cartilage and bone homeostasis.

We provided evidence that EVs released by mMSCs and hMSCs contain both TGFBI mRNA and protein, underlining a plausible common mechanism of action. Usually, EVs act on target cells after their internalization mediated by fusion of their membrane with the cell plasma membrane or after uptake. After uptake, EVs are addressed to the canonical endosomal pathway and can be targeted to lysosomes and degraded, or can discharge their cargo in the cytosol by fusion with the endosomal membrane [43]. A possible transfer of EVs to the plasma membrane for release might also occur. In our conditions, we could not detect the presence of human *TGFBI* mRNA in murine chondrocytes after 1 day of co-culture with hMSCs (data not shown), possibly because after uptake, the amount of *TGFBI* mRNA released in chondrocytes might have been too low to be detected, or rapidly degraded or translated into proteins. Further investigations are needed to determine the fate of EV cargoes in chondrocytes or synovial cells in the joint. Nevertheless, we previously demonstrated that IA injection of small-size and large size mMSC-EVs can protect mice from developing CIOA to a similar extent as mMSCs, suggesting that TGFBI mRNA and protein contained in EVs might play an important role [27]. The relative contribution of soluble TGFBI and EV-contained TGFBI needs to be investigated.

One of the main roles of TGFBI is to mediate cell adhesion and migration by acting as a linker that connects various matrix molecules and favours cell-collagen interactions. This has been shown mainly in tumour cells where TGFBI can act either as tumour promoter by increasing cancer cell invasiveness, or as a tumour suppressor by inhibiting cell adhesion leading to inhibition of cell proliferation and migration [44]. TGFBI also increases cell survival and proliferation during gastrointestinal tract tumorigenesis via activation of the FAK/AKT signalling pathway [45]. Moreover, TGFBI expression is a predictor of survival in patients with lung squamous cell carcinoma [46]. However, its physiological role is still unclear. Here, we demonstrated that TGFBI decreases chondrocyte adhesion and increases their proliferation. Therefore, TGFBI secreted by MSCs might mediate its therapeutic effect in joint tissues via a pro-survival and pro-anabolic role on chondrocytes.

TGFBI could also have an anti-inflammatory effect through its negative regulation of Toll like receptor-induced inflammation. For instance, TGFBI expression was increased in peripheral blood mononuclear cells in a model of lipopolysaccharide-induced endotoxin tolerance, resulting in lower activation of inflammatory actors, such as nuclear factor-κB, TNF-α and nitric oxide [47]. Moreover, a significant correlation has been observed between three single nucleotide polymorphisms in the *TGFBI* gene and type 1 diabetes, and TGFBI expression is lower in pancreatic islets from diabetic subjects. Interestingly, TGFBI can inhibit T-cell activation markers (CD44 and CD69) and the production of cytotoxic molecules, such as granzyme B and interferon-γ [48]. In addition, TGFBI-treated diabetogenic T cells cannot induce type 1 diabetes upon transfer in wild type mice, suggesting that TGFBI expression in pancreatic islets might contribute as a protective shield against cytotoxic T-cell attack. Our data indicated that TGFBI plays a role in MSC anti-inflammatory function and contributes to T lymphocyte proliferation inhibition. Therefore, the hypothesis that TGFBI released by hMSCs might exert an anti-inflammatory function in OA should be investigated *in vivo*.

Altogether, the present study highlights TGFBI role in hMSC chondroprotective effect in OA by inhibiting cartilage and bone degradation, while limiting calcification and osteophyte formation. Besides deciphering the mechanisms underlying hMSC therapeutic effect, it suggests the possibility of targeting TGFBI for therapeutic intervention.

Data availability

All experimental data required to reproduce the findings from this study will be made available to interested investigators.

Author contributions

DN, CJ designed the experiments. Experimental work was performed by MR, MM, KT, PR, DN. MR, MM, KT, PR, CJ, DN analysed the data and prepared the manuscript. All authors have contributed to writing or revising the manuscript and final approval.

Declaration of competing interest

The authors disclose any financial or personal conflict of interest.

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2006	Baccalauréat Série S avec Mention Très Bien

EXPERIENCES PROFESSIONNELLES :

2017 à 2021	**Master 2 puis Thèse Biologie Santé** (IRMB Montpellier) Doctorante Inserm U1183, groupe D Noël, équipe PrJorgensen Co-encadrement d'un étudiant en master 2
2013- 2016	**Internat de Médecine Interne** (Montpellier)
Nov 2015 - Mai 2016	Maladies Infectieuses et Tropicales (Pr Reynes)
Mai 2015 - Nov 2015	Immuno-Rhumatologie (Pr Jorgensen)
Nov 2014 - Mai 2015	Médecine Interne et Maladies Multiorganiques (Pr Le Quellec)
Mai 2014 - Nov 2014	Médecine Interne, Gériatrie et Néphrologie (Dr Oziol, CHR Béziers)
Nov 2013 - Mai 2014	Médecine Interne 2 (Dr Hallé, CHR Alès)
2008 – 2013	**Externat de Médecine** (Montpellier)
Juillet 2010	**Stage de Médecine interne** (Dr Nallam, Pondichéry – Inde)
Juillet 2009	**Stage d'initiation à la recherche, Master 1 Biologie Santé :** Biologie cellulaire et hormonale (Pr Maudelonde, Montpellier)

ENSEIGNEMENTS :

2015 - 2016	Conférences de préparation à l'iECN et Universitaires E. Delous
2014 - 2015	Conférences Universitaires E. Delous

PUBLICATIONS SCIENTIFIQUES :

Articles Originaux

Rozier P, Maumus M, Maria ATJ, Toupet K, Lai-Kee-Him J, Jorgensen C, Guilpain P, Noel D. Mesenchymal stromal cells-derived extracellular vesicles alleviate murine systemic sclerosis via miR-29a-3p and regulation of DNMT3A and PDGFRBB. Article en soumission

Rozier P, Maumus M, Maria ATJ, Toupet K, Jorgensen C, Guilpain P, Noel D. Extracellular vesicles from IFNγ-primed mesenchymal stromal cells improve lung fibrosis in murine systemic sclerosis. Article en préparation

Ruiz M, Toupet K, Maumus M, **Rozier P**, Jorgensen C, Noël D. TGFBI secreted by mesenchymal stromal cells ameliorates osteoarthritis and is detected in extracellular vesicles. Biomaterials. 2020, doi:10.1016/j.biomaterials.2019.119544. (IF:10.317)

Maria ATJ, Bourgier C, Martinaud C, Borie R, **Rozier P**, Rivière S, Crestani B, Guilpain P. [From fibrogenesis towards fibrosis: Pathophysiological mechanisms and clinical presentations] Rev Med Int. 2020, doi: 10.1016/j.revmed.2020.01.002 (IF :0.810).

Maria ATJ, **Rozier P**, Fonteneau G, Sutra T, Maumus M, Toupet K, Cristol JP, Jorgensen C, Guilpain P, Noël D. iNOS Activity Is Required for the Therapeutic Effect of Mesenchymal Stem Cells in Experimental Systemic Sclerosis. Front immunol. 2018, doi: 10.3389/fimmu.2018.03056 (IF:6,429)

Maria ATJ, Toupet K, Maumus M, **Rozier P**, Vozenin MC, Le Quellec A, Jorgensen C, Noël D, Guilpain P. Fibrosis Development in HOCl-Induced Systemic Sclerosis: A Multistage Process Hampered by Mesenchymal Stem Cells. Front Immunol. 2018, doi: 10.3389/fimmu.2018.02571 (IF:6,429)

Revues

Maumus M, **Rozier P**, Boulestreau J, Jorgensen C, Noël D. Mesenchymal stem cells derived extracellular vesicles: opportunities and challenges for clinical translation. Front in Bioengineering and Biotechnology. 2020, doi.org:10.3389/fbioe.2020.00997 (IF:3.644)

Boulestreau J, Maumus M, **Rozier P**, Jorgensen C, Noël D. Mesenchymal stem cells derived extracellular vesicles in aging. Front Biocell Dev. 2020, doi:10.3389/fcell.2020.00107. (IF:5.180)

Rozier P, Maria ATJ, Goulabchand R, Jorgensen C, Guilpain P, Noël D. Mesenchymal Stem Cells in Systemic Sclerosis: Allogenic or Autologous Approaches for Therapeutic Use? Front Immunol. 2018, doi: 0.3389/fimmu.2018.02938 (IF:6,429)

Maria ATJ, Partouche L, Goulabchand R, Rivière S, **Rozier P**, Bourgier C, Le Quellec A, Morel J, Noël D, Guilpain .P. Intriguing Relationships Between Cancer and Systemic Sclerosis: Role of the Immune System and Other Contributors. Front Immunol. 2019, doi: 10.3389/fimmu.2018.03112. (IF:6,429)

Rozier P, Fraisse T, Lauda M, Priner M, Forestier E, Paccalin M. Infections à Clostridium difficile en gériatrie. Cah Année Gérontol. (2014);6:107-113.

COMMUNICATIONS :

Communications orales :

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. MiR-29a plays a crucial role in the therapeutic effect of ASC-derived extracellular vesicles in systemic sclerosis. IFATS à Marseille (2019)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. Therapeutic effect of MSC-derived extracellular vesicles in systemic sclerosis and role of miR-29a. FSEV à Nantes (2019)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. Role of mir-29a in the therapeutic effect of extracellular vesicles isolated from MSC in systemic sclerosis. Retraite IRMB (2019)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. Role of mir-29a in the therapeutic effect of extracellular vesicles isolated from MSC in systemic sclerosis. 2ème journée FHU-Regenhab (2019)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. Effet thérapeutique des vésicules extracellulaires isolées de cellules souches mésenchymateuses dans la fibrose sclérodermique : implication potentielle de miR-29a. SNFMI à Montpellier (2019)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. Mesenchymal stem cell-derived extracellular vesicles:a novel therapeutic option in systemic sclerosis. (O021) EWRR à Genève (2018)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Guilpain P, Noel D. Extracellular vesicles released by mesenchymal stem cells represent a novel therapeutic option in systemic sclerosis. (OT03.01) ISEV à Barcelone (2018)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. MSC-derived extracellular vesicles: a novel therapeutic option in systemic sclerosis. Autoimmunity congress à Lisbonne (2018)

Rozier P, Maria ATJ, Rigau V, Le Quellec A, Guilpain P. Sarcoidosis and Sjögren's syndrome, a controversial association: about 7 cases and a systematic review of literature. Autoimmunity congress à Lisbonne (2018)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. Rôle des vésicules extra cellulaires isolées de cellules souches mésenchymateuses dans la sclérodermie systémique : rôle physiopathologique ou effet thérapeutique ? Conseil scientifique, SNMI à Grenoble (2018)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. Effets thérapeutiques des vésicules extracellulaires isolées de cellules souches mésenchymateuses dans la sclérodermie systémique. SNFMI à Paris (2017)

Communications affichées et interventions orales :

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. Therapeutic effect of MSC-derived extracellular vesicles in systemic sclerosis. 5th systemic sclerosis world congress à Bordeaux (2018)

Rozier P, Maumus M, Maria ATJ, Jorgensen C, Noel D, Guilpain P. Effet thérapeutique des vésicules extracellulaires de cellules souches mésenchymateuses dans la sclérodermie systémique. Congrès SFGM-TC à Montpellier (2018)

Rozier P, Schambach S, Veyssiere F, Sirvain S, Arnaud A, Tourrand B, Marty-Gres S, Fraisse T. Amylose hépatique AL révélée par un ictère chez un patient porteur d'une myopathie à fibres lobulées. 43ème Congrès National des biologistes des Hôpitaux à Marseille (2014) : a obtenu le Prix Poster pour la Biochimie

Rozier P, Ismail K, Gilbert O, Oziol E, Borlot F, Simorre B, Le Fahler G, Gomard-Mennesson E. Syndrome de Mac Duffie avec glomérulonéphrite extra-membraneuse : intérêt du Méthotrexate. Congrès SNFMI à Paris (2014)

Rozier P, Marty-Gres S. Animation d'un cas clinico-biologique « myopathie et ictère : quelles chaînes ? » pour la validation DPC de biologistes de la région LR. Journée annuelle de Biologie (2014, Nîmes)

Rozier P, Fraisse T. Présentation d'un cas clinique « un ictère révélateur » à la Journée régionale de Médecine Interne (2014, Nîmes)

Participations au congrès en dehors des communications :

Biomarker days - Edition spéciale COVID-19. Montpellier (2020)
1er Congrès français de médecine régénératrice et biothérapies (2020)

FINANCEMENTS-BOURSES-PRIX

2018 : Obtention d'une bourse ASF-Fai2R pour le 5th systemic sclerosis world congress
2017 : Obtention d'une bourse de la SNFMI.
2017 : Obtention d'un financement pour le projet de thèse allouée par l'ASF.
2017 : Obtention de 2 financements de bourse de thèse
- Bourse de thèse de 3 ans « MRT » (classée 2ème école doctorale CBS2) : acceptée
- Bourse de thèse de 2 ans « Poste Accueil Inserm » (classée 2ème) : refusée

2016 : Obtention de 2 financements de master 2
- Année recherche (ARS LR, CHRU Montpellier), classée major : acceptée
- Bourse Fondation pour la Recherche Médicale : refusée

2014 : Prix Poster pour la Biochimie au Congrès National de Biochimie.

COMPETENCES SCIENTIFIQUES

Biologie moléculaire – extraction d'ARN et microARNs, RT et qPCR gène et microARN
Biologie cellulaire – culture primaire en confinement P2 : isolement, amplification et caractérisation (ASC, cellules endothéliales, fibroblastes) ; transfection de cellules et manipulation d'OGM.
Biologie des EVs – ultracentrifugation, nanotracking analysis,
Western Blot, ELISA, immunofluorescence, cytométrie en flux,
Expérimentation animale – modèle ScS induit par l'HOCl, suivi clinique, injections intradermiques, euthanasie et prélèvements.
Microscopie optique, nanozoomer
Logiciels – microsoft office, graph pad prism, image J, Inkscape

DIVERS

Membre junior de la SNMI depuis 2013
Yoga
Musique

Effet thérapeutique des vésicules extracellulaires isolées à partir de cellules stromales mésenchymateuses dans la sclérodermie systémique

La sclérodermie systémique (ScS) est une maladie rare et sévère, caractérisée par une dérégulation des cellules immunitaires, une fibrose et une vasculopathie généralisées. Aucun traitement curatif n'est validé à ce jour. Les cellules stromales mésenchymateuses (CSMs), dont l'efficacité a été démontrée dans plusieurs modèles précliniques de ScS, sont actuellement évaluées dans des essais cliniques. Elles exercent leurs propriétés régénératrices au travers de la sécrétion de médiateurs libérés dans le milieu extracellulaire ou contenus dans des vésicules extracellulaires (EVs). Nous nous intéressons ici au rôle des EVs libérées par les CSMs dans leur effet systémique au cours de la ScS.

Dans un modèle murin de ScS induit par des injections d'acide hypochloreux, nous démontrons que les EVs stoppent l'évolution de la maladie au niveau cutané et pulmonaire sur le plan clinique, histologique et moléculaire. Nous identifions miR-29a-3p au sein des CSMs et de leurs EVs et prouvons qu'il est garant de leur effet thérapeutique, en ciblant *Dnmt3a* et *Pdgfr-bb,* deux nouvelles cibles identifiées dans la ScS.

Nous améliorons l'efficacité thérapeutique des EVs sur les anomalies pulmonaires en pré-activant les CSMs à l'aide d'IFNγ alors que l'augmentation de la quantité d'EVs injectées n'a aucun effet. L'IFNγ module l'expression de molécules immunosuppressives dans les CSMs et leurs EVs, potentialisant ainsi leurs propriétés thérapeutiques.

Alors que le rôle des CSMs de patients (CSMs-ScS) dans le développement de la maladie est sujet à débat, nous montrons que les CSMs-ScS inhibent *in vitro* la prolifération des lymphocytes T de manière similaire à des CSMs saines. En outre, elles sont capables d'améliorer le phénotype myofibroblastique de fibroblastes stimulés par du TGFβ1 qui présentent un comportement similaire à celui de fibroblastes de patients dans le modèle *in vitro* mis en place.

En conclusion, les EVs sont aussi efficaces que les CSMs pour traiter la ScS murine et le pré-conditionnement des CSMs par l'IFNγ améliore leur effet au niveau pulmonaire. Les CSMs de patients conservent leurs fonctions anti-fibrotique et immunosuppressive *in vitro* et une de nos perspectives est d'évaluer leur efficacité thérapeutique *in vivo*.

Mots clés : sclérodermie systémique, cellules stromales mésenchymateuses, vésicules extracellulaires, miR-29a-3p, TGFβ1, fibrose

Therapeutic effect of extracellular vesicles derived from mesenchymal stromal cells in systemic sclerosis

Systemic sclerosis (SSc) is a rare and severe disease characterized by generalized fibrosis, vasculopathy and deregulation of immune cells. No curative treatment is validated to date. Mesenchymal stromal cells (MSCs) are being evaluated in clinical trials as they are effective in several preclinical models of SSc. They exert their anti-fibrotic, pro-angiogenic and immunomodulatory properties through the secretion of mediators contained in extracellular vesicles (EVs). We investigate here the role of EVs released by MSCs in their systemic effect during SSc.

In a murine model of hypochlorous acid-induced SSc, we demonstrate that EVs can stop the disease course and improve histological and molecular parameters in skin and lung samples. We identify miR-29a-3p expression in both MSCs and their EVs and prove that miR-29a-3p is responsible for a large part of their therapeutic effect, targeting *Dnmt3a* and *Pdgfr-bb,* two novel targets identified in SSc.

We improve the therapeutic efficacy of EVs on lung abnormalities in SSc mice by stimulating MSCs with IFNγ before EVs production whereas increasing the amount of injected EVs did not have any benefit. IFNγ modulates the expression of known immunosuppressive factors in both MSCs and EVs, potentiating their therapeutic properties.

While the role of MSCs from SSc patients (SSc MSCs) in the development of the disease is debated, we show that SSc MSCs inhibit *in vitro* T lymphocyte proliferation and improve the myofibroblastic phenotype obtained after fibroblast stimulation using TGFβ1 which display a similar profile as fibroblasts from SSc patients in the *in vitro* model used here.

Overall, MSC-EVs are as effective as MSCs to stop the progression of murine SSc and EVs from IFNγ-preconditioned MSCs improve their beneficial effect in the lungs. SSc MSCs maintain their anti-fibrotic and immunosuppressive properties *in vitro* and one of our perspectives is to evaluate their therapeutic efficacy *in vivo*.

Key words: systemic sclerosis, mesenchymal stromal cells, extracellular vesicles, miR-29a-3p, TGFβ1, fibrosis