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Rôle de LRP-1 dans la prolifération des cellules issues de cancer du côlon en matrice tridimensionnelle de collagène de type I

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Role of LRP-1 in colon cancer cell proliferation in three-dimensional culture systems

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Rôle de LRP-1 dans la prolifération des cellules issues de cancer du côlon en matrice tridimensionnelle de collagène de type I

Le récepteur *low-density lipoprotein receptor-related protein-1* (LRP-1) est un récepteur d'endocytose multifonctionnel impliqué dans de nombreux processus physiologiques et pathologiques. Plusieurs études ont montré que LRP-1 joue un rôle crucial lors des processus de tumorigenèse et durant la progression tumorale, notamment en régulant l'expression de protéines membranaires. Des études antérieures ont montré l'implication des récepteurs du collagène de type I de la famille des *Discoidin Domain Receptors* (DDRs) dans la régulation de la prolifération des cellules cancéreuses en 3D. Le but de ce travail est d'étudier s'il existe une interaction fonctionnelle entre LRP-1 et DDR1 et si celle-ci pourrait moduler la prolifération des cellules de cancer colorectal (CRC) cultivées dans une matrice 3D de collagène de type I.

Nos résultats ont permis de montrer qu'une invalidation de LRP-1 ou une inhibition de son activité par l'utilisation d'antagonistes sélectifs (RAP, anticorps bloquants) altère la prolifération des cellules de CRC LS174T et HT-29, uniquement lorsque qu'elles sont intégrées dans une matrice 3D de collagène de type I. De plus, la surexpression de DDR1-GFP dans les cellules HT-29 (HT-29$^{DDR\text{-}GFP}$) diminue leur taux de croissance, tandis que l'inhibition de LRP-1 par RAP induit un arrêt du cycle cellulaire et une augmentation de l'apoptose dans les cellules HT-29 et HT-29$^{DDR\text{-}GFP}$. Nous avons montré que la quantité de DDR1 à la surface cellulaire était augmentée et que l'endocytose de DDR1 était réduite de moitié lors du traitement par RAP, mettant ainsi en évidence une nouvelle voie d'internalisation pour DDR1. De plus, LRP-1 et DDR1 co-immunoprécipitent ensemble indiquant que ces récepteurs sont fortement associés au sein d'un même complexe moléculaire dans les cellules de CRC.

Nos résultats mettent en évidence l'existence d'une interface fonctionnelle entre LRP-1 et DDR1 I soutenant la prolifération des cellules de CRC dans une matrice 3D de collagène.

LRP-1, DDR1, cancer colorectal, prolifération, matrice 3D de collagène de type I

Role of LRP-1 in colon cancer cell proliferation in three-dimensional culture systems

Low-density lipoprotein receptor related protein-1 (LRP-1) is a multifunctional endocytotic receptor mediating the clearance of various molecules from the extracellular matrix, including metalloproteases and various glycoproteins. Several studies have shown that LRP-1 plays crucial roles in tumorigenesis and during tumor progression. LRP-1 also functions as a main regulator of signaling pathway by interacting with other cell-surface receptors. Previous studies have highlighted the involvement of Discoidin Domain Receptors (DDRs), type I collagen receptors with tyrosine kinase activity, in the regulation of cancer cell proliferation in 3D experimental models. The aim of this work is to study the potential functional interplay between LRP-1 and DDR1 in order to investigate whether this interaction may modulate the proliferation of colorectal cancer (CRC) cells in highly relevant 3D type I collagen matrices.

In this study, we demonstrated that inhibition of LRP-1-mediated endocytosis using RNA interference or selective antagonists (RAP and R2629 blocking antibodies) impaired LS174T and HT-29 carcinoma cell proliferation, but only when embedded in a 3D collagen matrix. Using 3D cultures, DDR1-GFP overexpressing HT-29 (HT-29$^{DDR\text{-}GFP}$) reduced the colorectal carcinoma cell growth rate, whereas RAP treatment led to cell cycle arrest and induced apoptosis in both HT-29 and HT-29$^{DDR\text{-}GFP}$. By streptavidin/biotin-based immunoassays, we demonstrated that membrane-anchored DDR1 amount was increased upon RAP treatment while DDR1 uptake was reduced by a half upon LRP-1 inhibition, highlighting a new way for DDR1 internalization and dynamics. Consistently, co-immunoprecipitations confirmed the existence of a LRP1:DDR1 biomolecular complex at the cell surface of CRC cells.

Our results suggest a role for LRP-1 in promoting CRC cell proliferation in 3D collagen environment by mediating DDR1 endocytosis.

LRP-1, DDR1, colon cancer cell, proliferation, 3D collagen matrix

Discipline : SCIENCES DE LA VIE ET DE LA SANTE

Spécialité : Aspects moléculaires et cellulaires de la biologie

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List of Abbreviations

AAT	Alpha 1-antitrypsin
AD	Alzheimer disease
ADAM	A Disintegrin and Metalloproteinase
AMI	Acute Myocardial Infarction therapy
ApoE	Apolipoprotein E
APP	Amyloid Protein Precursor
Aβ	Amyloid beta
BBB	Blood-brain Barrier
BFT	*Bacteroides fragilis*
BM	Basement Membrane
CAF	Cancer-Associated Fibroblast
CAM	Cell Adhesion Molecule
CCL3	Chemokine (C-C motif) ligand 3
CDK2	Cyclin-dependent Kinase 2
cDNA	Complementary DNA
CRC	Colorectal Cancer
Dab1	Disabled-1
DC	Dendritic Cell
DDR	Discoidin Domain Receptor
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-mesenchymal Transition
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-regulated Protein Kinase
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3-β
HBMEC	Human Brain Microvascular Endothelial Cell
HGF	Hepatocyte Growth Factor
HNSCC	Head and Neck Squamous Cell Carcinoma
Hsp90	Heat shock protein 90
IFN	Interferon
IL	Interleukin
INL	Innate Lymphoid Cell
JNK	c-Jun N-terminal Kinase
LDLR	Low-Density Lipoprotein Receptor
LPS	Lipopolysaccharide
LRP-1	Low-Density Lipoprotein Receptor-related Protein-1
MAMP	Microbe-associated molecular pattern

List of Abbreviations (*continued*)

MAPK	Mitogen-activated Protein Kinase
MC	Myeloid Cell
MDSC	Myeloid-Derived Suppressor Cell
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
NF-κB	Nuclear Factor-κB
NMDA	N-methyl-D-aspartate Receptor
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PSDP95	Post-synaptic Density Protein 95
RAP	Receptor Associated Protein
RB	Retinoblastoma protein
RhoA	Ras homolog family member A
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SHG	Second Harmonic Generation
SHP2	Src Homology region 2 domain-containing Phosphatase-2
sLRP-1	Soluble LRP-1
SMC	Smooth Muscle Cell
STAT3	Signal Transducer and Activator of Transcription 3
TAM	Tumor-Associated Macrophage
TGF-β	Transforming Growth Factor-β
Th17	T-helper-17
Th2	T-helper-2
TLR4	Toll-like Receptor 4
TME	Tumor Microenvironment
tPA	Tissue-type Plasminogen Activator
TSP1	Thrombospondin 1
uPA	Urokinase-type Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
VBM	Vascular Basement Membrane
VEGF	Vascular Endothelial Growth Factor

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Introduction

Introduction Générale et Objectif (summary in French)

Le cancer colorectal (CCR) est un problème de santé publique majeur en France et dans le monde. Chaque année, 42 000 nouveaux cas de cancers colorectaux sont diagnostiqués en France. Tous sexes confondus, le CCR est le troisième cancer le plus fréquent après les cancers de la prostate et du sein et cette pathologie cause chaque année 17 000 décès. Les chances de guérison dépendent du stade de progression de la maladie : les patients ont 91% de chances de survie, cinq ans ou plus après la maladie lorsqu'elle est diagnostiquée au stade de tumeur primaire, alors que ce pourcentage est de 11% lorsqu'elle a atteint le stade métastasé. Le CCR est un cancer dont la fréquence augmente avec l'âge. Il est ainsi rarement diagnostiqué avant 45 ans. Les principaux facteurs de risque du CCR sont la consommation d'alcool, le surpoids, l'obésité, le tabagisme, l'alimentation pauvre en fibres, excessive en viande rouge ou en viandes transformées, la sédentarité et l'inactivité physique. En dépit des avancées dans la compréhension des mécanismes moléculaires qui sous-tendent l'apparition et la progression du CCR, et de l'émergence de thérapies ciblées, le traitement de cette pathologie demeure encore insatisfaisant. Ceci est particulièrement vrai pour les cancers colorectaux localement évolués ou compliqués d'emblée de métastases à distance.

Il est bien connu que la plupart des patients atteints de CCR développent des mutations (caractère sporadique). A cause de l'accumulation progressive de ces mutations qui activent les oncogènes et inactivent les gènes suppresseurs de tumeurs, les cellules épithéliales du côlon sont transformées en foyers de cryptes aberrants, entraînant ensuite la formation de polypes et la formation de cancers subséquents. L'analyse génétique a montré que le cancer colorectal sporadique contient des mutations somatiques dans les oncogènes et les gènes suppresseurs de tumeurs. Récemment, la plupart des mutations proto-oncogènes du cancer colorectal ont été élucidées. Des mutations activatrices de KRAS ont été identifiées dans 40% des cas de CCR. Une faible proportion de mutations NRAS a cependant été rapportée. Le gène codant pour la protéine BRAF, un effecteur en aval de la voie Ras, est muté dans le CCR sporadique avec une fréquence d'environ 5 à 10%. De plus, les mutations de BRAF ont été associées à une variation de la progression adénome-carcinome. D'autres mutations somatiques critiques ont également été trouvées dans la kinase PI3K avec des proportions entre 15 et 25% des CCR. Concernant le CCR héréditaire, les deux types de cette pathologie les plus courants sont le cancer du côlon héréditaire sans polypose (HNPCC ou syndrome de Lynch) et la polypose adénomateuse familiale (FAP). Le HNPCC présente des mutations dans plusieurs gènes tels que MSH2 et MLH1, tandis que plus de 90% des FAP contiennent des mutations du gène APC.

L'environnement des cellules cancéreuses a une influence majeure sur le développement de la tumeur. En effet, le caractère invasif d'une tumeur primitive est déterminé non seulement par le génotype des cellules tumorales, mais aussi par leurs interactions avec l'environnement extracellulaire ou stromal, qui contient de cellules stromales et des éléments de la matrice extracellulaire (MEC). La MEC est composée de facteurs dits solubles (cytokines, facteurs de croissances…) et de facteurs dits adhésifs. Les composants adhésifs majoritaires de la MEC sont représentés par la superfamille des collagènes, parmi lesquels, le collagène de type I constitue le composant structural majeur. Des études récentes basées sur des approches transcriptomiques et protéomiques ont pu identifier les marqueurs spécifiques qui sont dérégulés dans le microenvironnement lors des premiers stades CCR, mais aussi dans celui localement avancé ou métastatique. Il est intéressant de noter que ces études ont permis de mettre en évidence un remodelage significatif du microenvironnement, et en particulier de la MEC. Des acteurs, qui participent à ce remodelage tels que les protéases, oxydases et hydroxylases, ont été identifiés proposés comme des marqueurs de la progression du CCR.

L'analyse des signatures MEC dans les tumeurs du côlon des patients a montré que le collagène de type I est fortement exprimé. Par conséquent, une densité élevée du collagène de type I est un facteur de mauvais pronostic dans le carcinome du côlon. En effet, un environnement riche en collagène de type I est capable d'induire l'expression d'un phénotype mésenchymateux et par conséquent l'invasion tumorale. Outre la densité, la topologie du collagène (alignement des fibres) et l'élasticité (rigidité) semblent également associées au CCR et sa progression. En effet, le collagène dans la MECdes tissus du carcinome du côlon semble être plus rigide que celui des tissus normaux et que l'alignement des fibres de collagène est augmenté dans les tissus du carcinome du côlon par rapport aux tissus normaux. Les études biophysiques ont également montré différentes empreintes moléculaires pour les fibres de collagène dans les tissus du carcinome du côlon par rapport aux tissus normaux. Il est important de noter que l'hypoxie, qui est associée à la densité et à l'organisation du collagène, a été décrite pour avoir un impact sur la migration et l'invasion du CCR en induisant le mésenchymateux dans le CCR.

Dans le cas du collagène de type I, et en plus de sa fonction architecturale, celui-ci est capable de moduler également le comportement des cellules cancéreuses en interagissant avec celles-ci *via* des récepteurs spécifiques. Les récepteurs de collagène de type I les plus étudiés sont les intégrines α1β1, α2β1, α10β1 et α11β1. Pour leur activation, ces récepteurs reconnaissent la séquence GFOGER du collagène de type I. Une étude menée sur des patients a montré que parmi les hétérodimères cités ci-dessus, l'intégrine α1β1 était le récepteur le plus

exprimé dans le carcinome du colon. L'expression de l'intégrine β1 dans les tumeurs a été corrélée avec une diminution de la survie des patients atteints de CCR. Les récepteurs à domaine discoïdine (DDRs) représentent la seconde famille de récepteurs qui interagissent aussi avec le collagène de type I. Ces récepteurs reconnaissent la séquence GVMGFO du collagène de type I et, contrairement aux intégrines, ils ont la particularité de posséder une activité tyrosine kinase. De plus, contrairement aux autres récepteurs à activité tyrosine kinase tels que l'EGFR ou le PDGFR, qui présentent une activation rapide et transitoire, les DDRs présentent une activation longue et prolongée. En effet, leur phosphorylation n'est détectable qu'après une exposition prolongée au collagène (2 heures) et se maintient pendant plus de 16 heures. Le rôle des DDRs et leur interaction avec les voies de signalisation impliquées dans la survie et la prolifération des cellules tumorales a été étudié récemment, aussi bien *in vitro* qu'*in vivo*. Ainsi il a été montré que DDR1 et DDR2 étaient capables d'avoir à la fois un rôle dans l'augmentation de la prolifération cellulaire [1-7] mais aussi dans l'inhibition de celle-ci [8-11], en fonction du type cellulaire et du microenvironnement de la tumeur. Des résultats récents ont montré que l'expression de DDR1 et son activation confère aux cellules tumorales de colon des propriétés invasives et la capacité à former des métastases [12, 13]. Cependant, des données de notre groupe et de celui d'Erik Maquoi ont montré que le collagène de type I était capable en matrice 3D d'induire une diminution de la prolifération de cellules cancéreuses, alors qu'il n'en induit pas lorsque ces cellules sont cultivées sur du collagène déposé en 2D [9, 14-16]. De plus, nous avons pu montrer que les récepteurs DDR1 et DDR2 sont les principaux récepteurs impliqués dans ce processus alors que les intégrines n'y participent pas [9, 15, 16].

Le récepteur LRP-1 (*low-density lipoprotein receptor-related protein-1*) est un récepteur d'endocytose multifonctionnel régulant différentes voies de signalisation intracellulaire. Ses fonctions dans l'endocytose et le catabolisme de divers constituants associés à la MEC, indiquent un rôle crucial du récepteur dans l'assemblage, le renouvellement et le contrôle de l'organisation du réseau matriciel. Plusieurs études ont montré récemment son rôle dans la migration, l'invasion et la prolifération des cellules cancéreuses. Au cours des dix dernières années, plusieurs d'études ont montré l'implication de LRP-1 dans diverses voies de signalisation qui induisent la prolifération cellulaire. Il est bien connu que LRP-1 régule certaines voies de signalisation cruciales en aval qui sont associées, entre autres, avec la prolifération cellulaire. Cependant, cette fonction reste controversée et dépend des types cellulaires, des stimuli et des conditions qui sont étudiés.

LRP-1 semble participer dans diverses voies de signalisation avec différents acteurs moléculaires pour moduler la prolifération cellulaire. Par exemple, la liaison de l'α2-macroglobuline à LRP-1 induit une augmentation de la phosphorylation de ERK1/2, p38 et c-Jun, et favorise ensuite la prolifération cellulaire dérivée des macrophages J774 [17]. Une étude sur les cellules ostéoblastiques a montré que LRP-1 est également impliqué dans la prolifération cellulaire induite par la lactoferrine. Il a été suggéré que la lactoferrine stimule la mitogenèse des ostéoblastes en activant la voie MAPK p42 / 44 *via* LRP-1 et que la fonction endocytaire de LRP-1 est indépendante de sa fonction de signalisation [18]. De plus, l'activateur tissulaire du plasminogène (tPA) semble interagir avec LRP-1 en induisant une cascade d'événements de signalisation prolifératifs impliquant la phosphorylation de ERK1/2, p90RSK, GSK3β, et l'induction de la cycline D1 entraînant une progression du cycle cellulaire et une prolifération cellulaire [19]. En outre, LRP-1 induit la prolifération cellulaire des fibroblastes embryonnaires de souris après association avec ses ligands, en stimulant les cascades de signalisation pro-prolifératives en aval [20, 21]. En revanche, LRP-1 a été décrit comme un inhibiteur de la prolifération des cellules stellaires hépatiques humaines en diminuant par endocytose le niveau de molécules pro-prolifératives dans le milieu extracellulaire [22, 23]. Une étude récente sur les cellules endothéliales microvasculaires rétiniennes a montré que LRP-1 interagit directement avec la poly- (ADP-ribose) polymérase-1 (PARP-1), un régulateur de la progression du cycle cellulaire, induisant une inhibition de la prolifération des cellules endothéliales. LRP-1 régule également les activités de la kinase dépendante de la cycline 2 (CDK2) et du rétinoblastome, deux protéines qui jouent un rôle important dans la progression du cycle cellulaire et de l'angiogenèse [24].

Des travaux réalisés par notre équipe ont montré que ce récepteur d'endocytose régule les processus d'adhérence des cellules cancéreuses à la MEC afin de favoriser la progression tumorale [25, 26]. Nous avons notamment démontré que LRP-1 joue un rôle de mécano-senseur du microenvironnement tumoral en contrôlant la rigidité des cellules tumorales et en régulant les forces d'adhérence et de détachement de celles-ci [27]. Nous avons aussi montré que l'inhibition de l'expression ou de l'activité de LRP-1 induit des modifications des voies de signalisation intracellulaire (inhibition de l'activation de FAK, suractivation de RhoA…) ainsi qu'une réorganisation du cytosquelette d'actine, qui aboutissent à l'inhibition de la migration et de l'invasion tumorale de carcinomes thyroïdiens dans une matrice 3D de collagène de type I [28]. D'autre part, une étude translationnelle visant à évaluer pour la première fois l'impact clinique du niveau d'expression de LRP-1 sur la survie globale et/ou la survie sans progression de patients atteints de CCR a permis d'établir qu'une perte d'expression de LRP-1 est associée

à des caractéristiques cliniques et des profils morphologiques et moléculaires (instabilité des microsatellites, mutations BRAF…) défavorables qui concourent à un mauvais pronostic [29]. D'autre part, des mutations de LRP-1 ont été rapportées chez des patients ayant des métastases hépatiques [30]. Au vu de ces données, le rôle de LRP-1 dans le CCR nécessite d'être étudié de manière plus précise, en particulier au niveau moléculaire.

Concernant la prolifération cellulaire, des études ont montré que DDR1 induit l'apoptose de cellules de carcinome mammaire dans une matrice 3D de collagène de type I [12, 16, 31]. Dans le cas du CCR, des études récentes ont montré que le nilotinib, un inhibiteur spécifique de la phosphorylation de DDR1, réduisait fortement l'invasion des cellules de CCR induite par DDR1 ainsi que les métastases *in vivo* [32]. Ces travaux ont été effectués sur des cellules de CCR présentant un phénotype invasif. Concernant les cellules non invasives de type carcinome épithélial, une étude précédente a montré un effet inhibiteur de la prolifération cellulaire des cellules de CCR dans une matrice 3D. Cependant, le rôle de DDR1 dans un tel processus n'a pas été établi [15].

Dans le présent travail, nous avons étudié si LRP-1, grâce à sa fonction d'endocytose, peut réguler l'expression de DDR1 au niveau de la membrane plasmique et moduler le pouvoir de DDR1 en tant que suppresseur de la prolifération cellulaire. Nos données démontrent pour la première fois que LRP-1 peut induire une endocytose de DDR1 dans des cellules de CCR non invasives, diminuant ainsi la capacité de l'axe collagène de type I / DDR1 à inhiber la prolifération des cellules tumorales et à induire l'apoptose.

I. Colorectal Cancer Overview and Therapies

1. Colorectal cancer

The development of colorectal cancer is characterized by the change of normal colonic epithelium in carcinoma tissue, in most cases by the development of colorectal adenomas. Adenocarcinoma from glandular epithelial cells of the colon and rectum is known to be the major type of CRC and make up 90 percent of all colorectal cancer cases. CRC consists of squamous cell carcinoma, spindle cell carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma [33]. Approximately 60-65% of CRC cases are sporadic and arise through acquired somatic genomic alterations, whereas 25% of cases were reported as a family profile-associated CRC without a clear genetic cancer syndrome. Other cases were reported as hereditary cancer syndromes, genetic mutations or unknown inherited genomic aberrations. Among those, only 5% of CRC are hereditary cancer syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch syndrome) and familial adenomatous polyposis (FAP), due to inherited germline mutations (**Figure 1**, page 19) [34]. Besides, the biological and anatomical features of colorectum make a critical contribution to the incidence and malignancy of CRC. Results from meta-analysis studies of some systematic reviews indicated that tumors were dominantly found in the proximal (right) colon of older patients compared to the younger groups and are more common in women than in men. The patients with right colon cancer have lower survival rates than those with the distal (left) colon or rectum cancers [35, 36].

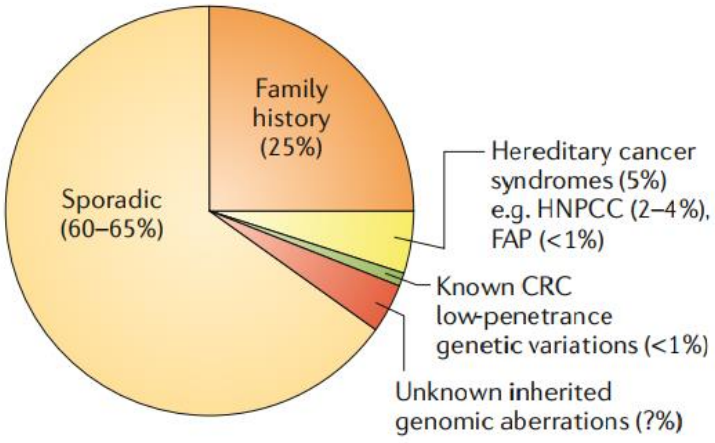

Figure 1. Classification of colorectal cancer based on sporadic and hereditary factors [34]

1.1 Epidemiology

1.1.1 Incidence and mortality

Colorectal cancer was reported as rare cancer in the 1950s, but its incidence has increased rapidly over the decades. CRC now accounts for about 10% of cancer-related mortality in western countries [37-39]. Recent data from the International Agency for Research on Cancer indicated that CRC is the second most common cancer diagnosed in women and the third most commonly occurring cancer in men worldwide (Figure 2) [40]. In 2018, CRC is the second deadliest cancer in the world, with over 1.8 million of new CRC cases and approximately 900,000 CRC-caused deaths. Many studies suggest that the increasing incidence rate of CRC is correlated with Western lifestyle, dietary patterns, industrialization and economic growth [41-43]. Indeed, more than 50 percent of prevalent CRC cases occur in countries characterized by high or very high human development index (**Figure 2**D, page 21) [44]. Analysis of age-standardized incidence rates of CRC in five continents showed that Oceania had the highest rate with more than 31 cases per 100 000 people, followed by Europe and North America with approximately 28 and 25 cases per 100 000 people, respectively. The lowest incidence rates were seen in Africa with less than 8 cases per 100 000 (**Figure 2**E, page 21). Global CRC statistics showed that the age-standardized incidence and mortality rates in men are higher than in women. The incidence was estimated at about 23.6 cases in men versus 16.3 in women per 100 000 people and estimated mortality was lower with 10.8 in men versus 7.2 in women cases per 100 000, respectively (Globalcan 2018, data not shown). To date, there is no strong evidence to show a significant difference in CRC incidence and mortality according to racial or ethnic disparities. However, extrinsic factors such as lifestyles and risk factors could contribute to CRC epidemical variation between races [34].

Although the incidence rate is higher in developed continents and areas, the ratio of deaths per number of new cases in these areas has been reported to be lower than those in developing areas (**Figure 2**E, page 21). It should be noted that healthcare systems made an undeniable contribution to that outcome. Indeed, screening programs including the increased use of sigmoidoscopy and colonoscopic polypectomy reduces CRC risk by detecting and removing adenomas, and increases survival and cure rates by earlier diagnosis [45, 46].

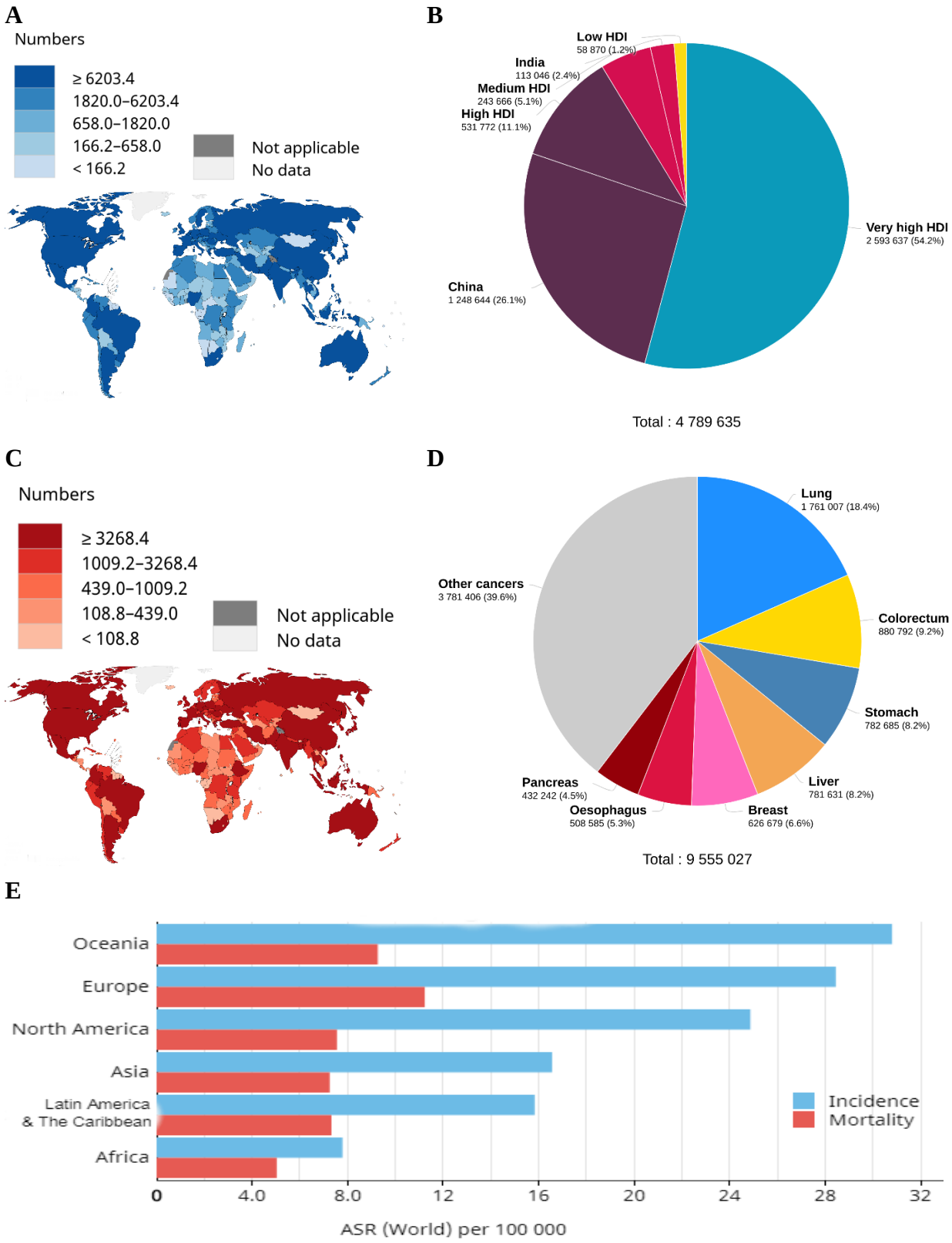

Figure 2. Global epidemiology of colorectal cancer in 2018. *(**A**) Number of new CRC cases worldwide including both sexes, all ages. (**B**) Estimated numbers of prevalent 5-year CRC cases. (**C**) Estimated number of deaths caused by CRC all over the world including both sexes and all ages.*

Figure 2. (Continued) *(**D**) Proportion of deaths caused by all cancers showing that CRC occupied the second leading cancer-related deaths including both sexes and all ages. (**E**) Estimated age-standardized rates (ASR-world, cases per 100000 individuals/year) of CRC incidence and mortality in five continents including both sexes and ages comprised between 0 and 74 years old. Data from GLOBOCAN database, 2018 [40].*

1.1.2 Prognosis

The prognosis of patients with colorectal cancer has improved over the past decades. The estimated 5-year survival rate reached approximately 70% in high-income countries such as Australia, Canada, Denmark, Ireland, New Zealand, and Norway (**Figure 3**, page 22), but it was under 50% in low-income countries [47]. In France, the estimated 5-year survival rate reached about 63.7 % between 2010-2014 (Data from Nuffieldtrust, UK). Tumor stage at diagnosis is an important prognostic factor, and survival is generally good for patients with early-stage CRC. According to data from the American Cancer Society in 2019, the relative survival rate for CRC is 65% at 5 years following diagnosis. However, only 39% of CRC patients are diagnosed with localized-stage disease, for which the 5-year survival rate is 90%; survival declines to 12% in patients diagnosed with stage IV CRC [48].

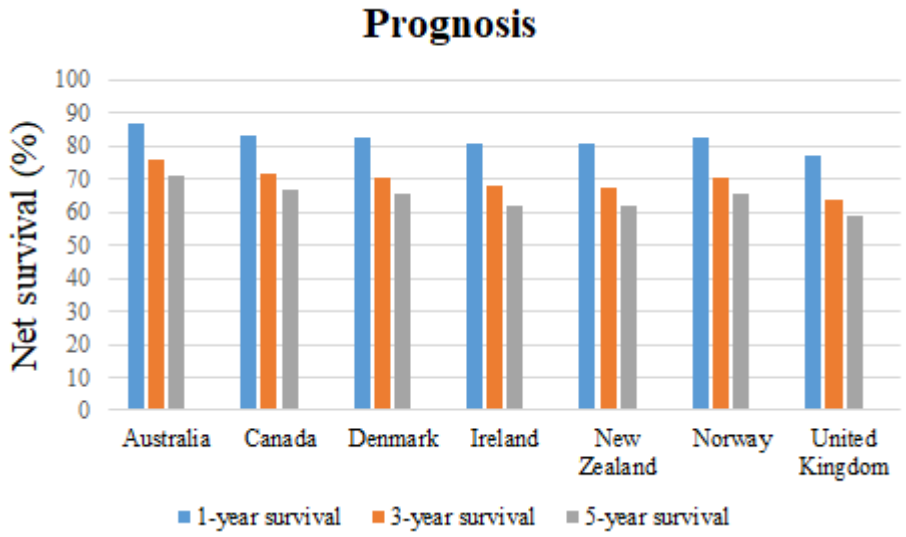

Figure 3. Estimated age-standardized 1-,3-,5-year net survival of colorectal cancer patients (both sexes,15–99 years) in some developed countries (2010-2014). *Data from Globalcan 2018.*

1.1.3 Risk and preventive factors

To date, many studies have indicated that both intrinsic (genetic) and extrinsic (environmental) factors partly contribute to the etiology of CRC. It should be noted that the extrinsic factors play undeniable roles on CRC formation. Diets and lifestyles have been strongly associated with CRC. Critical extrinsic risk factors including prolonged smoking [49, 50], excessive alcohol consumption [51], high consumption of red and processed meat [52] have been associated with a higher risk of CRC. Obesity is also a potential risk of CRC development [53]. Indeed, a higher Body-Mass Index (BMI) is associated with increased risks of CRC (**Figure 4**, page 23). Patients with diabetes [54] and long-term infectious diseases [55-57] are at a higher risk than the general population of developing CRC. Moreover, people with CRC family history or patients with inflammatory diseases have a greater risk of developing CRC [58, 59]. It should be noted that in about 5% of all cases, CRC is associated with a highly penetrant dominantly inherited syndrome, such as Lynch syndrome, familial adenomatous polyposis (FAP) or genetic mutations (<1%) (APC, MUTYH gene mutations) [34, 43, 60]. Interestingly, male sex (gender) is associated with an overall increased risk of CRC. Although gender is not a direct risk factor itself, different lifestyles may explain the variation in CRC causes between men and women. For instance, men tended to smoke and drink alcohol more regularly than women. Last but not least, older age is considered as the strongest risk factor due to the accumulation of risks during patients' lifetime (**Table 1**, page 24) [43].

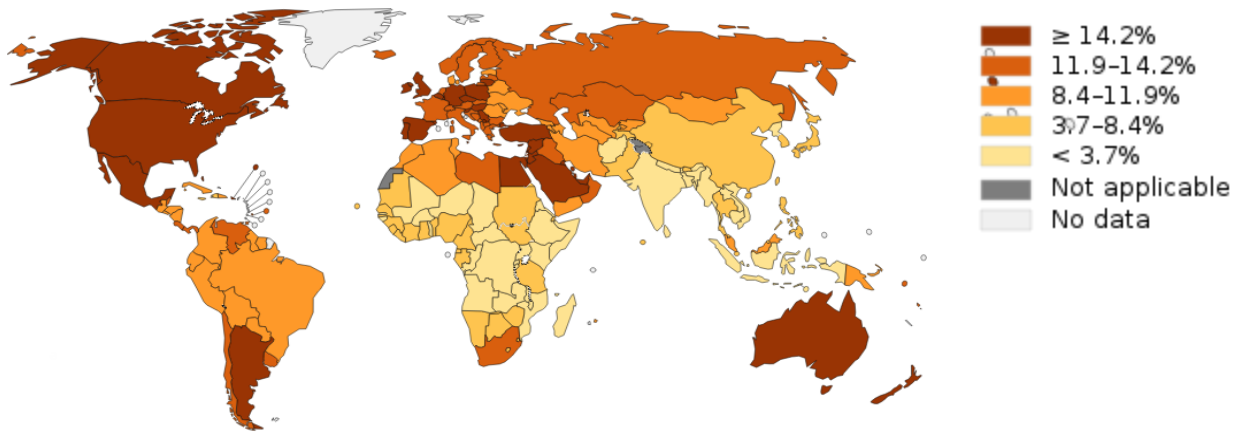

Figure 4. The percentage of all colorectal cancer cases among both sexes (worldwide) with the attribution of overweight*. Data from Globalcan 2012.*

On the contrary, many factors have been reported to be associated with a decreased risk of CRC. This includes physical activities, the use of hormone replacement therapy and aspirin, which were shown to decrease the risk of CRC by about 20-30% [43, 60]. The most effective

strategy for prevention of CRC is screening to detect precancerous polyps by endoscopy and remove them. [45, 46, 61, 62] (**Table 1**, page 24). Some studies suggested that the consumption of fresh fruits and vegetables, dairy products, cereal fibers and whole grains, as well as the intake of calcium, vitamin D, and multivitamins, could be beneficial to decrease risks of colorectal cancer [43, 60]. Finally, statins were considered as promising agents for colorectal cancer prevention, however ongoing clinical trials are still under investigation [63, 64].

Table 1. Overview of risk and preventive factors of colorectal cancer

Factors	Risk	Factors	Risk
Sociodemographic factors		**Lifestyle factors**	
Older age	↑↑↑	Smoking	↑
Male sex	↑↑	Excessive alcohol consumption	↑
Medical factors		Obesity	↑
Family history	↑↑	Physical activity	↑
Inflammatory bowel diseases	↑↑	**Diet factors**	
Diabetes	↑	High consumption of red and processed meat	↑
Helicobacter pylori infection	(↑)	Fruits and Vegetables	(↓)
Other infections	(↑)	Cereal fibre and whole grain	(↓)
Large bowel endoscopy	↓↓	Fish	(↓)
Hormone replacement therapy	↓	Dairy products	(↓)
Aspirin	↓		
Statins	(↓)		

↑↑↑= very strong risk increase, ↑↑= strong risk increase, ↑= moderate risk increase, ↓↓=strong risk reduction, ↓=moderate risk reduction. Parentheses "()" show probable risk factors, but are not fully established. The table is adapted from Brenner (2014) **[43]**.

1.2 Histopathological classification

To date, there are several staging systems used in oncology. However, the most clinically useful staging system is the tumor, node, and metastasis (TNM) staging system [65]. According to the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC), colorectal cancer stages are classified based on three factors "tumor-node-metastasis", which classified CRC into 3 main stages including invasive depth of primary tumor (T stage), regional lymph nodes (N stage), and presence of distant metastases (M stage) [65, 66]. Furthermore, the UICC created a new staging system based on TNM system to improve the prognosis and the therapeutic strategy (**Table 2**, page 25).

Table 2. Stages of colorectal cancer based on the classification of the International Union for Cancer Control.

Stages	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1/T2	N0	M0
Stage II	T3/T4	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
Stage III	Any	N+	M0
IIIA	T1–T2	N1	M0
	T1	N2a	M0
IIIB	T3–T4a	N1	M0
	T2–T3	N2a	M0
	T1–T2	N2b	M0
IIIC	T4a	N2a	M0
	T3–T4a	N2b	M0
	T4b	N1–N2	M0
Stage IV	Any	Any	M+
IVA	Any	Any	M1a
IVB	Any	Any	M1b

The table is adapted from Brenner and collaborators **[43]**

The factors used to define TNM staging system are summarized in **Table 3** (page 26).

Due to some limitations from TNM and UICC staging systems such as difficulties to predict the therapeutic response and outcome of individual patients [43], a future staging system is under investigation [65].

Table 3. Classification of colorectal cancers according to TNM staging system

	Definition
T stage	
Tx	No information about local tumor infiltration available
Tis	Tumor restricted to mucosa, no infiltration of lamina muscularis mucosae
T1	Infiltration through lamina muscularis mucosae into submucosa, no infiltration of lamina muscularis propria
T2	Infiltration into, but not beyond, lamina muscularis propria
T3	Infiltration into subserosa or non-peritonealised pericolic or perirectal tissue, or both; no infiltration of serosa or neighboring organs
T4a	Infiltration of the serosa
T4b	Infiltration of neighboring tissues or organs
N stage	
Nx	No information about lymph node involvement available
N0	No lymph node involvement
N1a	Cancer cells detectable in 1 regional lymph node
N1b	Cancer cells detectable in 2–3 regional lymph nodes
N1c	Tumor satellites in subserosa or pericolicor perirectal fat tissue, regional lymph nodes not involved
N2a	Cancer cells detectable in 4–6 regional lymph nodes
N2b	Cancer cells detectable in 7 or greater regional lymph nodes
M stage	
Mx	No information about distant metastasis available
M0	No distant metastasis detectable
M1a	Metastasis to 1 distant organ or distant lymph nodes
M1b	Metastasis to more than 1 distant organ or set of distant lymph nodes or peritoneal metastasis

This table is adapted from Brenner and collaborators **[43]** and Sobin and collaborators **[66].**

1.3 Molecular Genetics of Colorectal Cancer

It is widely known that most of patients with CRC develop gene mutations during their lifetime (sporadic CRC). Through the progressive accumulation of genetic mutations and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes, colon epithelial cells are transformed to aberrant crypt foci, then leading to polyp formation and subsequent cancer formation [34, 60, 67]. Genetic analysis has shown that sporadic colorectal cancer contains somatic mutations in both oncogenes and crucial tumor suppressor genes. For example, mutations reducing tumor suppressor function of APC were found in approximately 70-80 percent of sporadic adenomas and carcinomas [68, 69]. Similarly, most sporadic colorectal cancer contain p53-inactivating mutations [70-72]. Other common tumor-suppressor gene mutations are summarized in Table 4 (**Table 4**, page 28).

Recently, most of the proto-oncogene mutations in colorectal cancer have been elucidated. *KRAS* mutations are the most common recurrent somatic mutations in CRC. Activating *KRAS* mutations have been identified in 40% of CRC cases and primarily occur in codon 12 and 13 and rarely in codon 61. A small proportion of *NRAS* mutations in CRC was reported in codon 12, 13, or 61 [72-74]. The gene encoding for B-Raf protein, a downstream effector of Ras pathway, is mutated in sporadic CRC with a frequency of approximately 5-10% [75]. Moreover, *BRAF* mutations were associated with a variation on the usual adenoma-carcinoma progression and the altered DNA-methylation phenotype known as CpG island hypermethylation phenotype (CIMP) [76]. Other critical somatic mutations were also found in *PIK3CA* oncogene, in 15 to 25% of CRC [72, 77]. Subsequent studies showed that these mutations lead to activate PIK3CA kinase, which results in increased production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) [77], a key second messenger in cell proliferation, survival signaling and other processes [78]. Other common oncogene mutations are summarized in Table 4 (**Table 4**, page 28).

Concerning hereditary CRC, as mentioned above, which represents 5% of the cases, the two most common types of hereditary colorectal cancers are hereditary non-polyposis colon cancer (HNPCC or Lynch syndrome) and familial adenomatous polyposis (FAP). HNPCC contains mutations in several genes such as *MSH2 MLH1 PMS2 GTBP, MSH6* while more than 90% of FAP contains *APC* gene mutations [34, 43, 60, 79].

Table 4. Selected common recurrent somatic mutations in colorectal cancer.

Genes	Type of mutations	Estimated frequency
Oncogenes		
KRAS	Point mutations (codons 12, 13, 61)	40% (>75% at codon 12)
NRAS	Point mutations (codons 12, 13, 61)	<5%
PIK3CA	Point mutations activating kinase activity	15–25%
BRAF	Point mutations activating kinase activity (*V600E*)	5–10% (CIMP-positive CRCs)
EGFR	Gene amplification	5–15%
CDK8	Gene amplification	10–15%
CMYC	Gene amplification	5–10%
CCNE1	Gene amplification	5%
CTNNB1	Stabilizing point mutations and in-frame deletions	<5%
NEU/HER2	Gene amplification	<5%
MYB	Gene amplification	<5%
Tumor-suppressor genes		
p53	Point mutation, allele loss	60–70% (>95% missense)
APC	Frameshift, point mutation, deletion, allele loss	70–80% (truncated proteins)
FBXW7	Nonsense, missense, deletion	20%
PTEN	Nonsense, deletion	10%
SMAD4	Nonsense, missense, allele loss	10–15%
SMAD2	Nonsense, deletion, allele loss	5–10%
SMAD3	Nonsense, deletion	5%
TGFβIIR	Frameshift, nonsense	10–15% (>90% MSI-H CRCs)
TCF7L2	Frameshift, nonsense	5% (MSI-H and MSS CRCs)
ACVR2	Frameshift	10% (>80% MSI-H CRCs)
BAX	Frameshift	5% (~50% of MSI-H CRCs)

CIMP: CpG island hypermethylation phenotype; MSI-H: high frequency microsatellite instability; MSS: microsatellite stable; CRCs: types of colorectal cancer

The table is adapted from Fearon [48]

1.4 Mechanisms and pathophysiology of colorectal cancer

Like all other cancers, colorectal tumorigenesis and cancer formation have common mechanisms wherein the accumulation of environmental and genetic risk factors promotes the acquisition of cancer behaviors in normal epithelial cells, which lead to transform them to malignant cells with all hallmark features of cancer [80]. In particular, normal colorectal

epithelial cells can undergo several carcinogenic pathways before being transformed into malignant cells depending on accumulated genetic and epigenetic alterations [34, 60]. Indeed, genomic instabilities and/or gene mutations in colorectal cancer were observed and well described since the 1990s and the alterations in oncogenes and tumor suppressor genes were reported to drive the malignant transformation of colon cells [73, 81, 82].

A key feature of most colorectal carcinogenesis is the presence of a benign precursor polyp, a result of abnormal mucosal maturation in the lining of the large intestine, and its progression to invasive cancer can occur in 5 years or take more than 20 years. [83-85]. Adenomatous polyps (adenomas) and serrated polyps are two major subtypes that serve as direct precursors to most CRCs. CRC develops through three distinctive pathways: (*i*) adenoma–carcinoma sequence, (*ii*) serrated pathway and (*iii*) inflammatory pathway. Adenoma–carcinoma sequence model represents the classic pathway and explains the majority of sporadic CRC. In this model, initial genetic changes start in an early adenoma and accumulate as it transforms to carcinoma (**Figure 5**A, page 30). More precisely, carcinogenesis is initiated from inactivation of *APC* gene, regarded as the gatekeeper against colorectal neoplasms, leading to the over-activation of the Wnt/β-catenin signaling pathway, which results in dysregulated cell proliferation and adenoma development [86]. Furthermore, subsequent mutations of the oncogene *KRAS* inactivation of TP53 tumor suppressor gene promote adenoma formation [87].

In the serrated adenoma model, normal cells progress to hyperplastic polyp, to sessile serrated adenoma and, finally, to CRC (**Figure 5**B, page 30) [88, 89]. Two molecular events are particularly important in this pathway. Firstly, *BRAF* mutations lead to uncontrolled cell proliferation, which contributes to the formation of hyperplastic polyps. Then, the CpG island methylator phenotype (CIMP), as a consequence of epigenetic instabilities, frequently arises from this pathway [88, 89].

For the chronic inflammatory model, patients with inflammatory bowel diseases, particularly ulcerative colitis, have an estimated 2.4-fold higher risk of CRC compared with the general population [59]. In these patients, carcinogenesis progresses from no dysplasia to indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and, finally, to CRC (**Figure 5**, page 30) [90]. The timing and frequency of molecular events are distinct from other pathways. Contrary to the adenoma-carcinoma sequence, in which genetic mutations in *APC* and *TP53* respectively occur early and late in colorectal carcinogenesis, in the inflammatory pathway, TP53 mutations represent an early event, with APC mutations occurring infrequently and late in carcinogenesis [90].

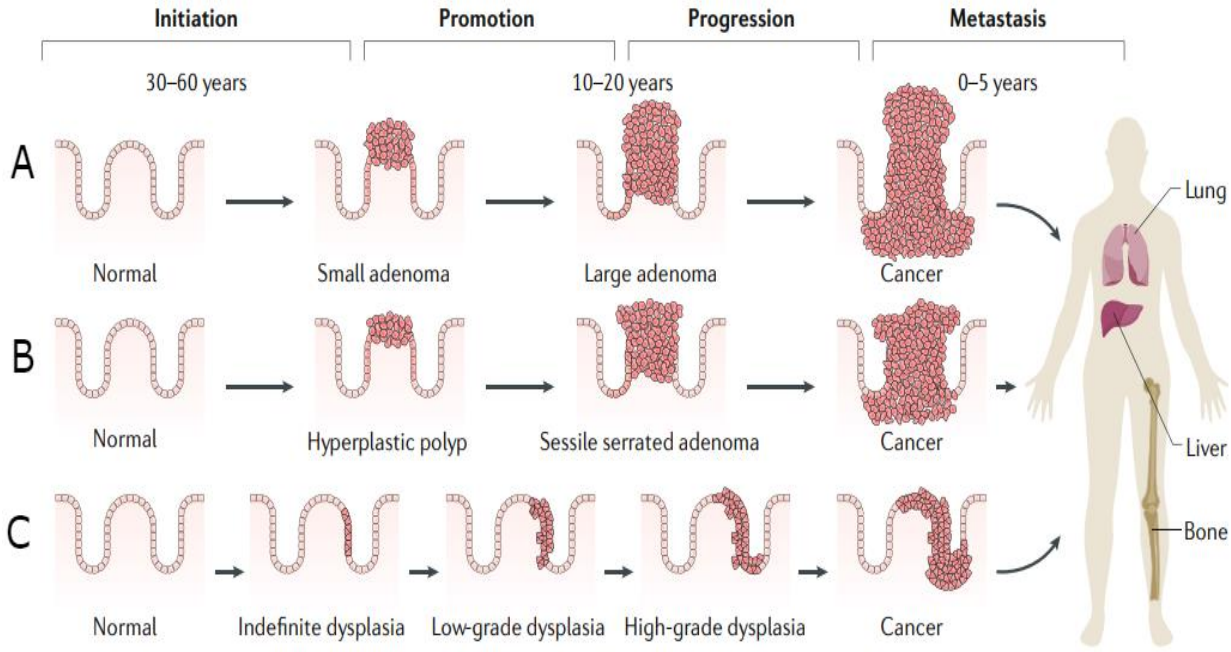

Figure 5. Presentation of colorectal carcinogenetic pathways. *Common sites of metastatic disease from CRC include the liver and lungs with bone metastasis.* ***(A)*** *Adenoma–carcinoma sequence pathway accounts for 85-90% of sporadic CRC. This pathway is highly associated with the development of the chromosomal instability (CIN)-positive subtype. (B) Serrated adenoma pathway makes up of 10–15% of sporadic CRC. In this pathway, the transformation of normal epithelial cells leads to hyperplastic polyp and sessile serrated adenoma formation. Then, the CpG island methylator phenotype (CIMP) further progresses adenoma to CRC. (C) The inflammatory pathway represents less than 2% of all CRC. The chronic inflammation promotes the progression of normal cells to several stages of dysplasia and finally to CRC.[34]*

1.5 Colorectal cancer diagnosis and screening

To date, several methods are recently recommended for CRC diagnosis and screening including endoscopic and radiologic examinations such as colonoscopy, sigmoidoscopy, computed tomographic colonography, double-contrast barium enema or stool-based tests like a guaiac-based fecal occult blood test, fecal immunochemical test, and FIT-DNA test (**Table 5**, page 33).

❖ **Colonoscopy**

Colonoscopy is the gold-standard method for the diagnosis of colorectal cancer over the past 20 years [34, 60, 91, 92]. This method not only allows physicians to see the whole large intestine, but it also enables to assess the tumor site for biopsy sampling. Hence, it can provide histological confirmation of the diagnosis and material post studies. Until now, this is the unique screening technique that provides both diagnostic data and therapeutic effect wherein polyps or adenomas can be removable during endoscopy examination using an endoscopic

polypectomy, which both reduces cancer incidence and mortality [60]. In addition, the examination is not required to be repeated in average-risk patients during a 10 years period if the screening results are normal. However, any positive results from other tests should be confirmed by a colonoscopy examination [92]. Long-term follow-up studies indicated that colonoscopy screening probably reduces CRC mortality by 53% and incidence by about 40% [93, 94]. Moreover, screening in the average-risk group was attributed to reducing risks for late-stage CRC incidence [95].

There are minority limitations of colonoscopy screening such as bowel tears and bleeding, especially in the case of polyp removal [84]. The serious bleeding occurs rarely with a ratio of 1 to 2 cases per 1,000 colonoscopy examinations [96-98]. In addition, colonoscopy screening potentially misses some small or flat adenomas such as sessile adenomas, which may progress to invasive colorectal cancers occurring before the next scheduled examination [99, 100].

❖ **Sigmoidoscopy**

Flexible sigmoidoscopy was a popular screening and diagnostic method for CRC before the widespread application of colonoscopy [92]. The mechanism of sigmoidoscopy is very similar to colonoscopy. However, this technique only allows visualizing the rectum and lower right colon [84]. In the case of a polyp or tumor detection, the patient is recommended for a colonoscopy examination. Due to this limitation, this method is not widely recommended nowadays [92]. Several studies indicated that sigmoidoscopy screening probably reduces approximately 20% of CRC incidence and 30% of mortality [101, 102].

❖ **Computed tomographic colonography** (CTC)

CTC, also known as a virtual colonoscopy, was introduced in the 1990s and enables to view the entire colon and rectum [84]. CTC is a non-invasive method that requires no recovery time and typically takes approximately 10 to 15 minutes to complete [103]. It has shown that CT colonography has a sensitivity of 96% for CRC detection [104]. Studies have indicated that CTC performance is similar to colonoscopy for the detection of polyps and invasive CRC. However, CT colonography has lower sensitivity for small polyps/tumors (6–9mm) and flat lesions [105]. In some countries, CTC has replaced the double-contrast barium enema examination (below) and used as an alternation for colonoscopy. However, CT colonography has not been accepted in Europe due to radiation exposure, cost-effectiveness, and efficiency while CTC is only recommended for people who are impossible for colonoscopy [106].

❖ **Double-contrast barium enema**

Double-contrast barium enema, also known as the barium enema with air contrast, an old technique, is less sensitive than colonoscopy for the visualization of small polyps or cancers. In the case of a polyp or tumor detection, the patient is recommended for a colonoscopy examination. Nowadays, the use of this method is very uncommon due to its efficiency and potential radiation exposure [92].

❖ **Guaiac-based fecal occult blood test** (gFOBT)

gFOBT is based on a chemical reaction to detect blood in the stool. Bleeding from CRC is possibly sporadic or undetectable so that accurate test results require annual testing of 3 consecutive stool samples. The benefits of this method are to detect the abnormalities at early stages without invasiveness that can provide the information for clinicians to decide further examinations such as colonoscopy with polypectomy. Thereby, precancerous polyps or adenomas can be removable during endoscopy examinations. Clinical data indicated that gFOBT could reduce the risk of CRC mortality by 32% and 20% for incidence [107, 108].

❖ **Fecal immunochemical test** (FIT)

FIT, also known as an immunochemical FOBT, or iFOBT, uses antibodies against hemoglobin to detect hidden blood in the stool. Current highly sensitive versions of this test were commercialized over 10 years. FIT is more convenient than gFOBT because it requires no dietary restrictions [92].

❖ **FIT-DNA test (Cologuard®)**

Cologuard®, a commercial test targeting multi markers, allows detecting blood and certain genetic mutations that are released into the stool by large adenomas and CRC. Patients with a positive result are recommended for a colonoscopy. This test has been shown to detect cancer and precancerous lesions better than FIT, but also results in more false-positive tests (for example, blood due to hemorrhoids), which can lead to unnecessary colonoscopies [109].

Table 5. Key indicators for available colorectal cancer diagnostic and screening techniques.

Methods	Benefits	Performance & Complexity	Limitations	Test Interval
Visual Examinations				
Colonoscopy	• Examines entire colon • biopsy and remove • Diagnoses other diseases • Confirms abnormal results from other tests	**Performance:** Highest **Complexity:** Highest	• Full bowel cleansing • Be expensive • Sedation and sports needed • Take time • Risks: bowel tears or infections	10 year
CTC	• Examines entire colon • Fairly quick • Few complications • No sedation needed • Noninvasive	**Performance:** High for large polyps **Complexity:** Intermediate	• Full bowel cleansing • No polyp removal or biopsies • Exposure to low-dose radiation • Colonoscopy necessary if positive	5 years
Double contrast barium enema	• Can view entire colon • Few complications • No sedation needed	**Performance:** High for large polyps **Complexity:** High	• Full bowel cleansing • Some false-positive test results • No polyp removal or biopsies • Exposure to low-dose radiation • Colonoscopy necessary if positive • Very limited availability	5 years
Sigmoidoscopy	• Fairly quick • Few complications • Less preparation • No sedation	**Performance:** High for rectum & right colon **Complexity:** Intermediate	• Partial bowel cleansing • Views lower right colon • No polyp removal • Risk of infection or bowel tear • Colonoscopy necessary if positive • Limited availability	5 years
Stool Tests (Low-sensitivity stool tests, such as single-sample FOBT are not recommended.)				
FIT	• No bowel cleansing • No sedation • Performed at home • Low cost • Non-invasive	**Performance:** Intermediate for cancer **Complexity:** Low	• Requires multiple stool samples • Miss most polyps • Possible false-positive test results • Colonoscopy necessary if positive	Annual
High sensitivity (gFOBT)	• No bowel cleansing • Performed at home • Low cost • Non-invasive	**Performance:** Intermediate for cancer **Complexity:** Low	• Requires multiple stool samples • Will miss most polyps • Possible false-positive test results • Pre-test dietary limitations • Colonoscopy necessary if positive	Annual
FIT-DNA test (Cologuard®)	• No bowel cleansing • Perform at home • A single stool sample • Non-invasive	**Performance:** Intermediate for cancer **Complexity:** Low	• Will miss most polyps • False-positive results • Higher cost than gFOBT and FIT • Colonoscopy necessary if positive	3 years

The table is adapted from the American Cancer Society: Colorectal Cancer Facts & Figures 2017-2019 with the combined information from Kuipers and colleagues **[60].**

1.6 Colorectal cancer treatment

Many colon cancer treatment options are available for colorectal cancer, including surgery, chemotherapy, radiation, and the emerging immunotherapy. Like all other cancer treatments, the approach of therapeutic methods is highly dependent on the pathophysiology of CRC such as stages, genetic and epigenetic features. Furthermore, colon cancer and rectal cancer are two distinct cancers that require different approaches.

❖ **Surgery**

Surgery is the mainstay curative treatment for patients with non-metastatic CRC. However, the outcome is strongly dependent on the quality of surgery, the quality of pre-operative staging [110, 111], as well as the location and complexity of tumors [111, 112].

Total mesorectal excision (TME) is a standard surgical procedure for rectal cancer treatment, which requires to remove completely the rectum, surrounding mesorectum, and mesorectal fascia. The complete removal is necessary to prevent recurrence and tumor deposits due to the possibility of the cancer cells to spread in lymph nodes [113]. Indeed, several studies showed that in patients with invasive rectal cancer called circumferential margin, the risks of local tumor recurrence and development of distant metastases are increased [114, 115]. Thereby, the clearance of tumors with a positive circumferential margin and its regional invasive sites clearly contributes to therapeutic outcome [114].

In colon cancer surgery, the removal of the tumor and the surrounding lymph vessels is considered as a conventional surgical procedure. The surgery can be extended depending on the localization of tumors and the supplying blood vessels [43]. Recently, the emerging method called complete mesocolic excision (CME) with central vascular ligation (CVL) has been developed. It consists in removing completely afflicted colon, its accessory lymphatic vessels and regional lymph nodes [116].

Nowadays, laparoscopic colon surgery has been shown to be as safe as the open alternative [60, 117], cost-effectiveness [118], and has the same long-term results as conventional methods with the exception of the long operation [117, 119-121].

❖ **Neoadjuvant therapy**

Neoadjuvant therapy is defined as therapy administered prior to definitive local treatment. There is no recommended neoadjuvant treatment for colon cancer. However, recent reports indicated that neoadjuvant chemotherapy improves surgical outcomes [122], and leads to

improve survival in patients with T4b colon cancer [123]. By contrast, neoadjuvant radiotherapy or chemo-radiotherapy are recommended for intermediate and advanced-stages of rectal cancer to reduce the rate of local tumor recurrence [60]. The treatment with a neoadjuvant radiotherapy or chemo-radiotherapy prior to a surgery have long been considered as a standard procedure for treatment of locally advanced rectal cancer, with a local recurrence rate of less than 10% at the fifth year [124-127]. In addition, both preoperative radiotherapy and chemo-radiotherapy are more efficient by reducing local tumor recurrence compared to postoperative treatments. Unfortunately, the effect of these treatments on overall survival rates remained unchanged [125, 128]. Furthermore, the combination of 5-fluorouracil and oxaliplatin treatment with radiotherapy has been studied. However, the results demonstrated no clear survival benefits and an increase of the toxicity of the treatments [129].

❖ **Adjuvant therapy**

Adjuvant therapy is defined by the National Cancer Institute (NCI) as "additional cancer treatment given after the primary treatment to lower the risk that the cancer will come back." The aim of this adjuvant therapy is to eliminate residual cancer cells left behind at surgery. Adjuvant therapy may include chemotherapy, radiation therapy, hormone therapy, targeted therapy, or biological therapy. For patients with stage III colorectal cancer, tumor has a local recurrence of 15% to 50%. An adjuvant chemotherapy with 5-fluorouracil is therefore commonly recommended for all patients with stage III colorectal cancer after surgery [130]. In addition, capecitabine, an oral prodrug of 5-fluorouracil, can be used as an alternative treatment to 5-fluorouracil with efficacy benefits maintained at 5 years and in older patients. [131]. To improve disease-free survival (DFS) and overall survival (OS) rates, some combined treatments were investigated [43].Among these treatments the combination of capecitabine and oxaliplatin (known as the XELOX protocol) [132], and 5-fluorouracil combined leucovorin and oxaliplatin (FOLFOX4 protocol) have shown a significant increase in both DFS and OS rates [133].

According to current guidelines, adjuvant (5-fluorouracil) chemotherapy is not recommended for stage II colon cancer patients with microsatellite instability-high (MSI-H) or defective mismatch repair (dMMR) due to the low risk of tumor recurrence in this CRC subtype [134-136]. The prevalence of MSI-H colorectal cancers depends on clinicopathological factors. This type of cancers are commonly found in elderly female patients (above aged 70 years) with poorly differentiated stage II colon cancers [137]. Nevertheless, combined chemotherapy has been demonstrated its beneficial treatment for colorectal cancer patients with MSI-H and/or

dMMR genotypes. The combination of three drugs including oxaliplatin, 5-fluorouracil, and leucovorin (FOLFOX4) has been reported to increase in relapse-free survival (RFS), FDS, and OS of stage II and III CRC patients with MSI-H/dMMR genotypes [138, 139]. In addition, treatment with FOLFOX improved survival outcomes in patients with dMMR tumors compared with those with MMR-proficient tumors [140, 141].

❖ **Treatments for patients with metastatic colorectal cancer**

The OS for patients with untreated metastatic CRC (mCRC) is about six months. However, a significant two years improvement in OS is observed in patients treated with the combination of chemotherapy and immunotherapy [142, 143]. Standard chemotherapy for patients with mCRC consists of 5-fluorouracil combined with other anticancer agents such as leucovorin and oxaliplatin (**Table 6**, page 36). Indeed, The combination of irinotecan, a topoisomerase I inhibitor, with 5-fluorouracil is associated with higher response rate, longer progression free survival, and longer OS [144-146]. Treatment with three drugs including 5-fluorouracil, leucovorin, and oxaliplatin as first-line therapy is associated with improvement in progression-free survival and response rate [147, 148]. In addition, FDA-approved therapy for mCRC treatment are summarized in Table 6 (**Table 6**, page 36).

Table 6. ***List of FDA-approved therapy for mCRC treatment [143].***

Therapeutic agent	Mechanism of action
5-Fluorouracil	Pyrimidine analog
Oxaliplatin	Platinum derivative, alkylating agent
Irinotecan	Topoisomerase I inhibitor
Regorafenib	Tyrosine kinase inhibitor of VEGFR1–3, TIE2, others
Bevacizumab	Monoclonal antibody to VEGF-A
Aflibercept	Recombinant protein, decoy receptor for VEGF-A, VEGF-B, and PlGF
Cetuximab	Monoclonal antibody to EGFR
Panitumumab	Monoclonal antibody to EGFR

Among these molecules, tumor-driven angiogenesis is an attractive target in mCRC. FDA has approved a total of four drugs that block angiogenesis (bevacizumab, aflibercept, ramucirumab, and regorafenib) (**Table 6**). Regorafenib and aflibercept are two recombinant proteins which inhibit either kinases of vascular endothelial growth factor receptors such as VEGFR1- VEGFR-2, and VEGFR-3 or the effect of placenta growth factor (PlGF) and vascular endothelial growth factors such as VEGF-A, VEGF-B, respectively. Bevacizumab, specific of VEGF-A, cetuximab and panitumumab (specific of epidermal growth factor

receptor (EGFR)) are monoclonal antibodies. Cetuximab and panitumumab are only recommended for patients without *RAS* mutations and are commonly used in combination therapy (**Figure 6**, page 37) [143].

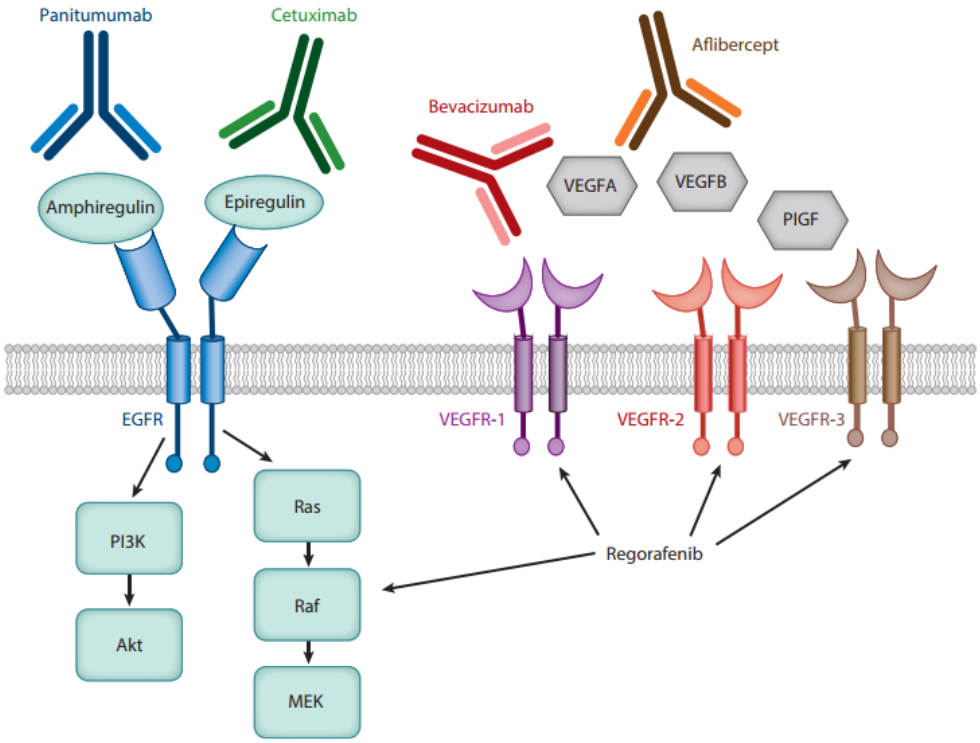

Figure 6. Crosstalk between therapeutic agents targeting EGFR and VEGFRs signaling pathways. *EGFR: epidermal growth factor receptor; MEK: mitogen-activated protein kinase kinase; PlGF: placental growth factor; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B [143].*

❖ **Immunotherapy**

Immune checkpoint therapy was approved in 2017 for the treatment of heavily mutated colorectal tumors with deficient mismatch repair (dMMR) and/or high rate of microsatellite instability (MSI-H) referred as dMMR–MSI-H colorectal cancer. However, treatments with current immune checkpoint inhibitors (ICIs) are inefficient in CRC patients with tumors containing proficient mismatch repair (pMMR), microsatellite stability (MSS), and in cases with a low rate of microsatellite instability (MSI-L) termed as pMMR-MSI-L colorectal cancer [149]. dMMR–MSI-H tumors are characterized by a diffuse immune infiltrate, composed of $CD8^+$ tumor-infiltrating lymphocytes (TILs), T helper 1, $CD4^+$ TILs, and macrophages and by a microenvironment rich in type I interferons [149].

dMMR–MSI-H genotype accounts for approximately 15% of all CRCs [150], and presents a lower risk of tumor recurrence at stage II than the patients with pMMR–MSI-L genotype

[151]. Although stage IV dMMR–MSI-H CRC represents only around 2–4% of all metastatic cases, they are associated with a worse prognosis [152]. However, recent studies have shown that the expression of both programmed cell death protein 1 (PD1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4/CD152) are up-regulated in dMMR–MSI-H colorectal cancer, which suggests a potential involvement of immune checkpoint blockade in this CRC type [153].

❖ **Immunotherapy for dMMR-MSI-H colorectal cancer**

Several clinical trials using monoclonal antibodies target CTLA4 (tremelimumab) or PDL1 (BMS-936559, nivolumab, pembrolizumab, and ipilimumab) in CRC patients with dMMR-MSI-H genotype. In most cases, the results of these trials showed a modest effectiveness of single antibody therapy, but presented a noticeable effect when combined with other treatments [149]. For instance, unlike nivolumab alone, combination of nivolumab and ipilimumab showed a robust and durable clinical benefit in CRC patients with dMMR-MSI-H genotype [154, 155]. Furthermore, pembrolizumab and nivolumab were approved by FDA in 2017 and were considered as the second-line treatment for patients with dMMR–MSI-H CRC. However, none of these molecules is currently approved by the European Medicines Agency (EMA) and phase III randomized controlled trials are needed to allow their use in Europe [149].

Recently, many monoclonal antibodies targeting PD1 and PDL1 in dMMR–MSI-H colorectal cancer that are currently in clinical trials are presented in Table 7 (**Table 7**, page 39).

Table 7. List of ongoing clinical trials targeting dMMr–MSI-H colorectal cancer

Checkpoint inhibitor	Trial type	Study treatment groups	Trial identifier
Atezolizumab	• Phase III • Stage 3 CRC	Adjuvant atezolizumab+FOLFOX versus FOLFOX alone	NCT02912559
	• Phase III • First-line metastatic CRC	Atezolizumab versus atezolizumab+FOLFOX +bevacizumab versus FOLFOX+bevacizumab	NCT02997228
Pembrolizumab	• Phase III • First-line metastatic CRC	Pembrolizumab versus standard-of-care chemotherapy	NCT02563002
	• Phase II • mCRC: refractory or ≥1 prior therapy	Pembrolizumab	NCT02460198
Avelumab	• Phase II • mCRC: >1 prior therapy	Avelumab	NCT03150706
Nivolumab ± ipilimumab	• Phase II • Refractory CRC	Nivolumab±ipilimumab or daratumumab or anti-LAG3 antibody	NCT02060188
Atezolizumab	• Phase I • Locally advanced or metastatic solid tumors	• Atezolizumab+bevacizumab • Atezolizumab+bevacizumab+FOLFOX • Atezolizumab+carboplatin+paclitaxel • Atezolizumab+carboplatin+pemetrexed • Atezolizumab+carboplatin+nab-paclitaxel • Atezolizumab+nab-paclitaxel	NCT01633970

Source from **[149]** with original data from *https://clinicaltrials.gov/ct2/home*

❖ Immunotherapy for dMMR-MSI-L colorectal cancer

For the vast majority of patients with mCRC whose tumors are dMMR-MSI-L, immunotherapy currently offers little to no clinical benefit. [149]. In fact, several clinical trials studied combined biological and/or biopharmaceutical agents (including approved/disapproved recombinant proteins and monoclonal antibodies) to target pMMR–MSI-L colorectal cancer. However, the results still show modest efficacy or confusing outcome [149]. So far, many clinical trials are still under investigation, which either combine the inhibition of MEK and PD1 or MEK inhibition with chemotherapy and are summarized in **Table 8** (page 40).

Table 8. List of ongoing clinical trials targeting pMMR–MSI-L colorectal cancer

Checkpoint inhibitor	Trial type	Combination treatment (target)	Trial identifier
Atezolizumab	• Phase I • mCRC	Cobimetinib (MEK) and bevacizumab (VEGFA)	NCT02876224
	• Randomized phase II • Refractory CRC	Capecitabine and bevacizumab (VEGFA)	NCT02873195
	• Phase III, mCRC	Cobimetinib (MEK) and regorafenib	NCT02788279
	• Phase II • First-line metastatic CRC	Cobimetinib (MEK)	NCT02291289
Durvalumab	• Phase I/II • Refractory CRC	Cediranib (VEGFR and KIT)	NCT02484404
Durvalumab± tremelimumab	• Phase I, mCRC	Radiation	NCT02888743
	• Phase II, mCRC	Radiation or ablation	NCT03122509
	• Phase II, mCRC	Radiation	NCT03007407
Durvalumab	• Phase II, mCRC	Trametinib (MEK)	NCT03428126
	• Phase II, mCRC	Azacitidine (DNMT)	NCT02811497
Nivolumab	• Phase I/II • CRC and solid tumours	Epacadostat (IDO1)	NCT02327078
	• Phase I/II • Locally advanced rectal cancer	Chemoradiation	NCT02948348
	• Phase II • Refractory CRC	TAS-102	NCT0280546
Nivolumab± ipilimumab	• Phase II • Refractory CRC	• Cobimetinib (MEK) • Daratumumab (CD38)	NCT02060188
	• Phase I/II • Metastatic pretreated CRC	Binimetinib (MEK)	NCT03271047
	• Phase II • CRC arm	Radiation	NCT03104439
	• Phase I/II • Metastatic pretreated CRC	Trametinib (MEK)	NCT03377361
	• Phase II • RAS-wild-type CRC	Panitumumab (EGFR)	NCT03442569
	• Phase II • Stage 1–3 CRC	Celecoxib (COX2)	NCT03026140
Pembrolizumab	• Phase I • Metastatic pretreated CRC	Oral azacitidine (DNMT) and romidepsin (HDAC1 and/or HDAC2)	NCT02512172
	• Phase Ib • mCRC	• Binimetinib (MEK) • ±FOLFOX or FOLFIRI	NCT03374254
	• Phase I/II • mCRC	Nintedanib (VEGFR, PDGFR and FGFR)	NCT02856425
	• Phase I/II • Refractory CRC and NSCLC	Azacitidine (DNMT) and epacadostat (IDO1)	NCT02959437
	• Phase Ib/II • Metastatic pretreated CRC	Cetuximab (EGFR)	NCT02713373
	• Phase II • GI tumors and CRC arm	Tumour-infiltrating lymphocytes, IL-2, cytoxan and fludarabine	NCT01174121
	• Phase II, mCRC	Binimetinib (MEK), FOLFOX and FOLFIRI	NCT03374254
PDR001	• Phase I • First-line metastatic CRC	FOLFOX and bevacizumab (VEGFA)	NCT03176264
	• Phase I • Metastatic pretreated CRC	Regorafenib (multikinase)	NCT03081494
Avelumab	• Phase II	eFT508 (MNK)	NCT03258398

Source from [149] with original data from *https://clinicaltrials.gov/ct2/home*

2. Colorectal Tumor Microenvironment

Like all other solid cancers, CRC cells possess all hallmarks of cancer [80] with a specific tumor microenvironment (TME) [156-160]. TME basically consists of cellular and non-cellular components. The main cellular components of colorectal TME are fibroblasts, cancer associated fibroblasts (CAFs) [161, 162], mesenchymal stem cells (MSCs), CRC non-stem and stem cells [163], and a complex inflammatory network with multiple immune cell types (**Figure 7**, page 41) [164-167]. However, unlike other cancers, colorectal TME contains numerous intestinal microorganisms, named gut microbiota that are commonly found in mucosal layer of intestine [161, 167, 168]. Non-cellular components are referred to extracellular matrix (ECM). To date, over hundred molecules of ECM have been identified in CRC tumors, which are classified into several groups such as glycoproteins, collagens, proteoglycans, ECM regulators, ECM-affiliated proteins, and secreted factors [169, 170]. The roles of some key components of CRC TME will be discussed in this section.

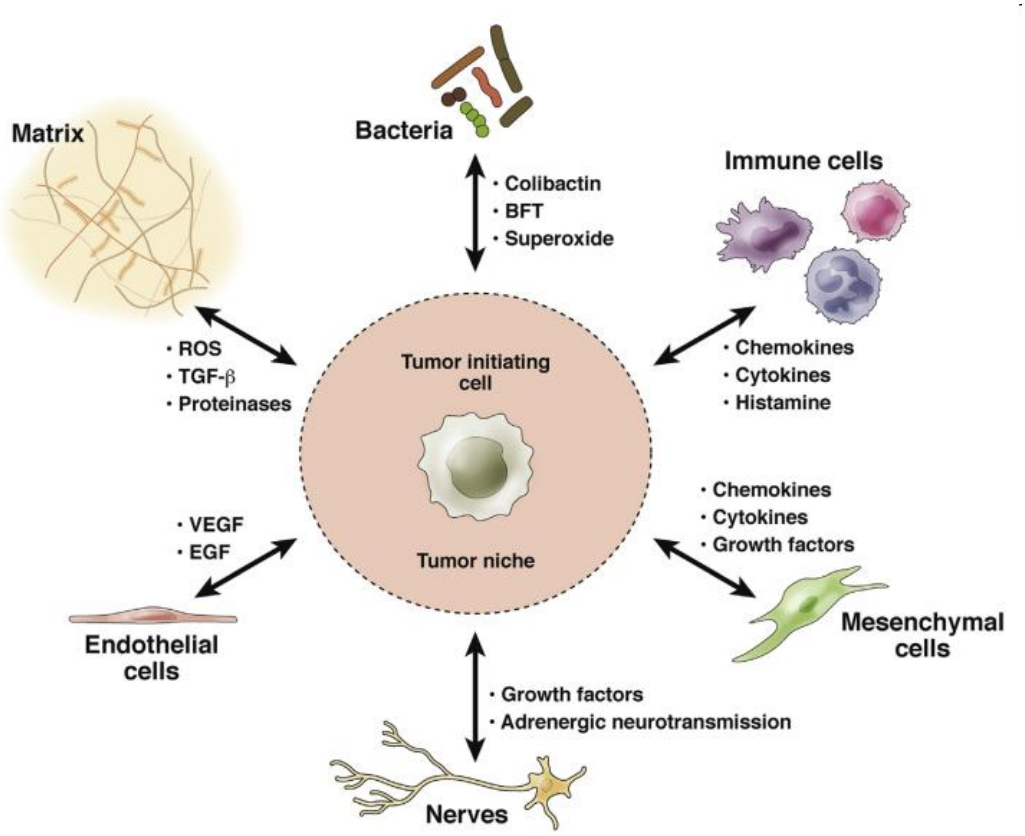

Figure 7. The overview of the crosstalk between colorectal tumor cells with other TME components in the tumor niche. *ROS: reactive oxygen species, BFT: Bacteroides fragilis, TGF-β: transforming growth factor-β, VEGF: vascular endothelial growth factor, EGF: epidermal growth factor* ***[161]****.*

2.1 Fibroblasts and CAFs

Fibroblasts are non-epithelial, non-vascular, and non-immune cells located in the fibrillar matrix of connective tissues [171, 172]. They are essential constituents of gastrointestinal tract, which form a cellular network under the epithelial basement membrane [173]. Fibroblasts not only play multiple roles in physiological conditions, but also contribute to pathogenesis. As main regulators of the ECM, fibroblasts mediate cell growth and differentiation through paracrine and juxtracrine signaling, and response to wound healing or tumorigenesis [18,20].

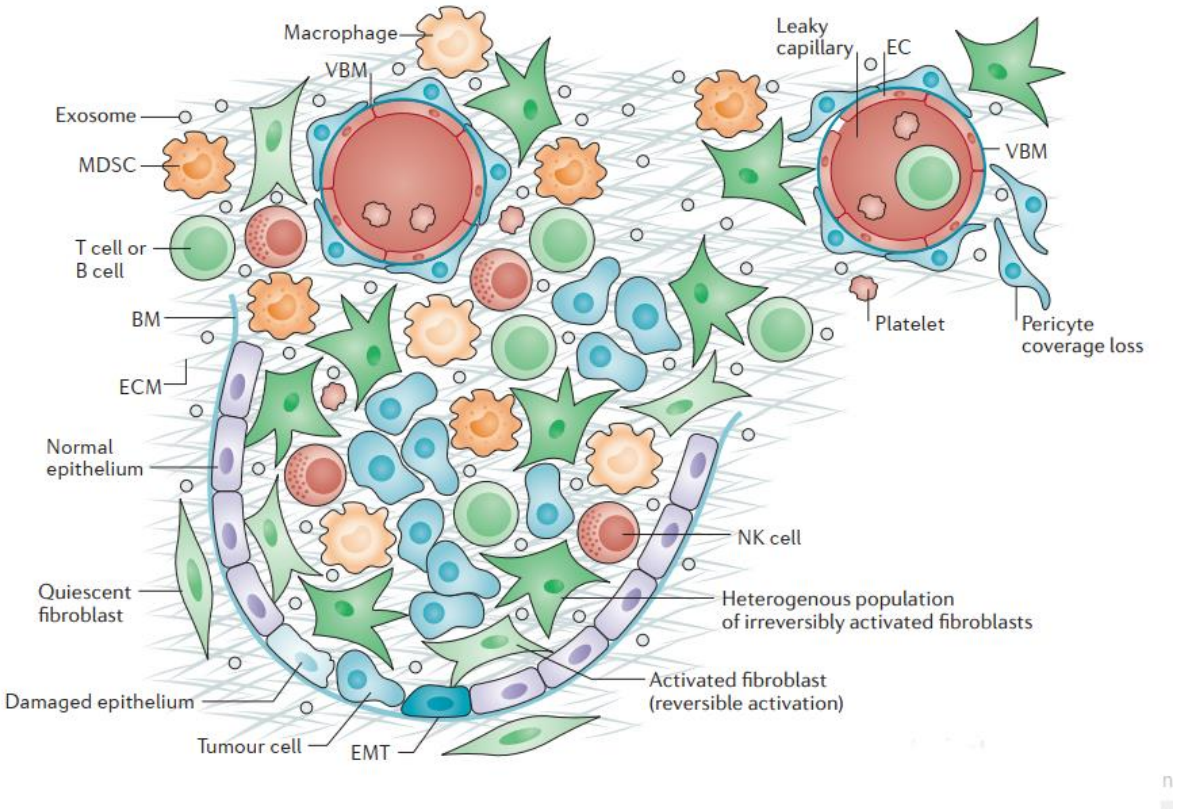

Figure 8. Fibroblasts heterogeneity in tumor microenvironment. *ECM: extracellular matrix, BM: basement membrane, VBM: vascular basement membrane, EMT: epithelial to mesenchymal transition, CAFs: cancer-associated fibroblasts, EC: endothelial cell, NK: nature killer. Source from [172].*

CAFs basically corresponded to all fibroblasts, which acquired phenotype modifications within TME and surrounding cancer cells (**Figure 8**) [171, 174]. CAFs population remains poorly specified regarding their origin, subtypes, and biology due to a high heterogeneity and a lack of specific markers [172, 175]. However, numerous studies have demonstrated that CAFs have emerged as important regulators of tumor progression. For instance, it has been shown that CAFs stimulate epithelial cell growth, maintain cancer stem cells and promote

tumorigenesis and cancer development, including in CRC [176-184]. Indeed, EGF family proteins secreted by hepatic myofibroblasts and colon cancer-attached CAFs promote cancer progression through ErbB receptors activation [181, 185]. Moreover, hepatocyte growth factor (HGF) secreted by CAFs is involved in maintaining colon cancer stem cells by enhancing Wnt signaling [177]. Fibroblasts have been shown to increase colon cancer cell proliferation and liver metastases [178, 186, 187]. In addition, stanniocalcin-1 secretion by PDGF-stimulated CAFs c can promote colorectal cancer cell intravasation and distant metastases [188].

2.2 Inflammatory microenvironment of colorectal tumors

In CRC tumor microenvironment, a network of immune cells have been well identified, which includes both innate and adaptive immune cells as well as gut microbiota (**Figure 9**, page 44). The innate immune cells comprise tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) or immature myeloid cells (iMCs), neutrophil granulocytes, dendritic cells (DCs), and mast cells (MCs) while the adaptive immune populations consist of T and B cells [189]. It is well known that immune cells are both involved in cancer progression and in anti-tumor properties. In fact, several innate immune cells (TAMs, MCs, neutrophils, and MDSCs) and adaptive immune cells such as T-helper-17 and -2 (Th17 and Th2) have shown to promote tumorigenesis and cancer development through the production of cytokines, growth factors, enzymes, and angiogenic mediators [189]. The density of T cells in colon tumors has been reported to be associated with patient outcomes, and was considered as a powerful prognostic indicator [190, 191]. Indeed, the high density of cytotoxic and memory T cells in tumor cores or at the margin of invasive tumor sites is predictive of a better response to chemotherapy in patients with liver metastatic from colon cancer [192]. In addition, the increasing number of M1 and M2 macrophages infiltrated in the TME correlates with the improved survival rate among CRC patients [193]. Interestingly, the presence of regulatory T (Treg) cells in tumors is a favorable prognostic indicator in patients with CRC [194, 195]. Notwithstanding, innate immune cells such as T-cell subsets were reported to promote CRC development. Similarly, high density of innate lymphoid cells was also known to correlate with colorectal tumorigenesis through interleukin-22 secretion. Whereas, the accumulation of Th17 cells at tumor sites has been correlated with poor overall survival in CRC patients [195]. The role of the gut microbiota in CRC will be discussed in the next section.

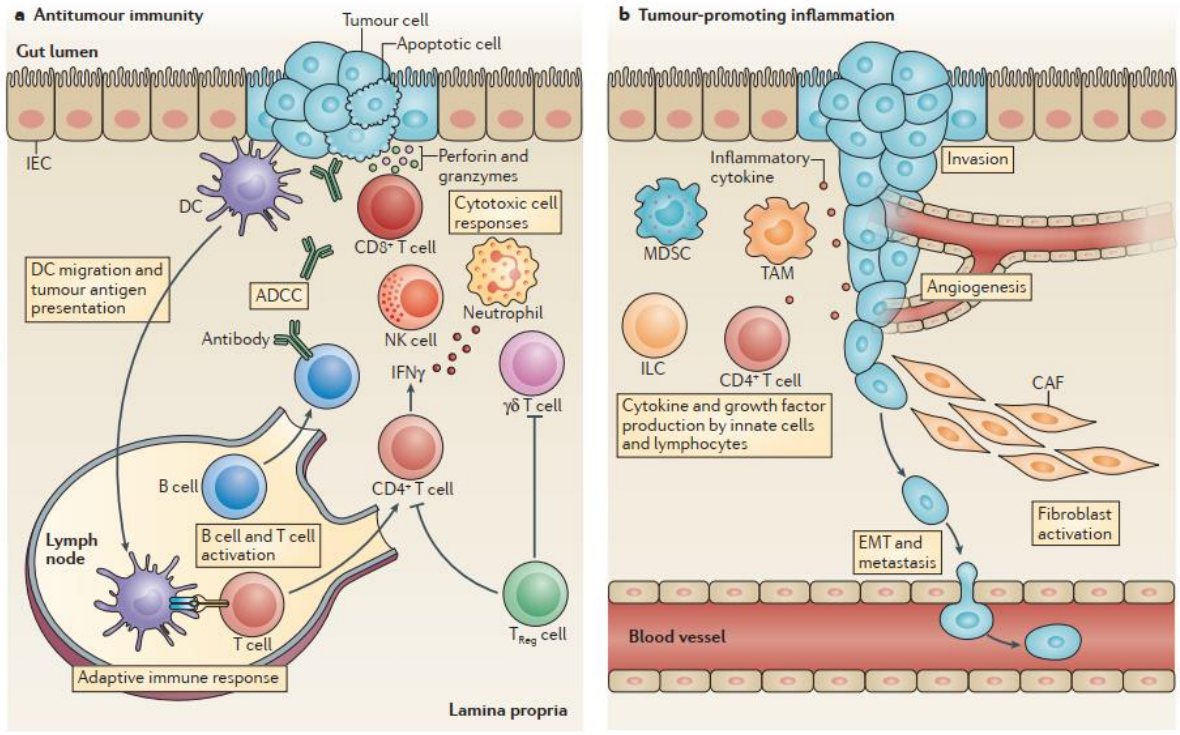

Figure 9. The immune response in colorectal cancer. *(a) The schema represents the antitumor immunity in CRC with the contribution of dendritic cells (DCs), tumor-specific B cells and T cells, Neutrophil, and Nature killer cells. In the contrary, (b) tumor promoting properties of immune cells are driven by CD4+ T cells, innate lymphoid cells (ILCs) and tumor-associated macrophages (TAMs) under gut microbial stimuli (not shown). Source from [167].*

2.3 Gut Microbiota in Colorectal Tumor Microenvironment

Microorganisms living in intestine are also known as gut microbiota and/or microbiome. These populations include both archaea, fungi, protozoa and viruses [196] that play vital roles in maintaining physiological conditions of the host. However, unbalanced growth or being invasive by harmful bacteria result in pathogenesis such as chronic inflammation, tumorigenesis, malignant transformation, etc [168, 197, 198]. In fact, several studies have indicated that specific alterations in the gut microbiome (termed as CRC gut microbiota) associated with CRC. In CRC gut microbiota, protective species such as *Roseburia* was found significantly less abundant while pro-carcinogenic species such as *Fusobacterium, Bacteroides, Escherichia,* and *Porphyromonas* were reported more abundant [199-201]. Interestingly, familiar pathogenic species such as *Shigella, Salmonella, Cronobacter, and Citrobacter* were abundantly found in normal adjacent tissues, but were rarer in colorectal tumor sites [202]. One of the most important findings was attributed to the clarification of *Fusobacterium spp* involvements in CRC [202]. In terms of geography, although the variations in gut microbiota were reported among populations [203, 204], several meta-analysis studies

have consistently indicated the involvement of gut microbiota in CRC across populations in different continents and territories [205, 206]. Indeed, a set of 29 core species has been identified to be significantly enriched in intestine of patients with CRC [207]. In colorectal adenomas, precursors of major CRC, modifications in ecological enrichment and alterations of microbial populations have been observed. For instance, *Fusobacterium* was abundantly found in patients with colorectal adenomas. Similarly, *F. nucleatum and Solobacterium moorei* were reported to be enriched across stages of colorectal carcinogenesis. However, in patients with multiple adenomas or intramucosal carcinomas, *Atopobium parvulum and Actinomyces odontolyticus* populations were only enhancing at early stages of carcinogenesis [208-211]. As mentioned above, viruses and fungi are also components of gut microbiota as well. However, the correlation of these components on CRC development remains unclear and controversial.

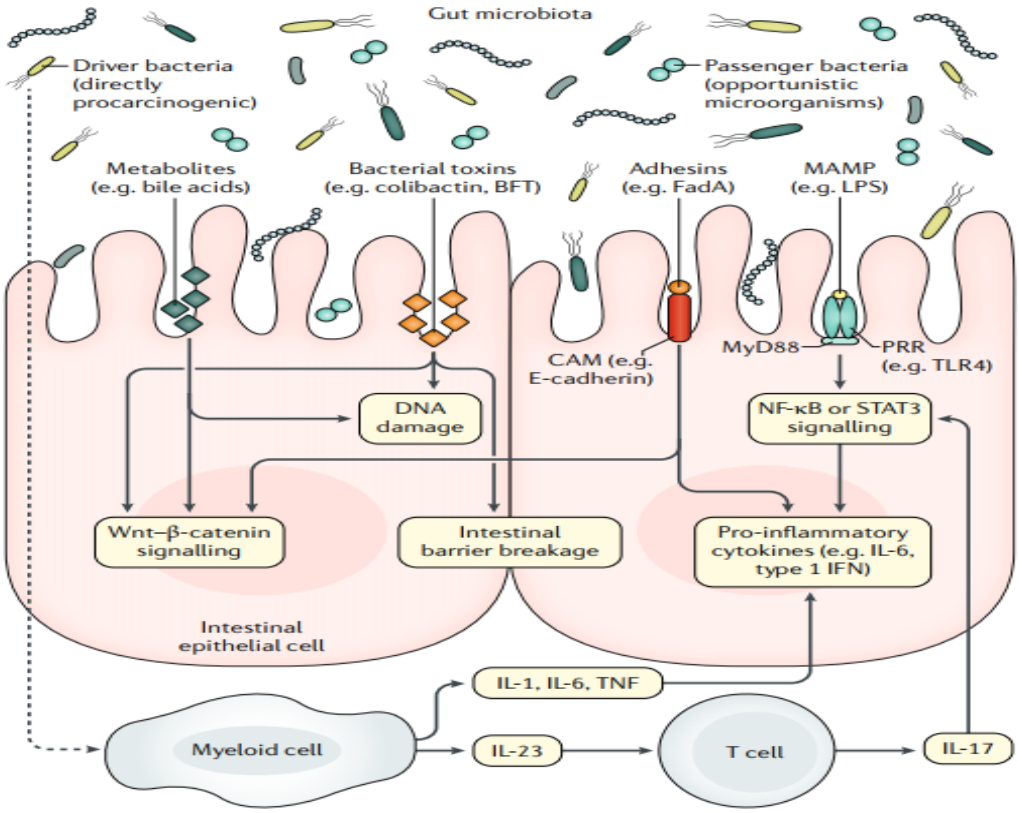

Figure 10. The association of gut microbiota in colorectal carcinogenesis. *The gut microbiota may participate in several colorectal carcinogenesis processes such as inflammatory pathways or metabolic pathways. In other cases, some bacteria can directly drive procarcinogenesis through stimulating cell proliferation in tumor microenvironment. BFT: Bacteroides fragilis toxin; CAM: cell adhesion molecule; FadA: Fusobacterium adhesin A; IFN: interferon; LPS: lipopolysaccharide; MAMP: microbe-associated molecular pattern; NF-κB: nuclear factor-κB; PRR: pattern recognition receptor; STAT3: signal transducer and activator of transcription 3; TLR4: Toll-like receptor 4. Source from [168].*

The role of gut microbiota in promoting colorectal carcinogenesis is complicated, which is partly explained by Tilg and collaborators [212] and described in **Figure 10** (page 45).

2.4 Extracellular Matrix of Colorectal Tumor Microenvironment

Composed of hundreds of different building blocks, the extracellular matrix (ECM) makes up the complex and dynamic, highly cross-linked, three-dimensional (3D) network of macromolecules that surround cells. The main components of ECM consist in collagens, laminins, fibronectins, glycosaminoglycans, and proteoglycans [213-218]. The composition and functions of ECM are different and highly dependent on the organization of tissue types [213-215, 217, 218]. In colorectal tissues, the main constituents of ECM are type I collagen (COL1A1, COL1A2), proteoglycan, perlecan, laminin, fibronectin, and nidogen [219]. Similarly, the basement membrane (BM), a highly specialized ECM structure, which physically separates mucous layers, contains the same components as previously, with the exception of type I collagen, which is substituted by type IV collagen [219].

In CRC, the remodeling and changes in ECM have been well characterized. Loss of BM integrity in colorectal primary tumors correlates with a higher metastatic potentiality and poor prognosis [220-222]. Extensive collagen remodeling including modification of collagen density and fiber alignment was reported in colorectal malignant samples [223]. In addition, collagen degradation was observed by the detection of collagen triple helical fragments in urine of patients with metastatic CRC [224]. The alteration of ECM protein expression was found in colorectal tumor samples [170, 225, 226]. Naba and collaborators have reported a specific expression of several common membrane-anchored proteins such as ADAM 9, 10, TSL1, and MMP1, 2, 9, 11, 12, as well as numerous unfamiliar ECM proteins in primary colon tumors, compared to normal tissues [170]. Indeed, high MMP-1, -2, -7, -9, and -13 levels in colorectal tumors were reported to correlate with worse clinical outcomes [227]. Moreover, some insoluble ECM proteins including myosin, keratin, and type IV collagen (a1, a2, and a3) were down-regulated in tumor biopsies of patients with CRC [228]. Unfortunately, the levels of some key growth factors in ECM of colorectal tumors such as VEGFA, FGFs, PDGFB, and TGFβ have not been fully identified so far.

II. Type I collagen receptors

1. Integrins

Integrins are membrane heterodimeric receptors that non-covalently combined of 18 α- and 8 β- subunits [229, 230]. In human, integrins enable to form 24 heterodimers [231, 232]. However, type I collagen-binding integrins are represented by four heterodimers of α1β1, α2β1, α10β1, and α11β1 which are able to recognize the GFOGER motif of type I collagen [233]. In tissue homeostasis, α2β1 integrin is responsible for thrombus formation while α10β1 is supposed to be important for cartilage formation. α11β1 integrin is necessary for tooth eruption [232]. However, in pathological situations, α1β1 and α2β1 integrins may be required for initiating immune response while α10β1 and α11β1 integrins are promising molecular targets for modulating regenerative processes (wound healing, cartilage repair). In tumor progression, α1β1 and α2β1 integrins are widely expressed by different tumors and stromal cells, including cancer-associated fibroblasts (CAFs). Recent studies have shown that α1β1 expression in cancer cells promotes tumorigenesis, invasion and metastasis [234, 235]. Pozzi et al. have reported that α1β1-integrin expression in endothelial cells promotes angiogenesis [236]. Enhancing α1 integrin expression was found in the majority of colorectal tumors suggesting a role for α1β1 in colorectal cancer progression [237, 238]. By contrast, loss of α2β1 integrin expression was reported to be associated with a poor prognosis in patients with breast carcinoma [239]. Recent studies have suggested that α10β1 integrin also potentially contributes to tumor progression [240, 241]. Lu et al. have shown that α11β1 integrin, expressed in fibroblasts, was able to promote tumorigenesis, migration, and invasion in lung adenocarcinoma [242]. Moreover, recent studies also have shown a role of this integrin in the collagen fiber remodeling [243, 244], as a consequence of the promotion of tumor growth and metastasis [244].

2. Discoidin Domain Receptors

Discoidin Domain Receptors (DDRs) are type I membrane tyrosine kinases receptors (***Figure 11***) [245]. DDRs have been identified and described by several groups in the early 1990s [246-252]. This tyrosine kinase receptor family consists of two members DDR1 and DDR2 [253]. *DDR1* gene, due to alternative splicing, encodes five different isoforms, including DDR1a, DDR1b, DDR1c, DDR1d, and DDR1e. However, *DDR2* gene encodes for unique receptor known as DDR2 [254-257]. DDRs were considered as orphan receptors until 1997. However, two independent studies later demonstrated that several types of collagen are

functional ligands of DDRs [258, 259]. In fact, the studies have also shown that DDRs are activated *via* dimerization and tyrosine phosphorylation upon type I collagen stimulation. They were the first membrane receptors of collagen harboring a kinase function [258, 259].

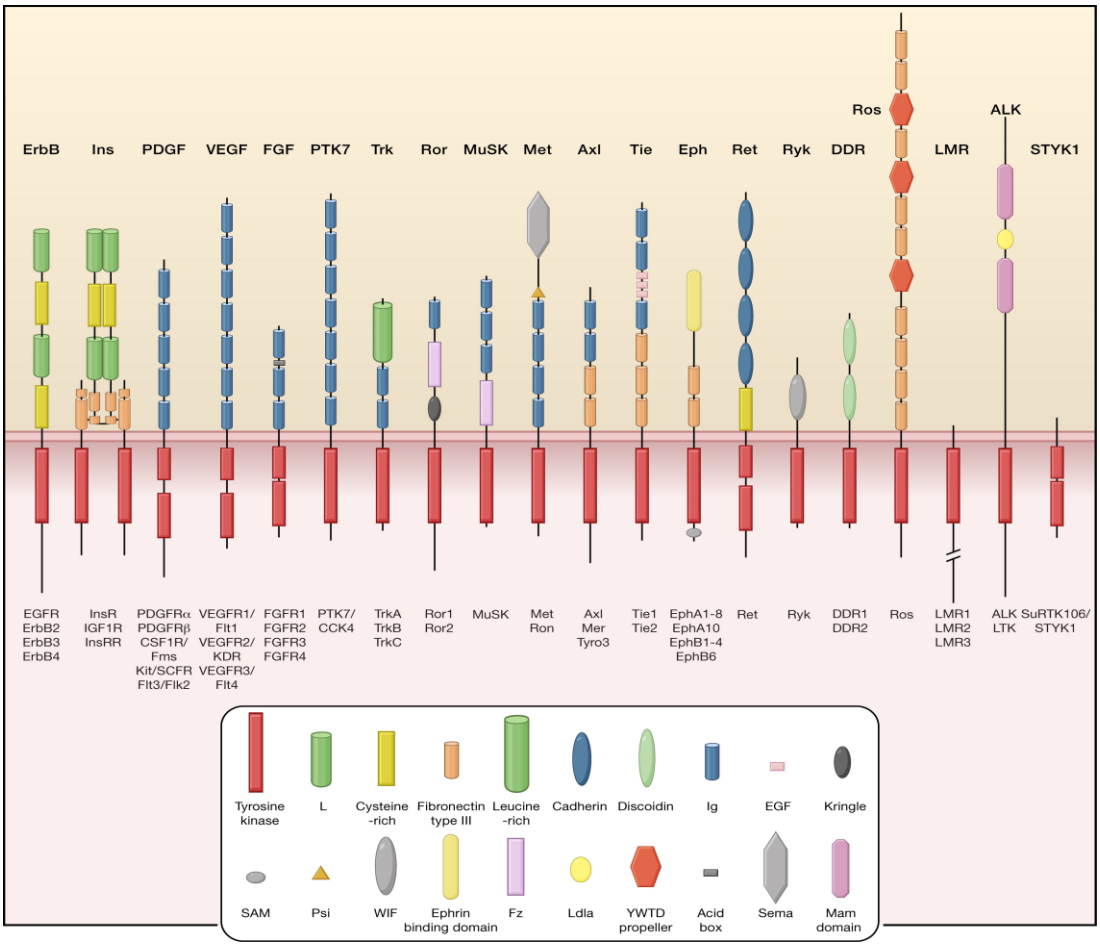

Figure 11. The overall structures of Receptor Tyrosine Kinase families *[245].*

2.1 The general structure of DDRs

DDRs consist of three crucial domains comprising an extracellular domain, a transmembrane domain, and an intracellular domain containing tyrosine kinase (***Figure 12***, page 49). In the extracellular domain, the N-terminal discoidin domain is able to interact with various types of collagen [260]. The juxtamembrane (JM) region makes up of about 50 and 30 amino acids for DDR1 and DDR2 respectively. The single transmembrane (TM) domain connects the extracellular domain with the cytoplasmic domain with up to 169 and 140 amino acids for DDR1 and DDR2 respectively(***Figure 12***, page 49) [257, 261]. The catalytic domains of DDR1 and DDR2 in the cytoplasmic domain contain 15 tyrosine residues and 14 tyrosine residues respectively [257].

Among the five isoforms of DDR1, only three isoforms, including DDR1a (97 kDa), DDR1b (101 kDa), and DDR1c (102 kDa), are encoded as full-length functional receptors while DDR1d (56 kDa) and DDR1e (86 kDa) are truncated encoding as a result of non-functional receptors [257].

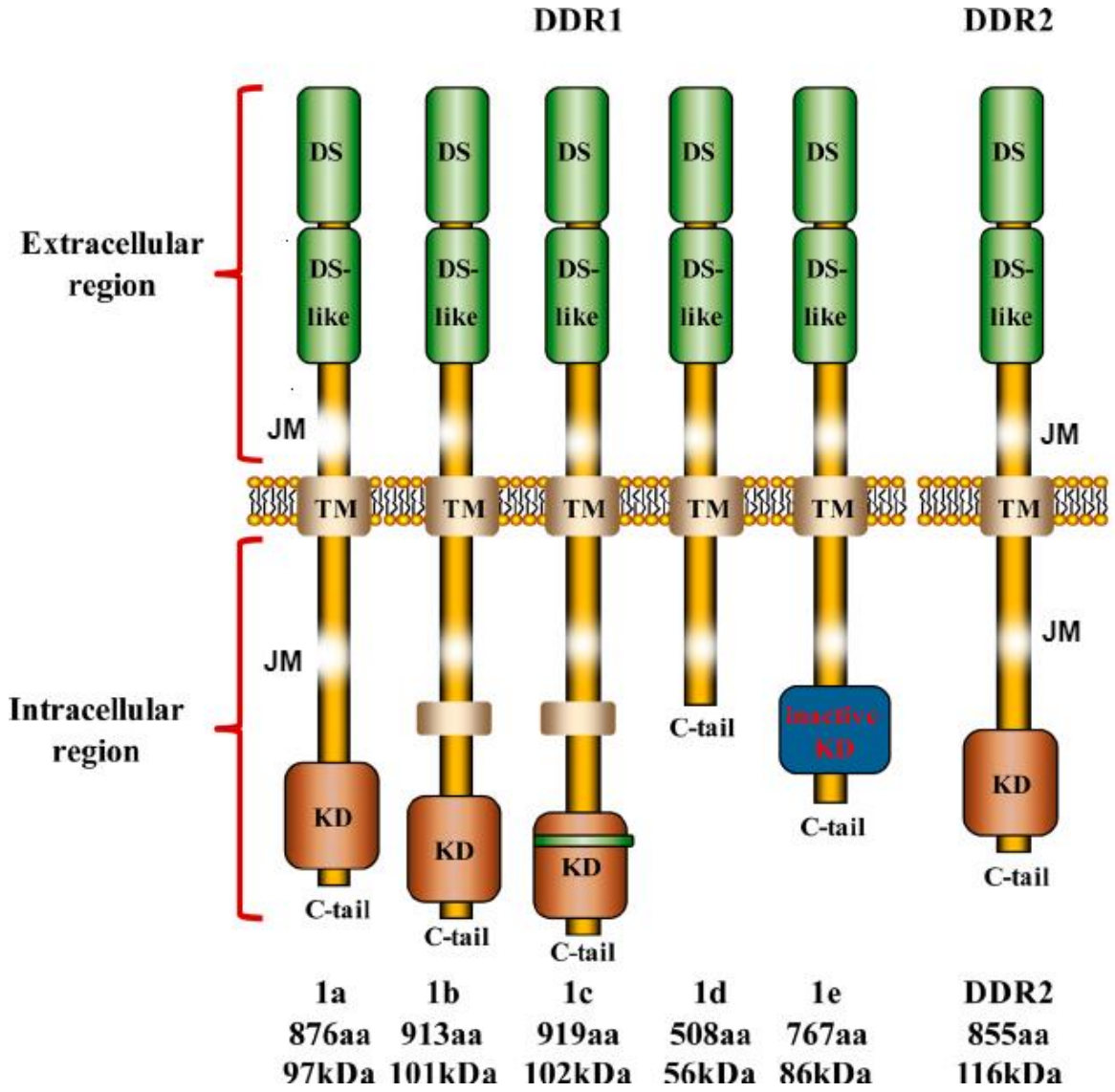

Figure 12. General Structure of DDRs*. The extracellular domain contains a discoidin subdomain (DS), a discoidin-like domain (DS-like), and an extracellular juxtamembrane region (JM). TM is for transmembrane domain. The intracellular domain contains an intracellular juxtamembrane region (JM) and a tyrosine kinase domain in DDR1a, DDR1b, DDR1c, DDR1e, and DDR2 [261].*

2.2 Collagen-binding site for DDRs

Both DDR1 and DDR2 are activated by fibrillar collagens such as type I collagen [258, 259]. However, there are some differences to distinguish the two receptors. In fact, only DDR1 can recognize non-fibrillar type IV collagen [258, 259, 262] while type X collagen likely activate DDR2 only [262, 263]. Besides, DDR1 can be activated by type VIII collagen, and this is not the case of DDR2 [264]. Many studies indicated that DDRs can be activated by almost collagen form, including fibrillar collagens [257, 260, 265], soluble collagen [258, 265, 266] or triple-helical peptides [265, 267, 268]. DDRs recognize collagen through its discoidin domain (DS) (**Figure 12**, page 49) [269]. The identification of the collagen-binding sequence of DDRs has been identified by using a collagen peptide library, known as a collagen Toolkit [270]. It is well documented that both DDRs can recognize the triple-helical collagen peptides containing GVMGFO motif (O for hydroxyproline) [271].

2.3 Mechanisms of DDR activation/phosphorylation

It is widely known that all RTKs typically undergo receptor auto-phosphorylation upon binding to their ligands [245, 272]. Unlike most RTKs, this process is unusually slow (up to 2 hours) and sustained (up to 18 hours) for DDRs [258, 259]. Collagen stimulation has been found to induce DDR1 aggregation into clusters, which further processes DDR1 internalization into early endosome vesicles. The internalized receptors are then recycled back to the cell surface instead of being digested in endosome (**Figure 13**, page 50) [273].

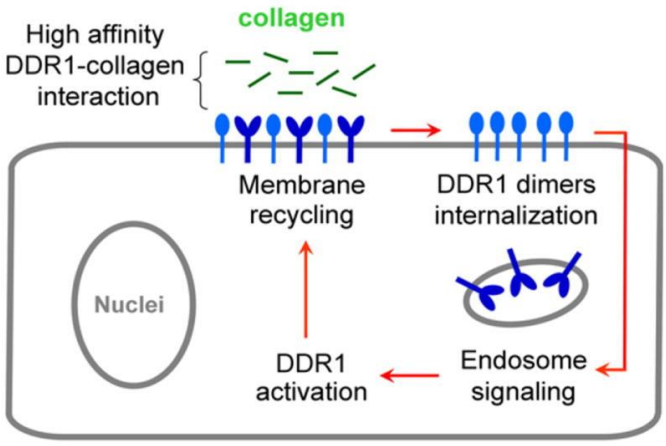

Figure 13. Proposed model of DDR1 activation mechanism. *DDR1 locates on the cell surface in both monomer and dimer forms. The high-affinity interaction between DDR1 and collagen induces DDR1 dimer internalization, followed by the activation of the endosomal signaling cascades, which further lead to activate the dimers. Activated DDR1 dimers are then recycled to plasma membrane [273].*

2.4 DDR Signaling Pathways

Cell signal pathways triggering by DDRs depends generally on the cell type and the nature of the collagen [258, 259, 274]. DDR1 is known to regulate several downstream signaling pathways in cancer cells. Some of them are represented in **Figure 14**. For instance, DDR1 activation by type IV collagen has been shown to be associated with the regulation of cyclooxygenase 2 (COX2) expression through activation NF-κB, and to induce cell survival in human breast cancer cells [2]. Several studies indicated that DNA damage induced by γ-irradiation or chemotherapeutic agents trigger p53 mediating DDR1 expression in cancer cells and chemo-resistance [2, 6, 275]. DDR1 expression in activated T cells is regulated by the ERK/MAPK signaling pathway and is involved in cell migration in 3D collagen [276, 277]. In macrophages, DDR1 activation by collagen induces an increase in the expression of nitric oxide synthase and the subsequent production of nitric oxide. This process requires the activation of NF-κB, c-jun N-terminal kinase (JNK), and p38 MAPK [278]. A very recent study has demonstrated that activated DDR1 can be translocated to nucleus and plays a role as a transcription factor regulating collagen synthesis and thus a key function in renal fibrosis [279].

DDR1 has also been reported to induce ERK1/2-MAPK cell signaling pathway, cell adhesion and proliferation in mesangial cells [280]. Similarly, DDR1-induced ERK1/2-MAPK phosphorylation mediates human vascular smooth muscle cells (SMCs) migration through a Src-mediated signaling pathway [281].

In the case of DDR2, Iwai *et al.,* (2013) had pointed out a direct crosstalk between DDR2 and the insulin receptor which enhances DDR2 phosphorylation in the presence of collagen [8]. Upon activation, DDR2 has been demonstrated to induce smooth muscle cell migration through activation of p38 MAPK pathway [282]. Several studies have shown that ERK1/2 and p38 MAPK are targeted by DDR2 to induce the transactivation of the transcription factor Runx2 and osteoblast differentiation and chondrocyte maturation [283, 284]. DDR2 was shown also to be involved in interleukine-12 (IL-12) production upon activation by type I collagen *via* NF-κB and JNK pathways [285]. In addition, studies had shown that DDR2 mediates the induction of IL-12 and T-cell reaction suggesting its role in immune response [286, 287]. A recent study indicated that DDR2 is considered as a collagen-aging sensor to regulate tumor cell growth. Upon activation by young collagen, DDR2 triggers SHP-2 phosphatase, which further dephosphorylate p-ERK1/2 signaling pathways in order to upregulate p21 expression and consequently abrogate cell proliferation [15]. In the case of old collagen, DDR2 is less

activated, which is unable to inhibit ERK1/2 phosphorylation, leading to a downregulation of p21 expression and a higher level of cell proliferation.

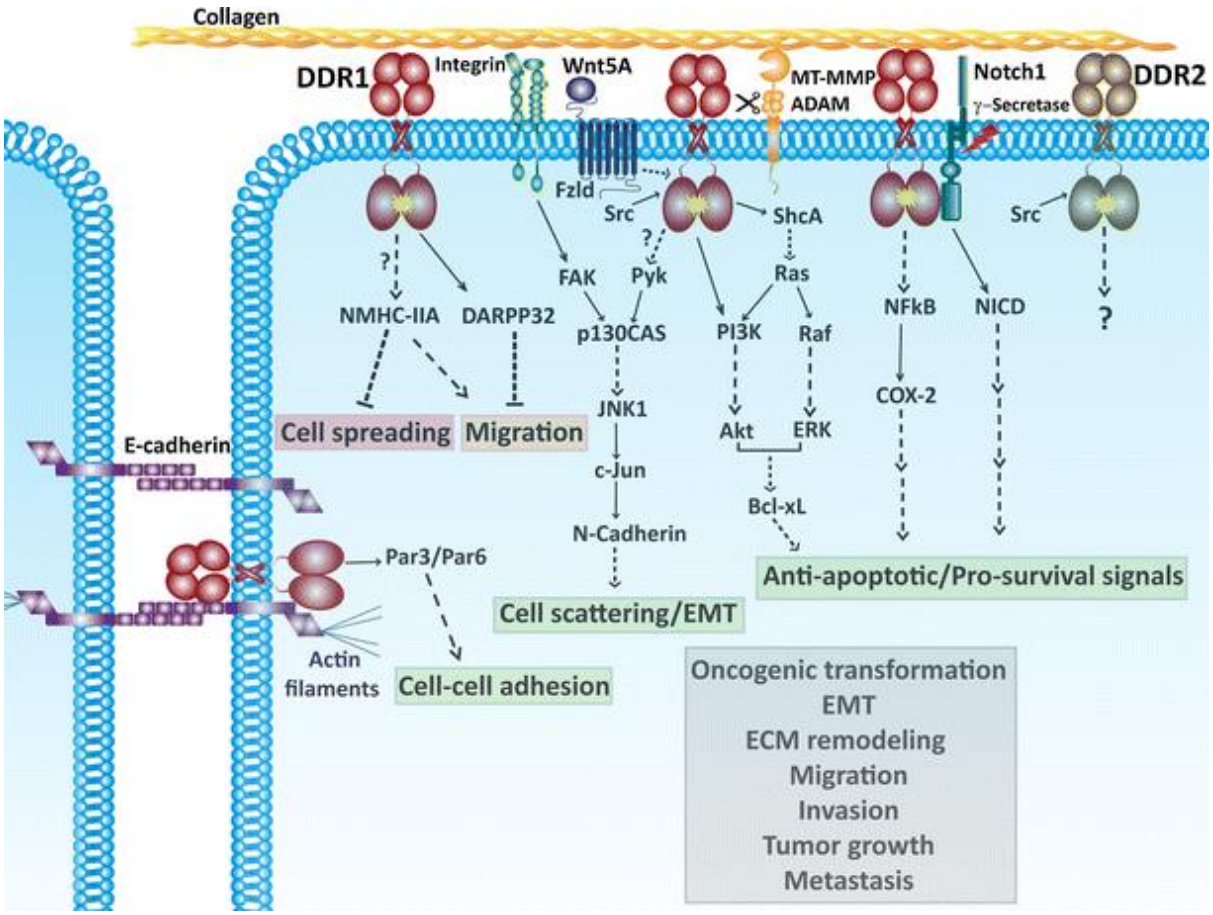

Figure 14. DDRs initiate several signaling pathways *[257].*

2.5 DDRs and cancer

2.5.1 DDRs expression and mutations in cancers

Analysis of DDR1 and DDR2 expression at mRNA and protein levels in different human tumor samples revealed that the association between DDR expression and tumorigenesis is controversial. In many some tumors, the increase of DDR1 or DDR2 expression correlates with a poor prognosis [257].

In breast cancer, DDR1 overexpression has been observed in primary and lymph nodes metastasis samples [288]. Other studies have shown a high level of DDR2 expression in invasive breast tumors, compared to normal tissue [289, 290]. In addition, Toy and collaborators have reported recently that DDR1 and DDR2 are coordinately deregulated in basal-like breast tumors [291]. Such observation is supported by a recent study published by

Werb's group showing that DDR1 expression is downregulated during epithelial-mesenchymal transition (EMT) [292].

DDR1 expression appears to be increased in other tumors. In fact, DDR1 expression is increased in 100% of patients carrying primary and metastatic brain tumors [293], and in 61 % and 64% of patients with primary and invasive lung adenocarcinoma respectively [294]. In renal cancer, DDR1 overexpression was shown to be involved in EMT suggesting that DDR1 is a promising biomarker for prognostic and target therapy in this type of tumor [295]. In gastric cancer, the presence of DDR1 was correlated with the expression of the EMT markers [296]; and DDR1 silencing results in an inhibition of cell migration, invasion of such tumor [297]. On the contrary, in an agreement with Werb's group for breast carcinoma, a recent study has shown a loss of DDR1 expression during EMT process in ovarian cancer cells by a CpG methylation process at the DDR1 promoter [298].

DDR1 and/or DDR2 mutations are much less common. To date, most of DDR mutations have been observed in lung cancer [299]. DDR2 kinase domain mutations were found in 3.8% of squamous cell lung carcinomas, which have been identified as a novel potential therapeutic target of the FDA-approved drug dasatinib [3].

2.5.2 Roles of DDRs in Cancer Cell Proliferation

In the last decade, several studies have investigated whether DDRs regulate cell growth. However, this function remains controversial. In fact, both DDR1 and DDR2 can either promote or suppress cell proliferation in tumors and such effect dependent on the nature of the pathology and composition of the stromal microenvironment [300]. A recent study has reported that pharmacological inhibition of DDR1 is able to reduce the proliferation of breast carcinoma [301]. By using DDR1-IN-1, a specific inhibitor of DDR1, Kim and collaborators have shown that such inhibition was able to induce a decrease in proliferation of solid tumors of several organs, including breast, lung, bone, colorectal, and uterus [302]. A recent study has shown that treatment of human lung adenocarcinoma xenograft with combined inhibition of DDR1 with dasatinib and Notch signaling induces inhibition of cell proliferation and an increase in apoptosis [303]. DDR1 knockdown has been also reported to reduce cell proliferation in several breast cancer cell lines *in vitro* [304-306]. *In vivo,* DDR1 knockdown has been shown to induce the expression of the transforming growth factor-β (TGF-β1) and to inhibit the growth of pancreatic tumor xenograft [7]. DDR1 overexpression promotes cell proliferation of gastric carcinoma and osteosarcoma cells of cancer cells *in vivo* [296, 307].

For DDR2, the S131C mutation in the receptor was shown to contribute to cell proliferation and invasion of squamous cell lung cancer (SCC) [308]. In addition, Kim *et al.* have reported that DDR2 inhibition results in an inhibition of cell proliferation and invasion in lung carcinoma [4].

2.5.3 Effects of DDRs on Cancer Cell Survival and Apoptosis

In breast cancer, DDR1 knockdown has been shown to reduce cell survival of MDA-MB-435 breast carcinoma cells [309]. DDR1 has been shown also to induce cell survival and resistance to chemotherapy in breast carcinoma [2]. A study carried on colorectal cancer has demonstrated that p53 induction by DNA damage was able to increase DDR1 expression. Consequently, this allows Ras/Raf/MAPK signaling pathway triggering, resulting in cell survival and resistance to apoptosis [6]. Conversely, DDR1 has been identified as a key factor in the induction of apoptosis by 3D matrix collagen in breast carcinoma by inducing an increase in the pro-apoptotic protein BIK [9, 16, 310].

DDR1 knockdown has been shown to induce a decrease in cell survival and collagen-induced resistance to etoposide in Hodgkin lymphoma cells [1]. A very recent study has shown that upon activation by collagen, DDR1 drives therapy resistance by modulating autophagy [311, 312].

2.5.4 Roles of DDRs in EMT and cell invasion

To date, many studies have shown the involvement of DDRs in cancer cell migration and invasion. For instance, a study has shown that DDR1 is required for invasion process of the basal-like breast cancer cells by inducing linear invadosomes [313, 314]. However, overexpression of DDR1 has been shown to reduce the invasive phenotype of the same breast carcinoma cells [315]. In gastric cancer, DDR1 overexpression has been shown to promote cell migration and invasion [296]. Moreover, DDR1 expression level was also correlated to the expression of EMT markers such as vimentin and Snail [296]. In addition, DDR1 silencing has been reported to inhibits multiple steps of metastasis cascade in gastric cancer [297]. In glioma, DDR1 was found to be able to promote cell invasion in association with matrix metalloproteinase-2 [316]. Moreover, DDR1 has been shown promotes cell invasion and to be associated with poor prognosis in non-small-cell lung cancer [317]. A recent study published by Serge Roche has demonstrated that DDR1 is able to phosphorylate BCR to maintain β-catenin transcriptional activity which is necessary for colon carcinoma cell invasion [12, 13].

In the case of DDR2, Zhang *et al.* have demonstrated a key role of DDR2 in supporting breast carcinoma migration and invasion through collagen matrix [290]. In hypoxic conditions, upregulation of DDR2 expression has been observed in several breast carcinoma cell lines. This supports the role of DDR2 in the positive regulation of cell migration and invasion [318]. Interestingly, DDR2 expression in CAFs also enhances collective invasion of metastatic breast carcinoma cells [319]. In metastatic murine melanoma, DDR2 inhibition results in a reduction of migration and invasion by suppressing MMP2/9 expression through downregulation of ERK/NF-κB signaling pathway [320].

Concerning EMT, it is well known that this process is important for the reactivation of the expression of several actors playing a crucial in metastasis program. Among these actors, we can cite Snail-1, which is involved in the invasion process of basal-like breast carcinoma cells [321]. Accordingly, some studies have shown that DDRs are involved in the EMT process. Whereas DDR1 activation by type I collagen has been reported to positively regulate the EMT process in pancreatic cancer cells [322], DDR2 activation has been reported to induce TGF-β-mediated EMT in renal and lung cancer cells [323]. In hypoxia condition, DDR2 positively regulates EMT process in breast carcinoma [318].

2.5.5 Roles of DDRs in Metastasis and Tumor Progression

In vivo, the role of DDR1 and DDR2 in metastasis were widely evaluated. Most of the experiments were based on xenograft models. DDR1 expression was reported to be correlated with metastasis process [296]. In gastric cancer, DDR1 silencing in metastatic cancer cells inhibits the metastasis process, especially to the host lymph node and the metastatic niche [324]. In pancreatic cancer, type I collagen-induced DDR1 activation has been shown to up-regulate N-cadherin and to promotes metastasis [325]. Kim and collaborators have shown that invalidation of DDR1 in human HCT116 colon cancer cells reduced tumor growth when implanted into the mouse subcutaneous tissue [5]. A recent study has shown that DDR1 inhibition by nilotinib or DDR1 knockdown lead to a reduction of the metastatic process of colorectal cancer cells [12, 13].

DDR1 has been reported also to be involved in bone metastatic process by non-small lung carcinoma [326]. Accordingly, clinical studies have shown that DDR1 expression was associated with poor prognosis of non-small lung carcinoma [294]. In the latest study, Miao's group has shown additionally that in non-small cell lung cancer, DDR1 was able to promote cell invasion *via* activating EMT process [317]. In breast carcinoma, DDR1 has been reported to promote multi-organ site metastatic activation [327].

In the case of DDR2, it was not only found important in promoting metastatic process of tumor cells but also plays biological roles in CAFs and other stromal cells [328]. Ren and collaborators have demonstrated that DDR2 knockdown in MDA-MB-231 breast cancer cells results in a decrease in lung metastasis. However, DDR2 deficiency has no affect on tumorigenesis [318]. In fact, it is demonstrated that DDR2-deficient 4T1 mouse breast cancer cells results in a decrease in lung metastasis while growth of the primary tumor is not affected [290]. Interestingly, another study has shown that DDR2 deficiency of the host inhibits both tumor angiogenesis and metastasis [329]. Other studies have shown that DDR2 was involved in the peritoneal dissemination of gastric carcinoma and growth of metastatic breast carcinoma [330-332]. DDR2 overexpression has also been reported in the metastatic process of head and neck squamous cell carcinoma (HNSCC) [333].

III. Low Density Lipoprotein Receptor-related Protein-1 and its main functions

1. LRP-1

LRP-1, for low-density lipoprotein receptor related protein-1, also known as CD91 or α2-macroglobulin receptor, is a member of Low-Density Lipoprotein Receptor (LDLR) family (**Figure 15**, page 58), which is ubiquitously expressed on the plasma membrane of both normal and malignant cells [334-336]. LRP-1 was first identified in 1988 as an abundant surface protein in liver with a structure close to LDLR [337]. Other members of LDLR family include LRP1B [338], LRP2 (megalin) [339], very low-density lipoprotein (VLDLR) [340], LRP3 [341], LRP4 (also known as Megf7) [342], LRP5 [343, 344], LRP6 [345], LRP8 (apolipoprotein E receptor 2) [346], LRP10 (murine LRP9) [347], LRP11 (sorLA-1) [348, 349], LRP12 (ST7/Mg13) [350], and the recent novel member LRAD3 [351]. Global deletion of the LRP-1 gene in mice leads to embryonic lethality at an early stage of development, demonstrating the essential role for LRP-1 in development [352]. LRP-1 is involved not only in ligand uptake, receptor mediated endocytosis and lipoprotein transport but also regulates pericellular protease activity, controls binding and cellular entry of several (glyco) proteins and acts on a wide variety of cell signaling pathways.

1.1 LRP-1 structure

LRP-1, with a mass of 600 kDa, undergoes a furin-mediated proteolytical cleavage in the Golgi apparatus during its biosynthesis. This cleavage results in two non-covalently bound subunits, a 515 kDa N-terminal fragment located extracellularly (the heavy α-chain) and an 85 kDa membrane-bound C-terminal fragment (the light β-chain) that form the mature LRP-1 [353]. The α-chain, primarily responsible of the ligand-binding activity of LRP-1, includes four clusters of complement-like repeats (CRs I-IV) and EGF-like domains which consist of two cysteine-rich EGF repeats (**Figure 15**, page 58 and **Figure 16**, page 59). These regions together with the β-propeller domain (YWTD) play an important role in releasing ligands at low pH conditions in the endosomal compartments [336].

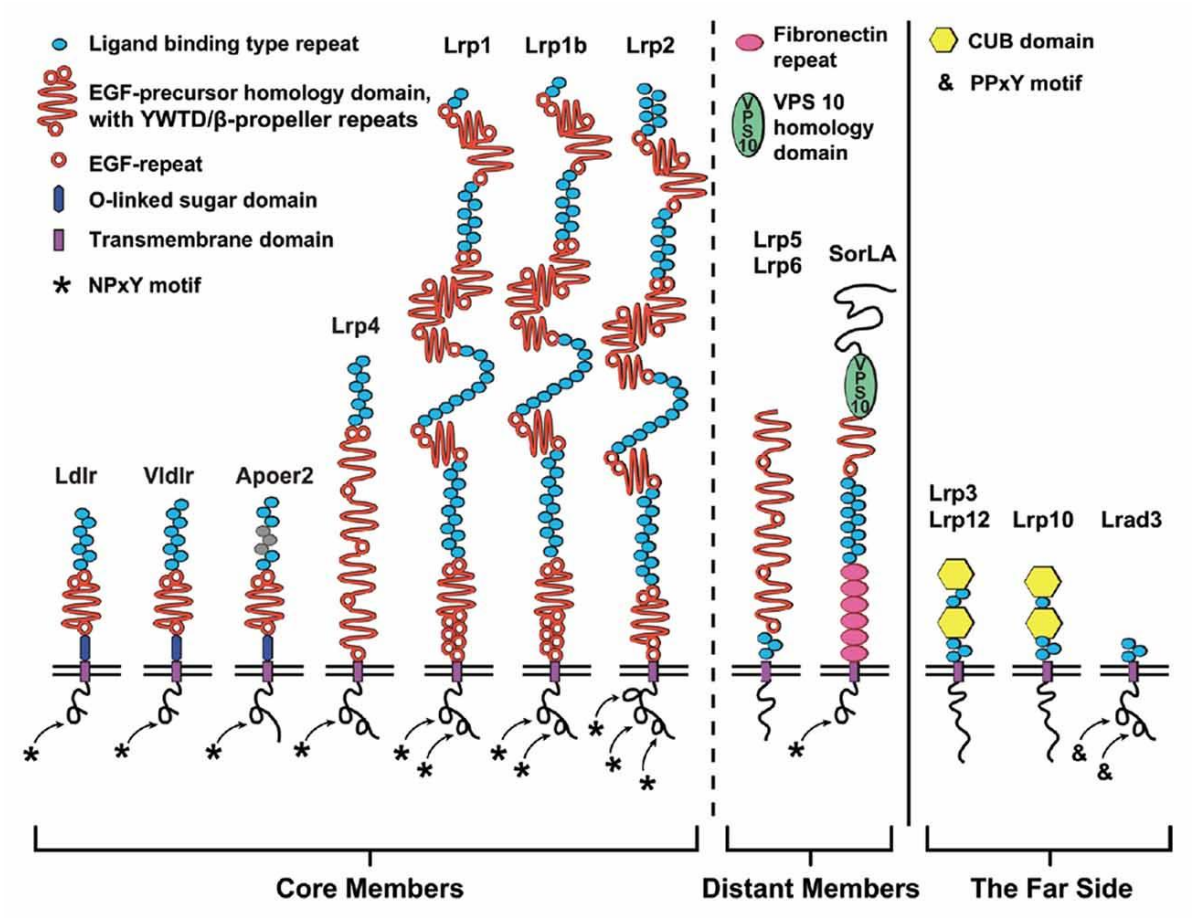

Figure 15. Modular domain organization of low-density lipoprotein receptor family. *The seven core members (left) are LDL receptor (Ldlr), very-LDL receptor (Vldlr), Apolipoprotein E (ApoE) receptor 2 (Apoer2/Lrp8), LDL receptor related protein-4 (Lrp4), Lrp1, Lrp1b and Lrp2. These members are classified as core members by the presence of at least one NPxY-motif (asterisk) and a combination of two classical LDL receptor domains. The more distant members (middle) are the NPxY-lacking Lrp5/Lrp6 and hybrid SorLA with additional Fibronectin repeats (pink) and importantly the VPS10p-sorting motif (green). Four very distant "far side" proteins (right, Lrp3, Lrp10, Lrp12, and Lrad3) only encode ligand binding-type repeats. Lrp3, Lrp10 and Lrp12 also contain atypical CUB-domain [354].*

The clusters (I-IV) contain 2, 8, 10, and 11 cysteine-rich complement-type repeats (CRs), respectively. The clusters II and IV are the major binding regions for ligands, including apolipoprotein E, the blood coagulation factor VIIIa, numerous proteinases and proteinase-inhibitor complexes (including matrix metalloproteases (MMPs) and plasminogen activators), bacterial toxins, viruses, and various extracellular matrix-associated proteins (**Figure 16**, right panel, page 59). In fact, the recent updated data indicate that over 80 representative molecules may interact with LRP-1 [355] and more than 40 ligands which bind directly to LRP-1 have been identified [336, 356].

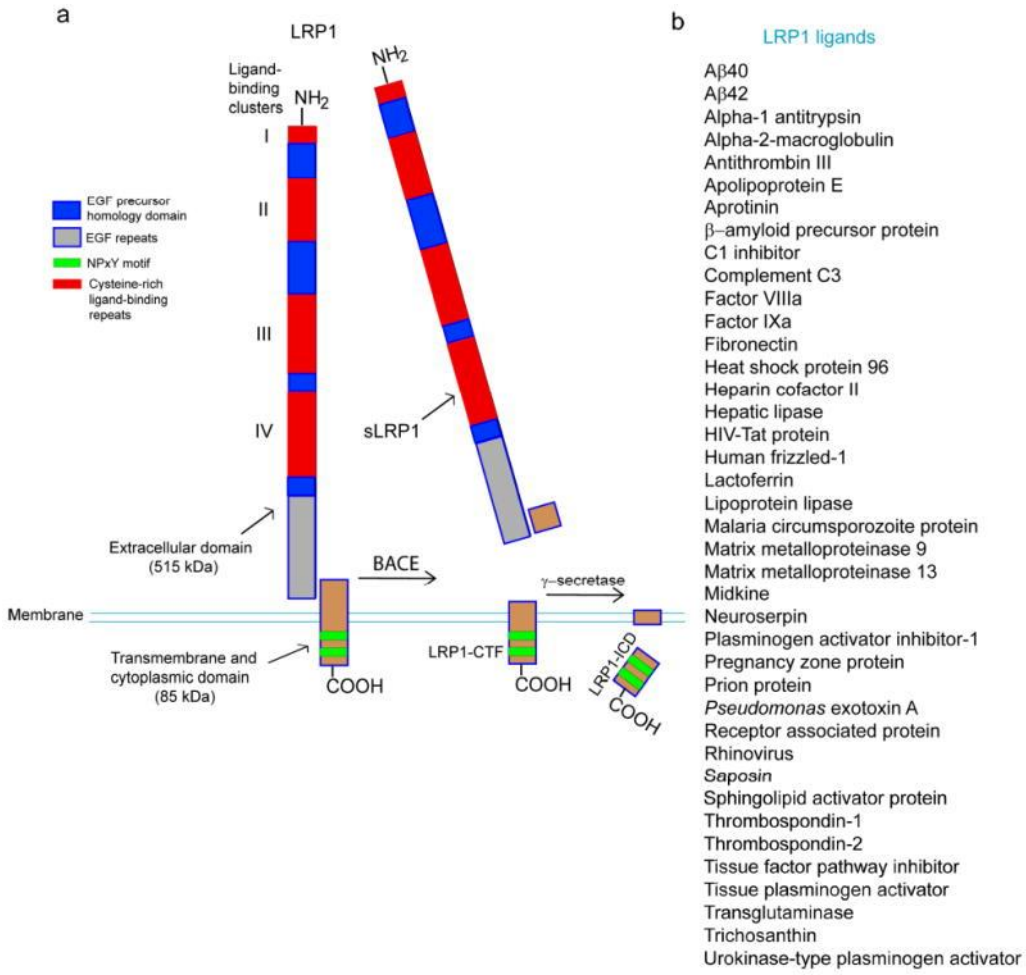

Figure 16. LRP-1 schematic structure and ligands. *(a) Full mature LRP-1 molecule can be processed by membrane resident proteinases. (b) A short list of well-known LRP-1 ligands [357].*

The LRP-1 α-chain is composed of ligand binding complement-type and EGF receptor-like cysteine-rich repeats, YWTD domains, a single transmembrane domain, and a cytoplasmic region. The β-chain cytoplasmic region consists of two NPxY motifs, one YxxL motif (a dominant signal transmitter that promotes a rapid internalization) and two di-leucine motifs that have been associated with the endocytotic functions of LRP-1 (**Figure 15**, page 58) [354, 358-360]. The β-chain also consists of docking sites for cytoplasmic adaptors or signaling proteins such as Disabled-1, Shc, FE65 or protein kinase C-α (PKCα), which mediate LRP1-dependent signal transduction (**Table 9**, page 60) [335, 336, 361]. LRP-1 initiates signaling by direct ligand binding or, through its co-receptors. Although the exact molecular mechanisms have been only partly elucidated, the tyrosine phosphorylation at the NPxY motifs appears necessary for LRP-1-mediated signal transduction [361, 362]. LRP-1 can additionally undergo an intramembrane proteolysis by β-secretase (BACE) that results in a shed extracellular LRP-1 fragment and a γ-secretase cleaved intracellular LRP-1 domain. Upon cleavage, the

intracellular LRP-1 domain may be translocated to the cell nucleus to activate gene transcription and signaling (**Figure 16**, left panel, page 59) [363, 364].

Table 9. Adaptor proteins known to bind to the cytoplasmic domain of LRP-1.

Adaptor protein	Functions
Dab1	Activation of downstream Src kinases; regulation of neurogenesis and neuronal motility
PKCα	Regulation of apoptosis, inflammation, proliferation, differentiation and motility
Shc	Activation of downstream tyrosine kinases
JIP-1, JIP-2	Activation of downstream MAPK kinases
GULP	Regulation of phagocytosis
PSD95	Coupling to NMDA receptors
FE65	Regulation of actin dynamics, APP processing, neuronal growth and migration

Dab1, disabled-1, PKCα, protein kinase C α; JIP, JNK (c-Jun N-terminal kinase)-interacting proteins; MAPK, mitogen-activated protein kinase; PSD95, post-synaptic density protein 95; NMDA, N-methyl-D-aspartate receptor.

1.2 LRP-1 maturation: the key role of RAP

The LRP-1 gene codes for a precursor protein that tightly binds to the receptor associated protein (RAP), an ER resident protein, functioning as a chaperone for several LDL receptors [365, 366]. RAP connects the ligand binding domains of the precursor [14] to prevent the binding of other ligands [367], thus ensuring its correct folding in the endoplasmic reticulum [368, 369]. In the ER, RAP functions as a chaperone to escort LRP-1 precursor to the trans-Golgi network. Under low pH environment in the trans-Golgi compartment, RAP is dissociated from the precursor receptor [365, 368, 370, 371] (**Figure 17**, page 61). Released RAP is recognized by ERD2 protein via its HNEL C-terminal sequence which allows its return to the ER [372]. LRP-1 precursor is then cleaved by furin-like endoproteases in the trans-Golgi complex to form the heavy 515-kDa α-chain coupled through noncovalent interactions to the 85-kDa β-chain [373], as mentioned above. LRP-1 fragments are then reorganized to construct a full mature LRP-1 finally transported to the plasma membrane.

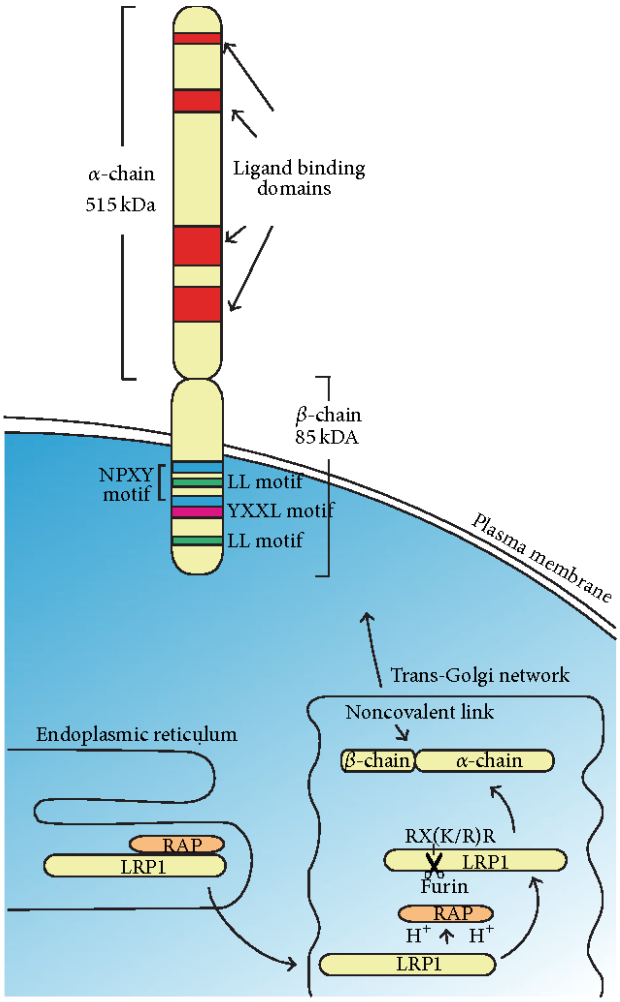

Figure 17. LRP-1 synthesis, processing and maturation [374]. *The LRP1 precursor protein is synthesized in the endoplasmic reticulum binding to its chaperone, the receptor associated protein (RAP). Then, the premature LRP-1 is transported to the trans-Golgi network where RAP is dissociated under low pH condition. In the Golgi, the protease furin cleaves the precursor LRP-1 at the RX(K/R)R consensus sequence to generate a large α-chain (515 kDa) and a light β-chain (85 kDa) that are linked noncovalently to each other to form the full functional LRP-1. Finally, LRP-1 is escorted to the plasma membrane where it is responsible for multifunctional tasks.*

RAP binds to LRP-1 with a strong affinity (K_D = 3 nM) on CR-II, III and IV. Furthermore, it is important to note that RAP is the only known ligand which interacts with CR-III [369, 375]. RAP is a modular protein that contains three independent bundle helical domains: D1, D2, and D3 [366, 376], which are connected by long flexible linkers but do not interact with each other allowing RAP to adopt various conformations to interact with LDL receptors (**Figure 18**, page 62). These three domains have distinct functions: the carboxyl-terminal domain (D3) of RAP is required for folding and trafficking of LRP-1, whereas the amino-terminal tandem D1D2 domains of RAP are required for blocking the binding of certain ligands, such as activated forms of α2-macroglobulin, to LRP-1 [375]. RAP binds LRP-1 via

two major high-affinity binding sites [377, 378]. The first binding site is located within domain 1 and 2 (D1, D2) [375, 379], that contains two lysine residues, Lys-60 in D1 and Lys-191 in D2 [380]. The second site is located within the D3. Two lysine residues in D3, Lys-256 and Lys-270 have been identified as critical residues for high-affinity binding of D3 to LRP-1 [379, 381]. Several studies indicated that RAP binds poorly to other LDL receptors due to the extreme weak affinity of D3 with CR34 structure (the 3rd and 4th CR module/region of the LDL receptors), compared to LRP-1 [381-383]. Indeed, the additional interactions between D3 and LRP-1 contribute to higher binding affinity between LRP-1 and RAP.

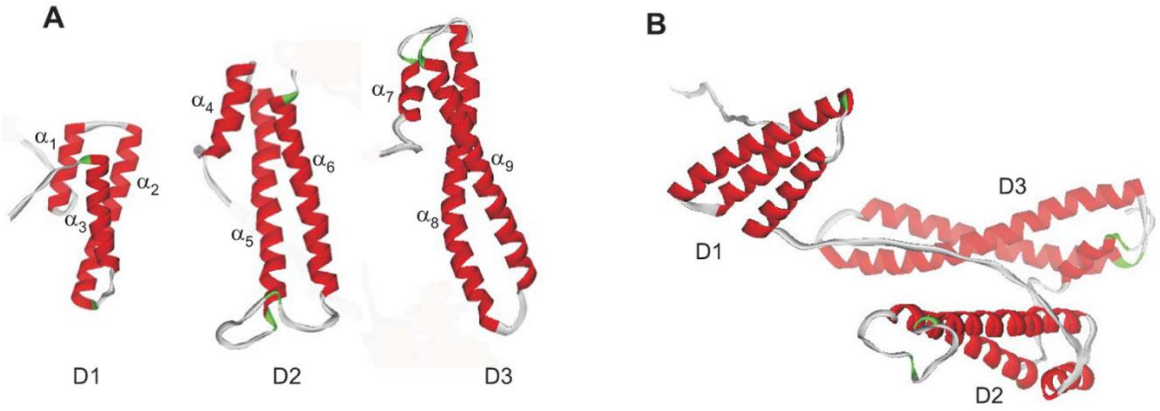

***Figure 18. Structural representation of RAP**. (**A**) NMR structure of RAP domains 1 (D1), D2 and D3. Each domain is composed of three helical bundle numbered as α_1 - α_9 (**B**) One of the possible conformations of RAP in solution. The organization of RAP shows the three independent domains of RAP connected by long flexible loops. [336].*

From an experimental point of view, RAP could be used as a competitive inhibitor of LRP-1. Indeed, if exogenous RAP is added in the culture medium, its high affinity for LRP-1 allows it to link preferentially to the membrane-anchored LRP-1 to the detriment of other extracellular ligands.

2. LRP-1, modulator of signaling pathways

LRP-1 is the most multifunctional member of the LDL receptor gene family. It has been implicated in two main biological functions: endocytosis of its numerous ligands and regulation of cell signaling pathways. The large variety of LRP1 ligands translates the multiple biological functions of this evolutionarily ancient receptor. Its ubiquitous expression, the noteworthy structural and sequence conservation among species and the lethality of the conventional knockout in mice reveal that LRP1 is essential for cellular physiology.

2.1 LRP-1 regulates apoptotic pathways and promotes cell survival

Several *in vitro* and *in vivo* studies have demonstrated that LRP-1 signaling is essential for inhibiting death pathways and promoting cell survival. Indeed, LRP-1 initiates and regulates several survival pathways, such as serine/threonine protein kinase (AKT), pro-apoptotic PI3K, NF-κB and JNK. For instance, *in vitro* studies have shown that in PC12 cells and neurons, the binding of tPA or α2-macroglobulin to LRP-1 results in Src family kinase (SFK) activation and SFK-dependent Trk receptor transactivation, followed by AKT activation [384]. AKT/PI3K signaling pathway is known to be one of the most important pathways which inhibits apoptosis and promotes cell survival [385].

Mounting evidence suggests that LRP-1 plays an important role in regulating cell survival and apoptosis. Indeed, knockdown of LRP-1 by short hairpin RNA in primary neurons results in a significant increase in caspase-3 activation and a decrease in the levels of AKT, insulin receptor phosphorylation [386]. In addition, LRP-1 silencing in Schwann cells by small interfering RNA also decreased PI3K/AKT activation and increased active caspase-3 levels [387]. In ischemic neurons, inhibition of LRP-1 by RAP decreased AKT and anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) activation and increased pro-death signaling pathways such as nuclear factor-Kappa B (NF-κB) and caspase-3 pathways [388]. *In vivo*, the important role of LRP-1 in regulating insulin receptor and AKT survival pathways was also demonstrated in LRP-1 forebrain knock-out mice [389]. In addition, Lutz *et al*., (2002) indicated that LRP-1 increases the location of c-Jun N-terminal kinases (JNKs) at the plasma membrane, inhibiting JNK translocation into the nucleus and nuclear activation of the JNK-dependent transcription factors Elk-1 and cJun [390]. The ability of LRP-1 to modulate cJun/Elk-1 transcriptional activity suggests that it may also be sensitive to negative signals that are involved in the induction of apoptosis. In summary, LRP-1 clearly plays important roles in regulating pro-survival signaling cascades and anti-programmed cell death. However, the understanding of how LRP-1 interacts and activates survival mediators remains unclear. Although Pallero *et al* have reported the involvement of LRP-1 in anoikis resistance by inducing tissue remodeling through TSP1/calreticulin/LRP1 signaling [391], other potential functions of LRP-1 in the regulation of distinct cell death processes such as necrosis and pyroptosis have not been studied.

2.2 Role of LRP-1 in cell proliferation

Over the past ten years, a series of studies revealed the involvement of LRP-1 in diverse signaling pathways leading to inducing cell proliferation. It is well known that LRP-1 regulates some crucial downstream signaling pathways such as Ras, c-Myc, MAPKs, and Akt/PI3K, which are associated with oncogenesis, cell proliferation, and survival [392] (**Figure 19**, page 64). However, the function of LRP-1 in cell proliferation remains controversial and depends on the cell types, stimuli and conditions that are studied.

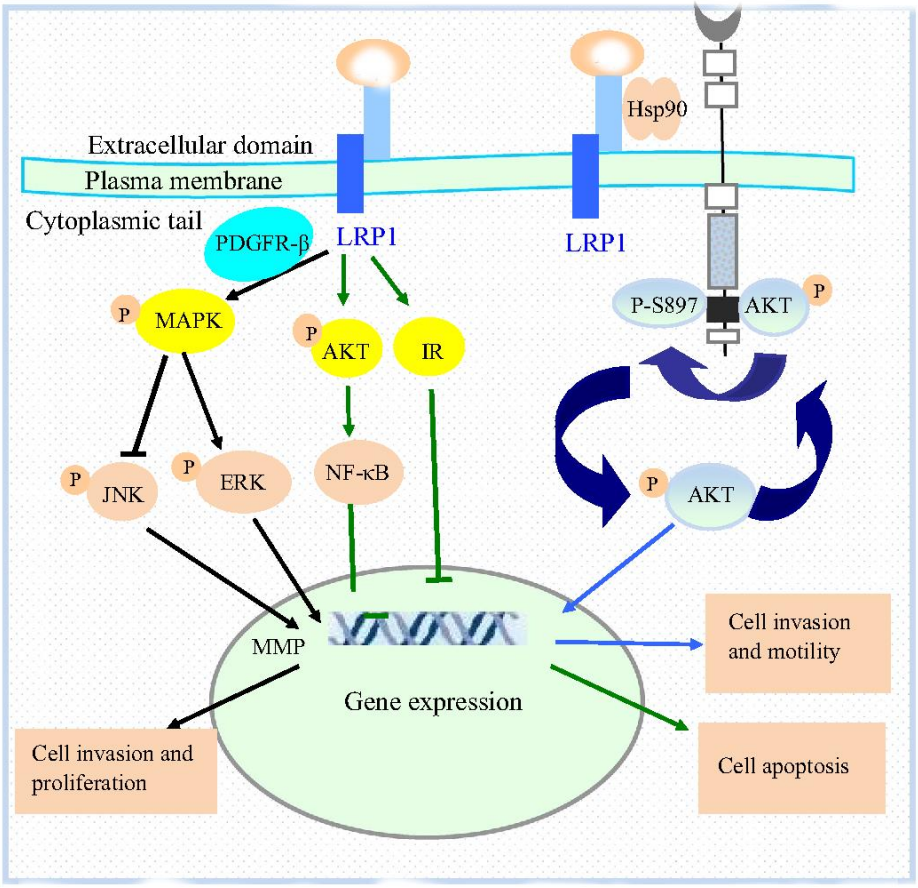

Figure 19. Proposed LRP-1 related signaling pathways in multiple processes of cancer development [356].

On one hand, LRP1 is involved in PDGF-mediated induction of ERK phosphorylation, which can increase smooth muscle cell and fibroblast proliferation. Interestingly, PDGF induces the phosphorylation of LRP-1-ICD in a process dependent on PDGF receptor activation. In details, LRP-1 functions as a co-receptor that modulates signal transduction pathways initiated by the PDGF receptor, which further regulates MAPK/ERK and Akt/PI3K pathways [393-395], and mediates the tyrosine phosphorylation of the LRP-1 at the intracellular domain (LRP-1-ICD) [394, 396]. Invalidation of LRP-1 in mouse vascular smooth

muscle cells (SMC) leads to PDGF receptor accumulation and abnormal activation of PDGF receptor signaling, followed by a disruption of elastic layer, SMC proliferation and migration, and aneurysm formation [393, 397]. LRP-1 was also found to bind Cbl, an ubiquitin-protein ligase necessary for lysosomal-mediated degradation of the PDGF receptor complex, resulting in a decrease in cell proliferation [398].

On the other hand, LRP-1 participates in other signaling pathways with different molecular actors to modulate cell proliferation. For example, α_2-macroglobulin binding to LRP-1 induces an increase of ERK1/2, p38 and c-Jun phosphorylation, and subsequently promotes J774 macrophage-derived cell proliferation [17]. A study in osteoblastic cells indicated that LRP-1 is also involved in lactoferrin-induced cell proliferation. It was suggested that lactoferrin stimulates osteoblast mitogenesis by activating p42/44 MAPK pathway through LRP-1 and that the endocytic function of LRP1 is independent of its signaling function [18]. In addition, Lin and colleagues demonstrated that binding of tissue plasminogen activator (tPA) to LRP-1 induces Tyr-4507 phosphorylation on LRP-1-ICD, initiating a cascade of proliferative signaling events involving phosphorylation of ERK1/2, p90RSK, GSK3β, and induction of cyclin D1 resulting in cell-cycle progression and stimulated cell proliferation [19]. Furthermore, studies in radial glia and neural stem precursor cells (NSPCs) showed that LRP-1 exhibits a positive effect on NSPC proliferation and survival, promotes oligodendroglial and neuronal differentiation, but negatively affects astrogliogenesis *in vitro*. Although this effect seems to be dependent of downstream activation of ERKs and PI3K/Akt signaling, the accurate molecular mechanisms by which LRP-1 mediates NSPC proliferation remains undefined [399]. Furthermore, LRP-1 induces cell proliferation of mouse embryonic fibroblasts after association with its ligands, by stimulating downstream pro-proliferative signaling cascades [20, 21]. By contrast, LRP-1 can suppress lung adenocarcinoma and human hepatic stellate cell proliferation by decreasing through endocytosis the level of pro-proliferative molecules in the extracellular medium [22, 23]. A recent study in retinal microvascular endothelial cells showed that LRP-1 interacts directly with poly-(ADP-ribose) polymerase-1 (PARP-1), a regulator of cell cycle progression, resulting in a negative regulation of endothelial cell proliferation and neovascularization in the hypoxic retina. LRP-1 also regulates cyclin-dependent kinase 2 (CDK2) and retinoblastoma activities, two proteins which play important roles in promoting cell cycle progression and angiogenesis [24].

Altogether, the role of LRP-1 on cell proliferation remains questioned and controversial and appears to be highly dependent on the microenvironment state and the time window of

observation. However, this is an issue that seems absolutely essential to clarify, particularly in the tumor context.

2.3 Role of LRP-1 on inflammatory signaling

Inflammatory is a natural adaptive immune response, which is triggered by harmful stimuli and by a variety of factors, including pathogens, damaged cells and toxic compounds [400]. However, inflammatory response could contribute to the pathogenesis of numerous chronic diseases such as arthritis, cardiovascular diseases, autoimmune disorders, and cancer [401]. In cancer, inflammation is a critical process during tumor progression and promotes different steps of malignant behaviors like proliferation, invasiveness, metastasis, or angiogenesis [80, 402]. It is well documented that inflammation is coordinated by a wide range of mediators, which are classified into seven groups such as vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines, and proteolytic enzymes [400]. Recent studies indicated that LRP-1 fragments can be involved in the regulation of inflammatory response. Indeed, the soluble extracellular domain obtained by shedding of LRP-1 (sLRP-1) constitutes potently a proinflammatory mediator [403] while the intracellular subunit of LRP-1 β-chain (LRP-1-ICD) serves as an anti-inflammatory factor neutralizing the inflammation [364, 403]. Moreover, studies clarified that LRP-1 shedding activities are involved in the regulation of inflammation. In fact, LRP-1 is shed by different membrane-anchored proteases. For instance, the membrane-anchored LRP-1 can be shed by either β-secretase to release sLPR-1 and/or intracellularly by γ-secretase to generate the LRP-1-ICD [357]. In addition, studies from human brain microvascular endothelial cells (HBMECs) and human Alzheimer's disease brains indicated that LRP-1 α-chain can be shed by both ADAM10 and ADAM17 [403-405]. Nonetheless, LRP-1 can also be shed by MMP-14 in inflammatory tissues such as osteoarthritic cartilages [406]. *In vivo*, LRP-1 shedding activities occur steadily in healthy conditions [407], but are strongly increased during enhancing inflammatory response such as in injured tissues of patients carrying rheumatoid arthritis and systemic lupus erythematosus [408]. It is agreed that LRP-1 regulates the inflammatory response through a two-way feedback loop signaling. Firstly, the inflammatory response induced by lipopolysaccharide (LPS) results in increased shedding of the LRP-1 extracellular domain and generation of sLRP-1. As mentioned above, sLRP-1 tends to support inflammatory events. By contrast, following γ-secretase-dependent cleavage, LRP-1-ICD can be translocated into the nucleus where it interacts directly with IRF-3 and separates it from CBP/P300. The complex LRP-1-ICD:IRF-3 is then exported out of the nucleus causing the interruption of LPS

targeted-gene transcription and expression of metalloproteases, resulting in decreasing inflammation response (**Figure 20**, page 67) [364].

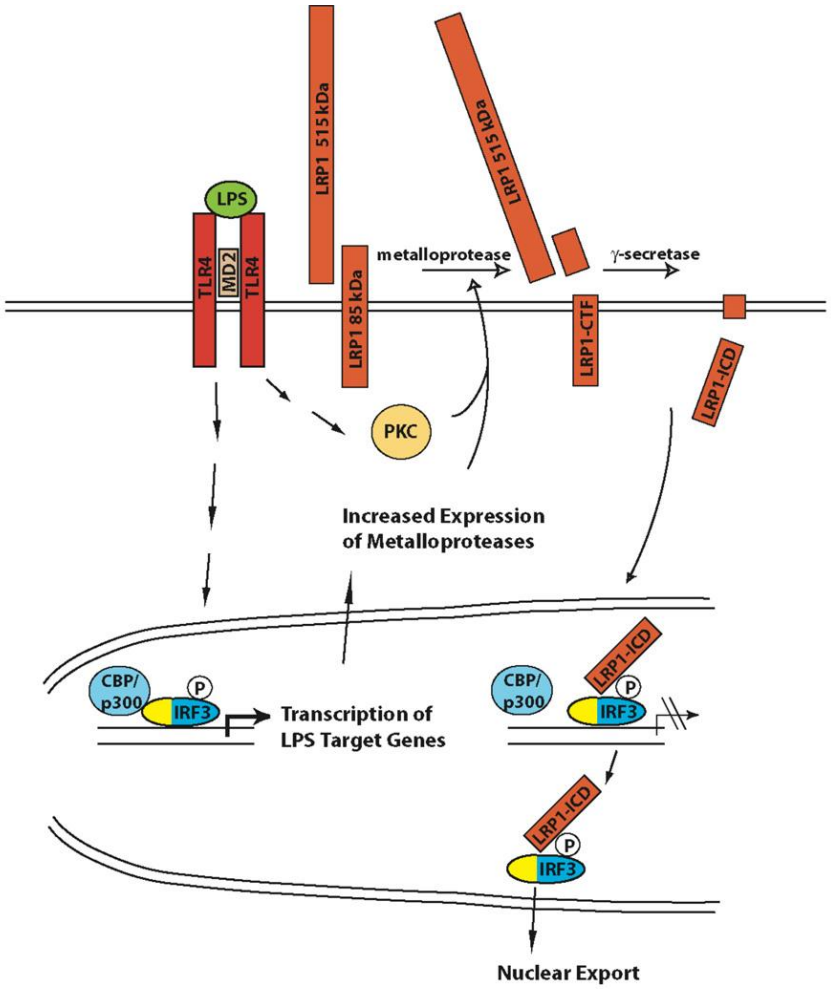

Figure 20. Regulation of LPS induced-inflammatory response by LRP-1. *IFR-3 activates a part of genes induced by LPS. Expression of metalloproteases are increased by IRF-3and other LPS-induced signaling pathways activation. In addition, PKC is activated by LPS. Shedding of LRP1 is therefore augmented. Increased proteolysis of LRP-1 leads to LRP1 ICD cleavage by γ-secretase and its release. The interaction of LRP1 ICD with IRF-3 facilitates its nuclear export and reduces the expression of IRF-3 target gene* ***[364]****.*

3. LRP-1 involvement in diseases

3.1 LRP-1 in neurodegenerative disorders and several chronic diseases

Dysregulation of LRP-1-dependent signaling events is involved in the development of multiple pathological events such as neurodegenerative disorders, atherosclerosis, tissue inflammation, and coagulation complications. In this part, I will describe few examples of LRP-1 involvement in Alzheimer's disease, atherosclerosis, and chronic diseases.

❖ **Alzheimer's disease**

LRP-1 and other members of LDLR gene family, including apolipoprotein E receptor 2 and the VLDL receptor, have been largely implicated in Alzheimer disease (AD) [409]. *In vivo* and *in vitro* experiments have demonstrated multiple mechanisms by which LRP-1 may regulate AD onset and progression. Notably, LRP-1 serves as an endocytic signaling receptor for apolipoprotein E and functions as a receptor for amyloid beta (Aβ peptide). Numerous clinical data suggested that the dysregulation of Aβ level, a crucial biomarker, is associated with functional and structural brain alterations and contributes to the major hallmark of AD [410]. Amyloid-β peptide accumulation and aggregation in the brain trigger neurodegenerative processes lead to the dementia observed in AD patients [411]. It should be noted that imbalance between Aβ clearance and its production is attributed to AD development in most cases [411, 412]. To date, numerous preclinical studies have demonstrated the involvement of LRP-1 in Aβ metabolism and degradation, as reviewed by Shinohara and collaborators [412]. It has been demonstrated that liver is the major organ responsible for Aβ clearance from plasma [413, 414] and LRP-1 is an important receptor for plasma Aβ clearance in the liver [415]. In addition, up-regulation of LRP-1 expression in the liver induced an increase of Aβ removing from the blood, and potentially enhances Aβ transport across the blood-brain barrier (BBB) [416]. A recent study indicated that BBB pericytes are able to clear Aβ aggregates in a LRP-1/ApoE isoform-specific interaction [417], which is similar to previous studies about LRP-1/ApoE-dependent Aβ endocytosis [412, 418]. **Figure 21** (page 69) summarized the different LRP-1 related pathways involved in Aβ production and clearance. Others studies have shown that LRP-1 expression is up-regulated in some cell types from brain of AD patients [419-421], and its expression is increased during neuroinflammation and postsynaptic damages [422]. To conclude, accumulating evidence from *in vitro* and *in vivo* studies demonstrated that LRP-1 not only regulates the metabolism of amyloid-β peptide (Aβ) in the brain, but also maintains brain homeostasis impairment which probably contributes to AD development in Aβ-independent manners. However, despite very promising results, the highly complex roles of LRP-1 in AD pathogenesis remains to be further clarified.

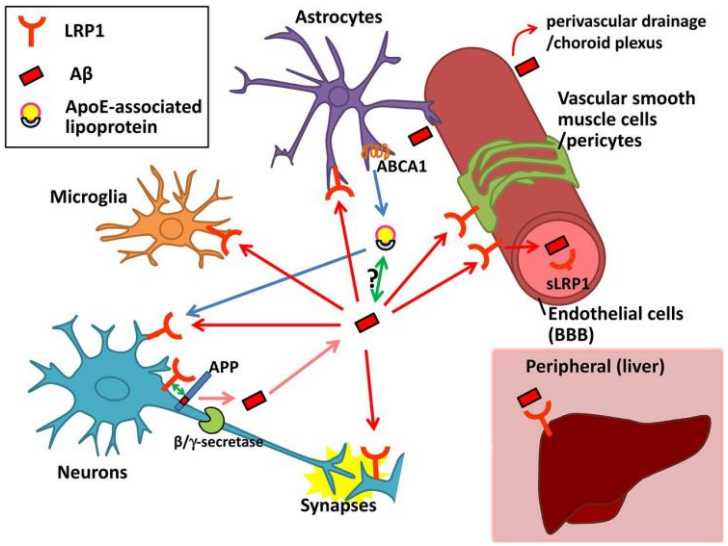

Figure 21. Proposed LRP-1-related pathways for Aβ clearance from circulating blood and Aβ accumulation in brains. *LRP-1 might regulate Aβ production from Amyloid Protein Precursor (APP) in neurons through its interaction with APP or competition with the β/γ-secretase cleavage of APP. After its secretion into the extracellular space in the brain, Aβ uptake is regulated by LRP1 in neurons, microglia, astrocytes, vascular smooth muscle cells, pericytes, endothelial cells, and the choroid plexus. After internalization, Aβ is either degraded in lysosomes or accumulated in the cells provoking cellular toxicity. A part of Aβ may be transported into the blood through LRP-1 dependent trancytosis. LRP-1 in the liver could accelerate the elimination of peripherally circulating Aβ [412].*

❖ **Metabolic disorders, atherosclerosis and inflammation**

Studies over the last decade have found that LRP-1 is implicated in the pathological mechanisms underlying metabolic disorders. In physiological conditions, LRP-1 regulates glucose and lipid metabolism in neurons [423, 424] and adipose tissue [425] as well as liver [426] whereas lower LRP-1 expression in visceral fat was reported in obesity patients [427]. Recent studies indicated that LRP-1 is involved in aggregated LDL uptake and lipid accumulation in vascular smooth muscle cells resulting in foam cell formation, which is attributed to atherosclerotic development [428-430]. It should be noted that the role of LRP-1 in atherosclerosis are cellular-dependent contexts. For instance, LRP-1/Wnt5a pathway regulates intracellular cholesterol homeostasis, which reduces the burden of cholesterol accumulation diseases such as atherosclerosis [431]. Moreover, LRP-1 modulates M2 to M1 macrophage transformation and production of proinflammatory factors that occur in the development of atherosclerotic lesions [432]. However, LRP-1 expression in macrophages has been shown to protect vessel wall and reduce neointimal formation [433]. In addition, LRP1 was reported to protect the vascular wall integrity and prevents atherosclerosis by modulating PDGFR activation [393].

Several studies reported also that LRP-1 is involved in other cardiovascular diseases including ischemic/non-ischemic heart failures, myocardial ischemia and reperfusion injury. The role of LRP-1 on these pathologies has recently been reviewed [434].

3.2 LRP-1 and cancers

LRP-1 is considered as a multifunctional regulator playing diverse roles in cancer-related events including adhesion, invasion, migration, as well as metastasis development. However, LRP-1 expression is highly variable depending on the tumor type and the stage of cancer progression. For instance, a clinical study in hepatocellular carcinoma (HCC) indicated that low level of LRP-1 is associated with tumor invasiveness, tumor recurrence, poorer prognosis, and decreasing overall survival rates [435]. LRP-1 expression is decreased during cutaneous melanocytic tumor progression, especially in the late stages. Similarly, low LRP-1 expression was also demonstrated in advanced stages of Wilms tumors [436]. A study in lung adenocarcinomas, using a cohort of 439 patients, showed that LRP-1 expression was reduced in both mRNA and protein levels and this lower expression of LRP-1 is correlated with less favorable clinical outcome [23]. In addition, Yamamoto *et al.* mentioned that LRP-1 is weakly expressed in primary lung adenocarcinomas and metastatic brain tumors from metastatic lung adenocarcinomas [437]. Recently, results from our team reported that loss of LRP-1 expression in colorectal tumors is highly correlated with advanced cancer stages, poor prognosis, and lower survival rates [29]. By contrast, LRP-1 overexpression was reported in breast cancer cells [438, 439]. Interestingly, although no evidence has been found to correlate LRP-1 expression with overall patient survival in these studies, it was suggested that overexpression of LRP-1 is correlated with increased cell proliferation, tumor recurrence, and invasiveness [439]. Supporting this way, recent data from our team indicates that LRP-1 participates to triple-negative breast cancer development by supporting tumor vascularization *in vivo* [unpublished results]. Moreover, increasing LRP-1 expression is correlated with high-grade tumors and advanced tumor stages in patients carrying endometrial carcinoma [440]. In prostate cancer, LRP-1 overexpression was found in the late stages of prostate tumors and metastasis lesions [441]. LRP-1 expression was reported in all types of cerebral tumors. Indeed, LRP-1 is highly expressed in invasive glial tumor cells, especially in glioblastoma and LRP-1 overexpression correlates with the malignancy grade of astrocytoma [437, 442, 443]. More, a recent study in pancreatic ductal adenocarcinoma demonstrated that LRP-1 overexpression is associated with poor prognosis and perineural invasion [444].

On one hand, it is well known that increased ECM degradation, including shedding mechanisms, is associated with advanced tumor stages that could partly explain how LRP-1 expression can be decreased during cancer progression. On the other hand, we need to consider that LRP-1-dependant endocytosis is mainly involved in the regulation of ECM remodeling in malignant cells by decreasing the level of certain pericellular proteases (**Figure 22**, page 72), such as matrix metalloprotease (MMP)-2 [445], MMP-9 [435], tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) [335, 446]. When the membrane-anchored LRP-1 amount and activity are decreased in tumors, this leads to even higher levels of these proteases at tumor sites contributing to support tumor aggressiveness and progression through a deleterious control loop. Many studies indeed demonstrated that LRP-1 inhibition or increased LRP-1 shedding resulted in the accumulation of MMP-2 and MMP-9 [447, 448]. For instance, LRP-1 inhibition leads to enhance invasion of low-metastasis human hepatocellular carcinoma cells due to decreased internalization of MMP-9 [435]. Indeed, several studies have demonstrated the involvement of MMP-2 in ECM remodeling and degradation during tumor progression [449-452], suggesting that the procees of LRP-1-mediated MMP-2 clearance appears absolutely essential to reduce cancer progression.

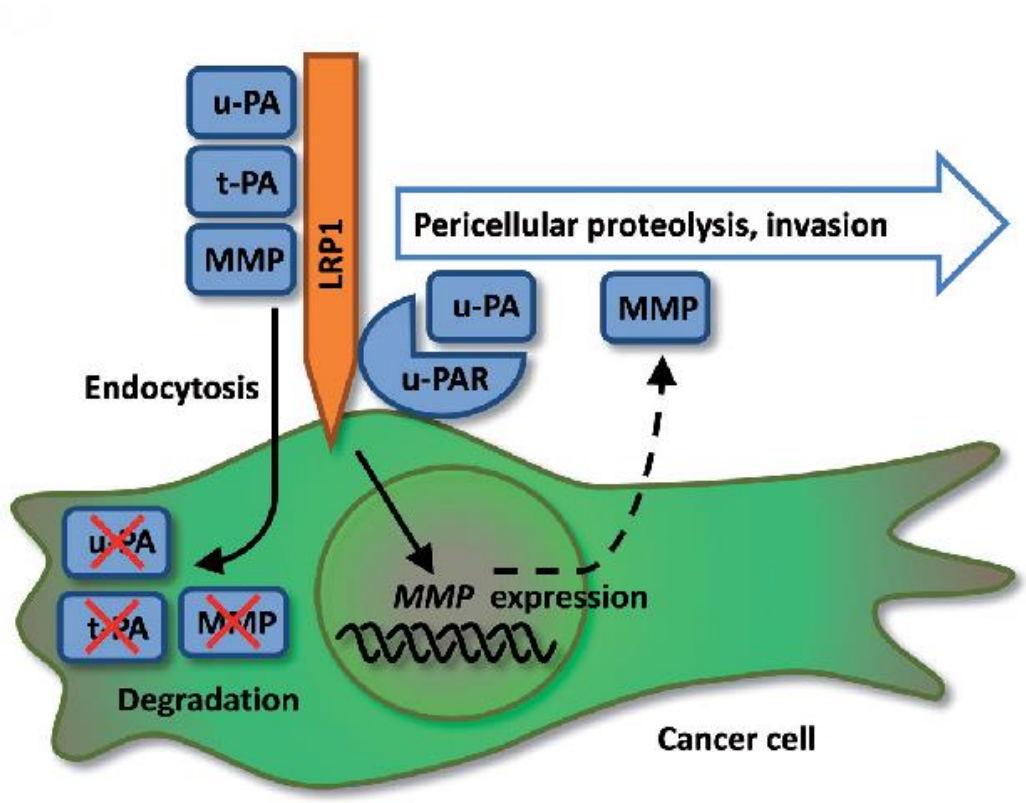

Figure 22. Contribution of LRP-1 to tumor invasion and migration. *LRP-1 regulates uPA, tPA, and matrix metalloproteases activities by endocytotosis causing membrane-anchored proteinases degradation, which results in cancer cell migration and invasion inhibition. On the contrary, LRP1-ICD fragment induces MMP expression which enhance matrix degradation and tumor invasion [453].*

In another context, urokinase receptor (uPAR) signaling is an alternative migration- and invasion-related pathway regulated by LRP-1 that can promote cell invasion and migration. The uPA-plasminogen activator inhibitor-1 (PAI-1) complex is a bivalent ligand that triggers uPAR internalization and regulates the uPAR signaling by bridging extracellularly uPAR and LRP1 [454-456]. Moreover, uPAR can associate with several integrins and binding to uPA:PAI-1:LRP-1 complex, thus stimulating formation of clathrin-coated pits and subsequent endocytosis [457]. This affects uPAR presence at the plasma membrane with consequences for ECM degradation via the plasminogen activation system and uPAR-integrin interaction, both important for supporting tumor cell migration and invasion (**Figure 23**, page 73) [458]. Indeed, uPA and its receptor participate in numerous signaling pathways that together play various functions in cancer-related events, including apoptotic suppression, extracellular matrix degradation, cell adhesion, migration, invasion, and metastasis [459, 460].

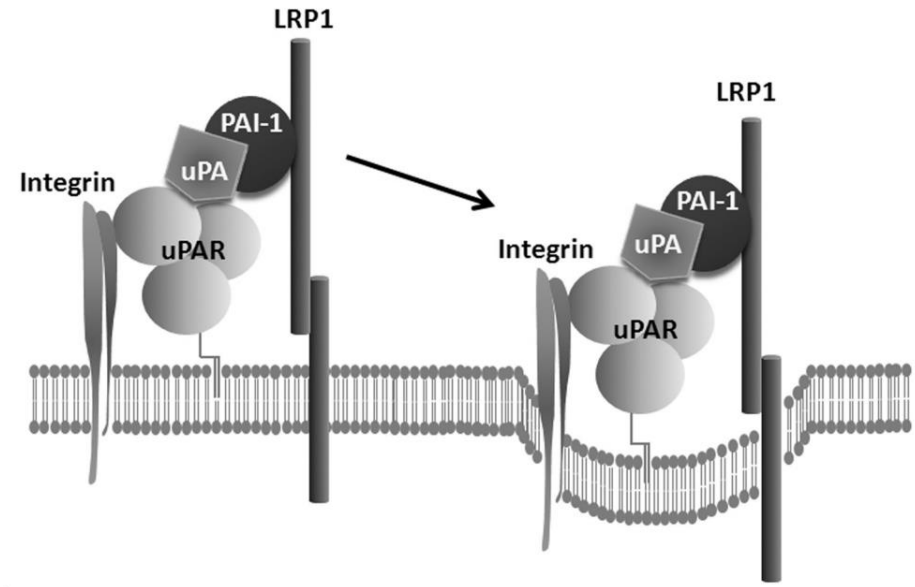

Figure 23. Endocytosis of uPAR complex by LRP1. *The ternary uPAR:uPA:PAI-1 complex associates to LRP-1. At once, various integrins associated with uPAR are subject to LRP1-facilitated endocytosis via clathrin-coated pits [458].*

Salama and collaborators recently highlighted the involvement of the tPA:LRP-1 complex in promoting cell migration and proliferation in melanoma [461]. From loss- and gain-of-function analyses, they proposed a model wherein LRP-1 enhances ERK activation, resulting in increased MMP-9 expression within the tumor (at both mRNA and protein level), accumulation of tPA and MMP-9 and changes the cancer-associated fibroblasts content to support tumor cell growth and metastasis [461]. Briefly, LRP-1-mediated endocytosis could be first associated with anti-tumor progression via the clearance of the pericellular or membrane-associated proteases (MMP-2, -9, tPA, and uPA). However, membrane LRP-1 could be post-transcriptionally regulated by other membrane-anchored proteases such as MMP-14, ADAM-10, and ADAM-17, as was mentioned in the previous section. Hence, by regulating the composition of the plasma membrane proteome, LRP-1 can also indirectly control activation of important cell-signaling pathways involved in each hallmark of cancer.

Many works indeed showed the contribution of LRP-1 in various signaling cascades involved in tumor progression regardless of LRP-1 expression amount. Our team reported that LRP-1 silencing prevents the invasion of follicular thyroid carcinomas despite increased pericellular proteolytic activities from MMP-2 and uPA [462]. In this previous work, we established that LRP-1 may coordinate the adhesion and de-adhesion balance of malignant cells to support tumor progression by regulating the amount, molecular composition and distribution of focal adhesion structures [462]. In addition, Langlois and collaborators reported that LRP-1 promotes thyroid carcinoma cell migration and invasion through concomitant activation of

ERK and inhibition of JNK pathways that stimulates focal adhesion disassembly [463]. These data were confirmed by a complementary study in three-dimensional type I collagen matrix. Indeed, inhibition of LRP-1 activity or expression leads to drastic morphological changes affecting cell-matrix interactions, reorganizations of the actin-cytoskeleton especially by inhibiting FAK activation and increasing RhoA activity and MLC-2 phosphorylation, thus preventing 3D cell migration [28]. Moreover, in glioblastoma, extracellular heat shock protein 90 (Hsp90) cooperates with LRP-1, activating Src and AKT phosphorylation to promote cell migration and stimulate the metastatic activities [443, 464]. In melanoma, it was shown that LRP-1 acts in response to apolipoprotein E (ApoE) as an endogenous suppressor of the metastatic phenotype [465]. It should be noted however, that LRP-1 silencing in xeno-transplanted CL16 breast cancer cells in mice has no effect on cell growth or on primary tumor formation. Montel and colleagues reported that LRP-1 expression is notably increased by hypoxic conditions and stimulates metastasis development in the lung [466]. Similarly, recent work from Leslie and collaborators demonstrated that LRP-1 expression plays a crucial role on colorectal cancer cell survival and is regulated by p53 under stress conditions [467]. Thereby, under sub-lethal conditions, p53 increases both LRP-1 mRNA and protein expression, which further induces an increase in tumor cell survival. Interestingly, lethal doses of p53-activating stress inhibit LRP-1 expression through a translational repression mechanism regulated by miRNA 103 and 107. This inhibition results in cell death [467]. On the contrary, it is reported that stable transfection of H1299 human lung carcinoma cells with full-length LRP-1 cDNA restores the sensitivity to growth inhibition [468]. However, this mechanism needs to be further elucidated by additional studies.

Many studies reported that LRP-1 in monocytes and macrophages plays a critical role in cancer progression by acting as a regulator of inflammation. As inflammation is a critical feature in cancer, the activities of macrophages in tumor microenvironment make a crucial contribution to cancer progression, as described elsewhere [402, 469]. Indeed, the presence of LRP-1-deficient monocytes into subcutaneous and orthotopic pancreatic tumors were significantly increased [470]. The secretion of chemokines, particularly CCL3, is enhanced in LRP1-deficient macrophages, resulting in an increased number of tumor-associated macrophages (TAM) at the tumor site. The authors provided evidence that the LRP1-deficient TAM contribute to the higher VEGF amount into the tumor microenvironment, leading to increased tumor angiogenesis. [470]. Moreover, a recent study in which our team participated, indicated that LRP-1 contributes to the activation and trafficking of CXCR3 and correlates with poor prognosis in glioblastoma patients [471].

In summary, LRP-1 functionalities during cancer development and progression appears highly dependent of the temporality and tumor context and remains, despite numerous studies, extremely difficult to understand as a whole. As a multifunctional cell surface receptor, LRP-1 can internalize diverse biological ligands and regulate many signaling pathways. To accurately decipher LRP-1 functionalities in the tumor context, we have to systematically take into account the cell types, the state of the cellular microenvironment, the co-receptor distribution and expression, and even the assortment and local concentration of soluble ligands. In cancer cells, LRP-1 expression is not only dependent on the cellular properties but also on the experimental conditions of cell cultures. For example, LRP-1 remains undetectably in HCT116 colon cancer cells under conventional cell culture conditions. Its expression, however, is strongly increased upon adjusting a sub-lethal stress [467]. In addition, LRP-1 is expressed at very low or nearly undetectable levels in some cancer cells such as 2C5, CL16 and MDA-MB-468 when cells are cultured in conventional conditions. In opposite, LRP-1 expression in these cells is very abundantly in tumor xenografts. Furthermore, it is well documented that LRP-1 expression is reduced in normoxic cell culture conditions in many cancer cell lines, compared to hypoxic cell cultures mimicking tumor environment [466]. Further in-depth studies will be necessary in the future to better understand the function of this receptor in malignant diseases and the emergence of more complex and realistic experimental models (such as organoids and organ-on-a chip) should be helpful.

4. LRP-1 in preclinical and clinical trials

Several preclinical and clinical trials have been performed to target acute myocardial infarction therapy (AMI). Alpha 1-antitrypsin (AAT), a naturally occurring non-selective/specific LRP-1 agonist [472, 473], was first considered as a potential target for clinical trials. Indeed, AAT is known as an abundant serine-protease inhibitor that protects tissues from degradation by inhibiting proteolytic activities from proteinase-3, cathepsin G, and neutrophil elastase [474, 475]. Preclinical trials showed that AAT treatment reduces significantly myocardial injuries in AMI mouse model [476]. Reports from a clinical feasibility trial (VCU-α1RT pilot study, numbered NCT01936896, available online at https://clinicaltrials.gov/) showed that treatment with AAT in patients with acute myocardial infarction is well-tolerated without serious adverse events [477, 478]. Moreover, data indicated that plasma-derived AAT is associated with a blunted acute inflammatory response after AMI and no heart failure occurs in treated patients the year following treatment. Although the obtained data need to be further elucidated, these studies offers promising prospects to LRP-1

agonists as new cardiac therapeutic target. Furthermore, preclinical studies using a synthetic peptide agonist of LRP-1, SP16 (Ac-VKFNKPFVFLNleIEQNTK-NH2), demonstrated a cardioprotective effect of this peptide in AMI (**Figure 24**, page 76) [479]. These extremely interesting data allowed initiation of a phase I clinical trial testing SP16 in healthy volunteers (numbered NCT03651089, available online at https://clinicaltrials.gov/). The results show that SP16 is not toxic and well tolerated.

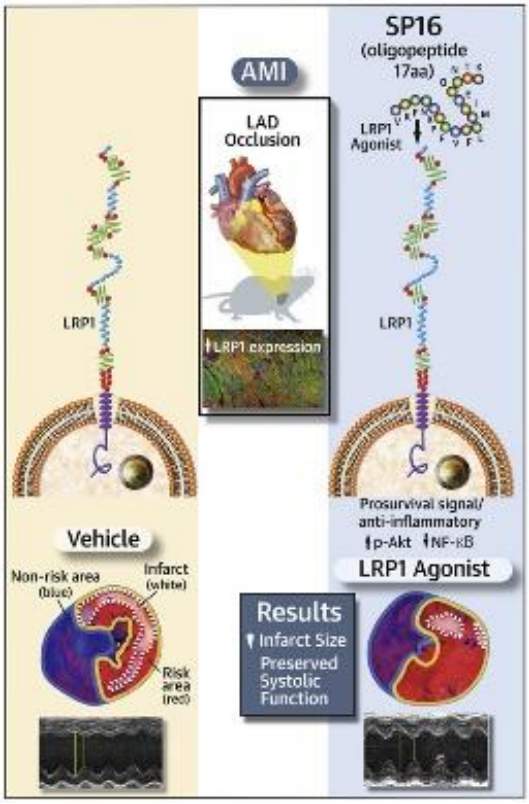

Figure 24. Role of LRP-1 in acute myocardial infarction*. Effect of a short peptide LRP1 agonist SP16 in AMI mouse model. LRP1 activation with SP16 during experimental AMI leads to a cardioprotective signal, reducing infarct size and preserving cardiac systolic function in mice. SP16 represents a first-in-class pharmacologic agent exploring an entirely novel approach of cardioprotection in AMI [479].*

To date, despite very promising results from preclinical studies, no LRP-1-based clinical trial for AD therapy is underway. However, numerous evidences from preclinical and clinical studies have indicated potentialities for LRP-1 targeting in AD treatments and have recently been reviewed by Shinohara *et al.* [412].

In cancer, no therapeutic strategy targeting LRP-1 is currently developed. Only therapeutic strategies using LRP-1 as a cargo receptor to transport chemotherapy agents across the blood-brain barrier have been proposed. Indeed, it was shown that LRP-1 is able to mediate bidirectional transcytosis of amyloid-β across the BBB [480]. This ability of LRP-1 to mediate the transport of molecules through the BBB is therefore a valuable and expected tool for

delivering chemotherapeutic agents to which the BBB was previously impermeable. According to this principle, a molecule named ANG1005 or GRN1005 has been developed. GRN1005 is composed of three molecules of paclitaxel covalently linked to Angiopep-2, a peptide designed to exploit the LRP-1 transport system to cross the BBB or BCB (blood-cerebrospinal fluid barrier) (**Figure 25, page 78**) [481]. This Angiopep-2 peptide derived from a Kunitz domain presents an important ability of transcytosis across the BBB *in vitro* [482]. This strategy was also shown to be effective *in vivo* [483]. ANG1005/GRN1005 have been tested in clinical Phase I in patients with progressive grade II to IV glioma undergoing treatment [481] and in patients with brain metastases from different types of cancer (melanoma, breast, lung,.., etc) [484]. These studies reported promising results with good tolerance and a therapeutic response (stability or decrease of the lesions) in 1/3 to half of the patients (**Figure 25**, page 78) [481, 484].

It is obvious that there is considerable potential to exploit the endocytosis properties of the receptor or to inhibit its deleterious functions for clinical studies, especially in the field of malignant diseases. However, the development of original pharmacological approaches and therapeutic strategies will require a better decoding and understanding of the overall functionalities associated to this endocytic receptor.

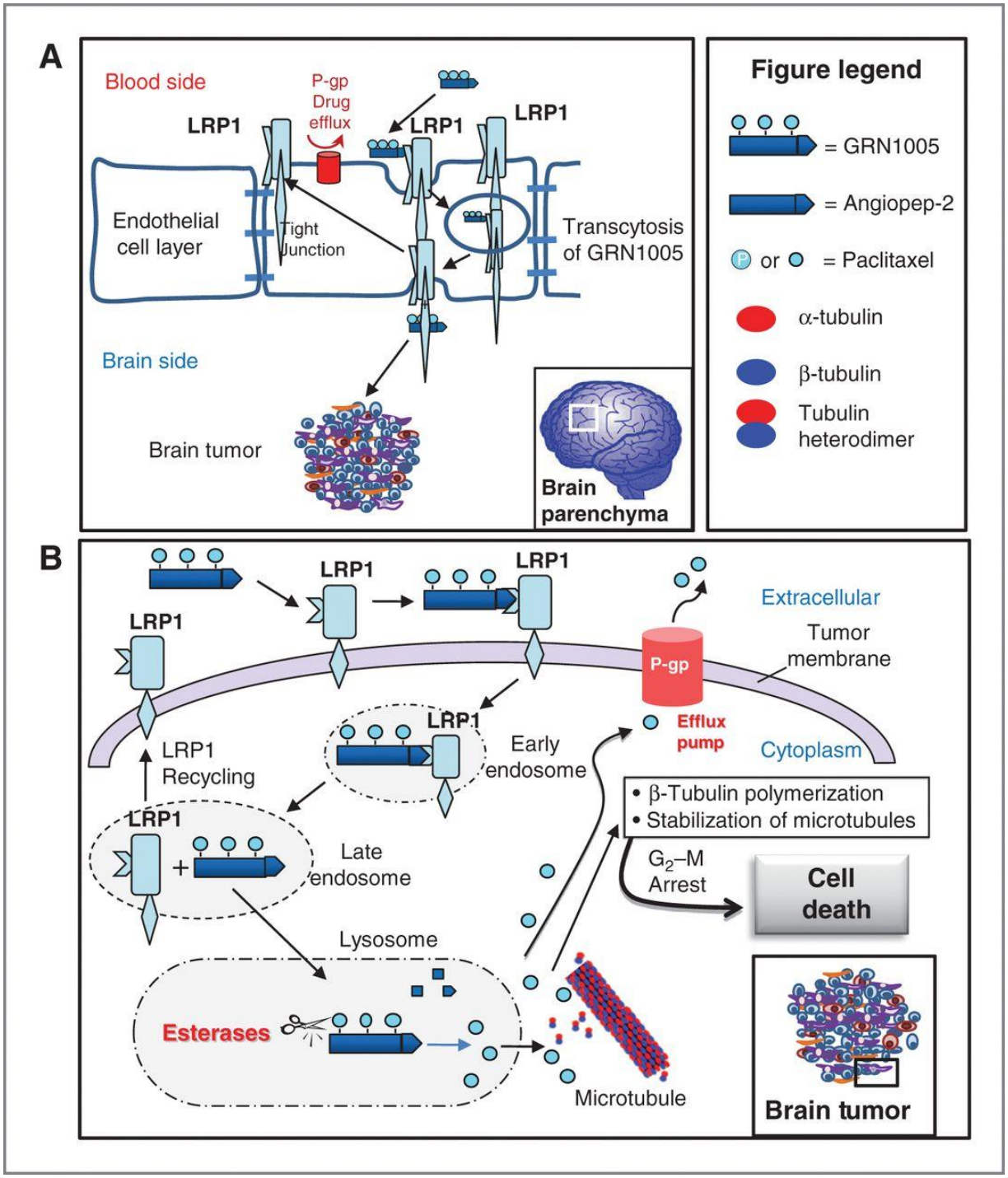

Figure 25. Using LRP-1 as a cargo receptor to transport GRN1005chemotherapy agent*. (**A**) Transcytosis through the BBB using Angiopep-2 targeting LRP-1. (**B**) LRP-1-mediated uptake of GRN1005 into a tumor cell to induce cell death [481].*

Results

Scientific goal statement and presentation of the scientific article

The use of *in vitro* 3D collagen matrices to mimic *in vivo* cellular environment has been developed over the past two decades to improve our understanding of cell growth, survival, migration, and cell-ECM interactions that may occur *in vivo* under physiological and pathological conditions.

Among the cellular interactions within the tumor microenvironment, the interactions between cancer cells and collagen is known to contribute to tumor initiation, progression and metastasis in colorectal cancer (***see our review***). Collagen constitutes a key molecule that influence tumor cell behavior through two main receptors that are integrins and DDRs. DDR1 is expressed in colon carcinomas and appears involved in invasion processes and metastasis development. Data from our laboratory and Dr. Maquoi's group have reported that the proliferation of tumor cells is decreased in type I collagen 3D matrices, but not when using a conventional 2D coating. DDR1 and DDR2 were identified as being the main collagen receptors involved in these processes, while integrins were not engaged.

LRP-1 mediates the endocytosis of a series number of extracellular ligands, which play crucial roles in tumorigenesis and tumor progression. In addition to its endocytic function, LRP1 also mainly regulates signal transduction pathways and can interact with a lot of cellular receptors, themselves involved in intracellular signaling. In the field of cancer, LRP-1-mediated endocytosis was first associated with antitumor properties but original data from our team shed light on the ability of LRP-1 to coordinate the adhesion-deadhesion balance in malignant cells to support tumor progression. LRP-1 has recently been identified as a hub within a biomarker network for multi-cancer clinical outcome and distinct studies reported that the ECM could influence the ability of LRP-1 to modulate the composition and the dynamics of the membrane proteome in the tumor context. Now the vision we have of this receptor, previously considered only as a scavenger receptor, is much more complex than originally thought. Its functionalities clearly vary from one tumor type to another and are highly dependent of the temporality of the tumor evolution and development and the state of the tumor microenvironment. Indeed, several studies have reported that low LRP-1 expression was closely related to advanced tumor stages and poor survival in several solid tumors, such as hepatocellular carcinoma, lung adenocarcinoma, melanoma and Wilms tumors, whereas high LRP-1 expression was related to advanced tumor stages in endometrial, breast and prostate

carcinomas. Furthermore, recent data obtained in our team showed that both LRP-1 mRNA and protein levels were significantly lower in colon adenocarcinoma cells compared with colon mucosa and stroma cells obtained after laser capture microdissection, thus suggesting that loss of LRP-1 expression is associated with worse colon cancer outcomes. However, the role of LRP-1 in CRC remains poorly understood and deserves to be further studied, especially to gain molecular insights.

Therefore, **in my PhD works**, we have investigated how LRP-1 may contribute to CRC development. We investigated how the extracellular matrix from CRC may influence LRP-1 functionalities by using a 3D relevant culture system. As DDR1 is involved in cell proliferation and tightly interacts with type I collagen, we focused our study on the putative interplay that may occur between LRP-1 and DDR1 for the regulation of colon carcinomas proliferation and survival in 3D matrices. The expression of these two receptors were first assessed by qRT-PCR and western-blot in distinct CRC (Caco2, RKO, LS174-T and HT-29 cells). Three cell lines were selected to evaluate the role of LRP-1 in cell proliferation: LS174T and HT29 cells that expressed both LRP-1 and DDR1, and RKO cells expressing only LRP-1. LS174T, HT-29 and RKO cells were seeded in type I collagen 3D matrices or in plastic-based cell culture in presence or in absence of RAP (receptor-associated protein), the LRP-1 antagonist, in order to efficiently inhibit LRP-1-mediated endocytosis. Results from 3D cell cultures highlighted that RAP treatment in LS174T or HT-29 cells led to reduced proliferation, while no effect was observed using plastic-based cell culture systems or classical 2D coating. Interestingly, RAP treatment has no effect on RKO cell proliferation. To confirm these results, we examined the effect of the R2629 polyclonal antibody, a validated LRP-1 blocking antibody. Interestingly, treatment with R2629 decreased the proliferation of both HT29 and LS174T cells to the same extent as using RAP. These experiments were confirmed by LRP-1 knock-down in HT-29 cells. Taken together, these results suggested that LRP-1 supports colon cancer cell proliferation, but only in a 3D type I collagen network.

Since cell proliferation was down-regulated under LRP-1 antagonization only in 3D collagen environment, we hypothesized that DDRs could be involved in this molecular regulation. DDR1 overexpression was conducted in HT-29 using lentivirus encoding for DDR1-GFP. Our results clearly showed that DDR1 overexpression in HT-29 cells results in decreased cell growth in our 3D collagen matrix. Additionally, the contribution of DDR1 on CRC proliferation was reinforced by using treatment with nilotinib, a tyrosine kinase inhibitor. Indeed, we observed a significant increase in CRC proliferation under nilotinib treatment that drastically decreased DDR1 phosphorylation and activation.

We then assessed the potential molecular interaction between LRP-1 and DDR1 using especially co-immunoprecipitation assays. Our data demonstrated that these two cell-surface receptors coexisted within the same molecular complexes in colon cancer cells. Interestingly, DDR1 was found accumulated at the plasma membrane of HT-29 cells when LRP-1-mediated internalization was prevented. Using a biochemical endocytosis assay, we demonstrated that DDR1 uptake was impaired when LRP-1 was antagonized, suggesting that LRP-1 promotes CRC proliferation through DDR1 internalization, and that this function cannot be compensated. To further characterize the role of LRP-1, we investigated whether RAP treatment affects the cell cycle of colon carcinomas using flow cytometry. Cell cycle analysis revealed that RAP treatment led to cell cycle arrest at G0/G1 phase. Interestingly, RAP treatment and LRP-1 blocking antibody induced a higher accumulation of tumor cells at G1 phase when DDR1 is overexpressed.

The inhibition of breast cancer cell growth in type I collagen 3D matrices has been previously attributed to a strong DDR1-dependent induction of apoptotic cell death. To evaluate whether type I collagen/DDR1 axis can induce apoptosis of colon carcinomas, cells apoptosis was evaluated by Annexin V staining and flow cytometry. RAP treatment induces an increase in apoptosis in both wild-type and DDR1-overexpressing HT-29 cells. Furthermore, using a second harmonic generation approach, we found that LRP-1 participates to collagen fiber/fibrin remodeling. This finding provides a crucial information which will require further exploration of collagenase activities and collagen fiber structure, especially using Raman spectroscopy imaging. This will certainly contribute to a better understanding of how LRP-1 may influence the ECM organization.

So, our recent results shed light on a new role of LRP-1 in increasing colon carcinoma proliferation in collagen-rich network through mediating DDR1 endocytosis and regulating cell cycle regulation and apoptosis.

Article

LRP-1 stimule la prolifération des cellules cancéreuses du côlon en induisant l'endocytose de DDR1 dans des matrices 3D de collagène de type I

Article (summary in French)

Introduction

Le récepteur low-density lipoprotein (LDL) receptor-related protein 1 (LRP-1) est un membre de la famille des récepteurs aux LDL. Cette famille est composé de douze protéines transmembranaires participant à plusieurs processus physiopathologiques [485]. LRP-1 est un récepteur d'endocytose multifonctionnel exprimé dans plusieurs tissus et semble être impliqué dans des processus clés tels que le métabolisme des lipoprotéines, l'inflammation, la coagulation etla régulation de la protéolyse matricielle. Initialement synthétisé sous forme d'un précurseur de 600 kDa, LRP-1 est clivé dans l'appareil de Golgi par une furine-convertase pour former une sous-unité de haut poids moléculaire (chaîne α, 515 kDa) et une sous-unité de faible poids moléculaire (chaîne β, 85 kDa) qui seront toutes deux liées de manière non covalente. L'endocytose médiée par LRP-1 est étroitement couplée à la régulation des voies de signalisation [394, 486, 487]. Il a été démontré que le processus d'endocytose dépendant de LRP-1 et les événements de signalisation cellulaire associés jouent un rôle essentiel dans différentes pathologies telles que les maladies de Parkinson et d'Alzheimer, les troubles du métabolisme, l'athérosclérose et le cancer. Concernant la prolifération des cellules tumorales et les processus d'invasion et de métastase, la contribution moléculaire de LRP-1 reste mal connue et dépend fortement du microenvironnement tumoral. Bien que l'expression de LRP-1 et son rôle dans la progression du cancer soient désormais établis dans le gliome [471], le mélanome [461], la thyroïde [25, 28, 488] et le carcinome du sein [489, 490], peu de choses sont connues sur le rôle de LRP-1 dans le développement et la progression du CCR. Des études antérieures ont montré une diminution de l'expression de LRP-1 dans les cellules de CCR [491, 492]. Une étude clinique récente de notre équipe a montré que l'expression de LRP-1 était significativement plus faible dans les cellules de CCR que dans les cellules épithéliales du tissu colique normal [29]. De plus, des mutations de LRP-1 ont été rapportées chez des patients atteints de métastases hépatiques [493].

Le collagène de type I est la protéine matricielle la plus abondante du microenvironnement cellulaire dans de nombreux tissus et joue un rôle crucial dans la progression tumorale de plusieurs tumeurs solides, en particulier du CCR [494]. Cette protéine est sur-exprimée dans le CCR et y présente généralement une densité importante ainsi qu'une topologie associée à une agressivité tumorale [495]. Deux types de récepteurs membranaires peuvent interagir avec le collagène de type I, les hétérodimères de l'intégrine β1 et les récepteurs à domaine discoïdine (DDR). Quatre hétérodimères d'intégrine, α1β1, α2β1, α10β1 et α11β1 sont activés par le

collagène de type I et ont un impact sur la progression de tumorale [496, 497]. DDR1 et DDR2 sont les seuls récepteurs du collagène de type I possédant une fonction tyrosine kinase [263, 300, 498]. L'activation de DDR1 et DDR2 est relativement lente et soutenue par rapport aux récepteurs à fonction tyrosine kinase classiques [259, 499]. L'expression de DDR1 a été associée à une augmentation de l'agressivité de nombreuses tumeurs humaines [296, 305, 306, 500]. Le rôle de DDR1 dans la régulation de la prolifération des cellules tumorales et de l'apoptose reste cependant controversé. Dans le cancer du sein, DDR1 semble être associé fonctionnellement au récepteur dit *insulin-like growth factor-1 receptor* (IGFR) pour stimuler la prolifération cellulaire [305]. Dans les cellules cancéreuses de poumon, il a été montré que l'inhibition de DDR1 diminue la prolifération cellulaire [306]. Cependant, d'autres études ont montré que DDR1 inhibe la prolifération cellulaire et induit l'apoptose dans les cellules de carcinome mammaire [9, 16, 310].

Dans le cas du CCR, des études récentes ont montré que le nilotinib, un inhibiteur spécifique de DDR1, induit une diminution du pouvoir invasif des cellules de CCR ainsi que les métastases *in vivo* [12]. Ces travaux ont été effectués sur des cellules de CCR présentant un phénotype invasif. Concernant les cellules non invasives de type carcinome épithélial, une étude précédente a montré un effet inhibiteur de la prolifération cellulaire des cellules de CCR dans une matrice 3D. Cependant, le rôle de DDR1 dans un tel processus n'a pas encore été établi [32].

Dans ce travail, nous avons étudié si LRP-1, grâce à sa fonction d'endocytose, peut réguler l'expression de DDR1 au niveau de la membrane plasmique et moduler le pouvoir de DDR1 en tant que suppresseur de la prolifération cellulaire. Nos données démontrent pour la première fois que LRP-1 peut induire une endocytose de DDR1 dans des cellules de CCR non invasives, diminuant ainsi la capacité de l'axe collagène de type I / DDR1 à inhiber la prolifération des cellules tumorales et à induire l'apoptose.

Résultats et discussion

Dans un premier temps, et parmi plusieurs lignées cellulaires de carcinome colique, les cellules HT-29 et LS174T, ont été sélectionnées pour leur niveau d'expression de LRP-1 et DDR1. La prolifération des cellules HT-29 et LS174T a été étudiée en matrice 3D et en dépôt 2D de collagène de type I, en présence ou en absence de RAP (*Receptor Associated Protein*), un antagoniste de LRP-1, ou d'un anticorps bloquant spécifique de LRP-1 (R2629). Nos résultats montrent que l'inhibition de LRP-1 entraîne une diminution de la prolifération des cellules cancéreuses d'environ 50%, mais uniquement lorsque les cellules sont cultivées en

matrice 3D de collagène de type I. Afin de confirmer ces données, l'expression de LRP-1 a été invalidée dans les cellules HT-29 grâce à une stratégie d'interférence ARN qui a permis une diminution de l'expression de LRP-1 supérieure à 90%. La prolifération des cellules HT-29 invalidées pour LRP-1 est diminuée d'environ 50% en matrice 3D de collagène de type I alors qu'aucune diminution de la prolifération n'a été observée en 2D, par comparaison avec des cellules infectées avec un shRNA témoin. L'ensemble de ces résultats montre que LRP-1 soutient la prolifération des cellules de cancer colorectal et que cet effet ne se produit que dans un environnement 3D incluant le collagène de type I.

Nous avons alors émis l'hypothèse que LRP-1 pourrait interagir avec DDR1, un des récepteurs clé du collagène de type I, pour exercer ses effets sur la prolifération. L'inhibition de l'endocytose médiée par LRP1 *via* l'antagoniste RAP provoque une augmentation de la présence de DDR1 à la surface des cellules d'environ 50%. D'autre part, des expériences d'endocytose ont permis de démontrer que l'inhibition de LRP-1 diminue de 40% l'internalisation de DDR1. Ces résultats semblent indiquer que LRP-1 pourrait réguler par endocytose la présence de DDR1 au niveau membranaire et ainsi modifier les fonctionnalités de ce récepteur. LRP-1 est connu pour posséder de très nombreux ligands, plus d'une quarantaine ont à l'heure actuelle été répertoriés, mais il peut également, par l'intermédiaire de ceux-ci interagir de manière indirecte avec de nombreuses autres protéines. C'est pourquoi, nous avons décidé de déterminer si LRP-1 et DDR1 étaient suffisamment proches pour interagir et co-exister au sein d'un même complexe protéique. Pour cela, des expériences de co-immunoprécipitation ont été réalisées. Nos résultats montrent que DDR1 co-immunoprécipite avec LRP-1 et inversement, ce qui confirme notre hypothèse de départ.

Des données précédentes ont montré que la prolifération de cellules cancéreuses est inhibée par le collagène de type I dans des cellules cancéreuses mammaires, et ce de manière dépendante de l'activation de DDR1 par le collagène [9, 310]. Etant donné que LRP-1 régule la prolifération cellulaire des cellules HT-29 et l'endocytose des récepteurs DDR1 uniquement en matrice 3D de collagène de type I, nous avons proposé l'hypothèse selon laquelle le niveau d'expression de DDR1 à la membrane plasmique pourrait constituer un paramètre clé du contrôle de la croissance et de la survie des cellules cancéreuses du côlon. Afin de la vérifier, une stratégie de surexpression de DDR1 dans les cellules HT-29 a été adoptée et a permis de montrer que l'augmentation de l'expression de DDR1 dans les cellules HT-29 en matrice 3D de collagène de type I diminuait la prolifération des cellules d'environ 40% par rapport aux cellules sauvages. D'autre part, l'inhibition de LRP-1, soit par RAP soit par l'anticorps R2629, réduit la prolifération des cellules HT-29 surexprimant DDR1 d'environ 60%. De plus,

l'inhibition pharmacologique de l'activation de DDR1 par un agent pharmacologique, le nilotinib, induit une augmentation de la prolifération des cellules HT-29 surexprimant DDR1.

Afin de mieux caractériser le rôle de LRP-1 dans la régulation de la prolifération cellulaire des cellules HT-29, une étude du cycle cellulaire a été effectuée sur des cellules HT-29 traitées ou non par RAP ou R2629. L'ensemble des données montrent que l'inhibition de LRP-1 entraine une augmentation du nombre de cellules en phase G1, suggérant ainsi un arrêt du cycle cellulaire. L'inhibition de la prolifération des cellules de carcinome mammaire induite par les matrices 3D de collagène de type I a précédemment été attribuée à une forte induction de l'apoptose induite par DDR1 [9, 310]. Une étude de l'apoptose a donc été effectuée par marquage à l'Annexine V puis analyse par cytométrie en flux. L'inhibition de LRP-1 par RAP induit une forte augmentation de l'apoptose des cellules HT-29. De plus, la surexpression de DDR1 dans les cellules HT-29 augmente la quantité de cellules apoptotiques par rapport aux cellules contrôles.

Original article

LRP-1 promotes colon cancer cell proliferation in 3D collagen matrices by mediating DDR1 endocytosis

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Abstract

Low density lipoprotein receptor related protein-1 (LRP-1) is a large ubiquitous endocytic receptor mediating the clearance of various molecules from the extracellular matrix. Several studies have shown that LRP-1 plays crucial roles during tumorigenesis functioning as a main signal pathway regulator, especially by interacting with other cell-surface receptors. Discoïdin Domain Receptors (DDRs), type I collagen receptors with tyrosine kinase activity, have previously been associated with tumor invasion and aggressiveness in diverse tumor environments. Here, we addressed whether it could exist functional interplays between LRP-1 and DDR1 to control colon carcinoma cell behavior in three-dimensional (3D) collagen matrices. We found that LRP-1 established tight molecular connections with DDR1 at the plasma membrane in colon cancer cells. In this tumor context, we provide evidence that LRP-1 regulates by endocytosis the cell surface levels of DDR1 expression. The LRP-1 mediated endocytosis of DDR1 increased cell proliferation by promoting cell cycle progression into S phase and decreasing apoptosis. In this study, we identified a new molecular way that controls the cell-surface expression of DDR1 and consequently the colon carcinoma cell proliferation and apoptosis and highlighted an additional mechanism by which LRP-1 carries out its sensor activity of the tumor microenvironment.

Keywords

LRP-1, DDR1, colon cancer cell, proliferation, 3D collagen matrix

Introduction

The low-density lipoprotein (LDL) receptor-related protein (LRP) superfamily contains twelve transmembrane proteins participating in a wide range of physiopathological processes (1, 2). Belonging to this family, LRP-1 is widely expressed in a large variety of tissues and exhibits functionalities in controlling key biological processes such as pericellular protease activities and extracellular matrix (ECM) function. This protein consists of a large functional endocytic receptor firstly synthesized as a 600-kDa precursor cleaved to an extracellular ligand-binding subunit of 515 kDa and a transmembrane 85 kDa part containing a 100 amino acids cytosolic tail. LRP-1-mediated endocytosis is tightly coupled to regulation of signaling pathways (3-5). LRP-1 can indeed regulates mitogen-activated protein kinases (MAPK) as well as the survival-associated PI3K/Akt signaling pathway (6-8). LRP-1-dependent endocytosis and signaling-related events have been shown to play critical roles in severe pathologies including both Parkinson's and Alzheimer's diseases, metabolism dysfunction and cancer. Regarding tumor growth and metastasis, the molecular contribution of LRP-1 remains misunderstood and be highly dependent of the tumor microenvironment. Although LRP-1 expression and its role in cancer hallmarks are now well referenced in glioma (9), melanoma (10), thyroid (11-13), and breast carcinoma (14, 15), little is known about LRP-1 functionalities in colorectal carcinoma (CRC). LRP-1B, a member of LDL-R family highly homologous to LRP-1, is downregulated in the colon cancer tissues and inhibits the growth, migration and metastasis of colon cancer cells (16). Previous studies based on few colon adenocarcinoma samples have shown a frequent loss of LRP-1 immunohistochemical expression in adenocarcinomatous cells (17, 18). A recent clinical study from our team demonstrated that LRP-1 expression was significantly lower in colon adenocarcinoma cells compared to colon epithelial cells and stromal cells and that this decrease in LRP-1 expression is associated with worse patient outcomes (19). Moreover, LRP-1 mutations have been reported in patients with liver metastasis (20). In the light of these data, the role of LRP-1 in CRC remains poorly understood and deserves to be further studied, especially to gain molecular insights.

Type I collagen is one of the main components of the cellular microenvironment in many mammalian tissues and plays a crucial role in tumor progression in several solid tumors, particularly in CRC (21). This protein is highly expressed in CRC with infiltrative growth phenotype (22). Two cellular groups of membrane receptors can interact with type I collagen, β1 integrin heterodimers and discoidin domain receptors (DDRs). DDR1 and DDR2 are the only receptors of collagen harboring a tyrosine kinase function (23-25). Upon collagen binding, activation of DDR1 and DDR2 are associated with a slow and sustained self-phosphorylation in comparison to other tyrosine kinase receptors (26, 27). Indeed, tyrosine residues of DDR receptors are phosphorylated after two hours and can be maintained for several hours. DDR1 expression has been associated with an increase in tumor invasion and aggressiveness of many human tumors, including esophageal cancer (28), gastric cancer (29), glioma (30), breast cancer (31), and lung cancer (32). The role of DDR1 in the regulation of tumor cell proliferation and apoptosis remains poorly documented and somewhat controversial. In breast cancer, DDR1 activates the insulin-like growth factor I receptor (IGF-IR) to support several IGF-IR-mediated biological responses such as cell proliferation (31). In lung cancer cells, DDR1 knockdown has been reported to decrease ERK and Akt phosphorylation leading to a downregulation of cell proliferation (32). However, other studies have demonstrated that, in breast carcinomas, DDR1 promotes apoptosis through induction of pro-apoptotic protein BIK1 (33-35). In the case of CRC, recent studies have shown that nilotinib, a specific inhibitor of DDR1 phosphorylation, strongly reduced DDR1-mediated CRC cell invasion and metastasis in mouse models (36), and that the use of antibody-drug conjugates targeting DDR1 exhibits antitumor effects in a mouse

model of CRC (37). These works have been carried out on CRC cells harboring invasive-like phenotype. Concerning the non-invasive epithelial-like carcinoma cells, a previous study reported a down-regulation of cell proliferation using 3D matrix, but the role of DDR1 in such a process was not explored (38).

In the present work, we investigated whether LRP-1 may control DDR1 expression at the plasma membrane in non-invasive CRC and influence its ability to regulate tumor cell proliferation upon its activation by type I collagen. Our data demonstrate for the first time that LRP-1 can induce endocytosis of DDR1 in CRC, thus decreasing the ability of the 3D collagen matrix/DDR1 axis to inhibit tumor cell proliferation.

Materials and methods

Cell lines

LS174T (Duke's type B) and HT-29 CRC cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). LS174T cells and HT-29 cells were grown in Eagle's Minimum Essential Medium (EMEM) (ATCC 30-2003) or in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) (Thermo scientific) respectively. Culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Dutscher, France) and 1% penicillin-streptomycin (v/v, Invitrogen, Cergy-Pontoise, France). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 (v/v). Cells were routinely passaged at preconfluency using 0.05% trypsin, 0.53 mM EDTA (Invitrogen, 25300) and screened for the absence of mycoplasma using PCR methods.

Vectors, transfection and infection.

DDR1-GFP overexpression was performed using pLVX-CMV-DDR1-GFP construct which was a generous gift from Frederic Saltel (INSERM, UMR1053, BaRITOn Bordeaux Research in Translational Oncology, Bordeaux, France). DDR1-GFP lentiviral particles were generated by transient co-transfection of 293T with pCMV ΔR8.91 (gag-pol) and phCMVG-VSVG (env) expression constructs using the FuGene 6 transfection reagent (Promega) according to manufacturer's recommendations. Three days after transfection, the supernatant containing lentiviruses was collected, filtered through 0.45 µm filter, mixed with fresh medium (1 of 4) and hexadimethrine bromide at 8 µg/ml (Sigma) and used to infect HT-29 recipient cells. GFP control cells were processed in the same way. Infected cells were selected using puromycin (Invitrogen) at 3 µg/ml. LRP1 knock-down was achieved using shRNA sequences previously described (39) that were purchased from Sigma. shRNA LRP1 lentiviral particles were produced in 293T cells using FuGene 6 transfection reagent (Promega) and used to infect HT-29 recipient cells as described above. HT-29 cells expressing control shRNA were generated in the same way. Infected cells were selected using puromycin (Invitrogen) at 3 µg/ml.

2D and 3D cell culture

Fibrillar native type I collagen was extracted from tail tendons of 2-month-old rats and prepared as already described (40). For 2D cell cultures, each well was coated with 5 µg/cm² of collagen solubilized in 0.018 M acetic acid. Coated substrates were dried overnight at room temperature (RT) under sterile conditions. Thereafter, wells were washed two times with PBS (Invitrogen) before cell plating. In cell proliferation studies, cells were seeded on the coated surfaces at a density of 15 x 10^3 cells/well or 5 x 10^3 cells/0.33 cm^2 (24 well plates). In other studies, cell density was adjusted depending on the confluence. For 3D culture, cells were seeded at a final density of 15 x 10^3 cells/mL. For that, 3×10^4 cells were resuspended in 100 µl of FBS and mixed with a solution containing 100 µl of 10X DMEM culture medium for HT-29 cells or

EMEM for LS174T cells, 100 µl NaHCO3 (0.44 M), 90 µl NaOH 0.1 M, 10 µl sodium pyruvate, 10 µL Ampicillin + Streptomycin, (and 10 µl glutamine 200 mM for MEM culture medium), the premix is adjusted to 500 µl with sterile ultrapure water. After that, the mix containing cells is gently homogenized with 500 µl of collagen 3 mg/ml to finalize the collagen-based medium. Then, 1 ml/well of this pre-solidified medium was deposited in 24-well plates, and collagen gel solidification was performed at 37°C during 30 min. Finally, 1 ml of complete culture medium was added on top of each gel and the plates were incubated at 37°C. Covering medium is changed every 2 days. After 3 or 5 days, the covering medium was removed, and gels were digested with 2 mg/ml collagenase P (Roche). Viable cells were determined by phase contrast microscopy using Kova Glasstic Slides (Kova International Inc, Garden Grove, CA, USA).

Antibodies and recombinant proteins

Anti-LRP1 β-chain antibody (clone EPR3724) was purchased from Abcam (Cambridge, UK). Rabbit monoclonal antibodies against DDR1 (D1G6), phospho-DDR1 (Tyr792, 4G10), GFP (D5.1) and GAPDH (14C10) were purchased from cell signaling. IgGs used as a negative control for immunoprecipitation was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Blocking LRP-1 polyclonal antibody (R2629) was a generous gift from Dr. D.K. Strickland (Department of Surgery, University of Maryland School of Medicine, Baltimore, MD, USA) (41). Histidine-tagged RAP was purified as previously described (39).

RNA isolation and qPCR

Total mRNAs were extracted using TRIzol reagent (Thermofisher), isolated from other cellular materials by chloroform/isoamyl alcohol (24:1) precipitation before centrifugation (12,000 × g, 4°C, 15 min), as described previously (12). 250 ng total mRNAs were reverse-transcribed using VERSO cDNA kit (Thermofisher) according to the manufacturer instructions. Real-time PCR was then performed using an Absolute SYBR Green Rox mix (Thermofisher) and a CFX 96 real time PCR detection system (Bio-Rad, Hercules, CA, USA). The cycle threshold (Ct) values were recorded using Bio-Rad CFX Manager 3.0 software (Bio-Rad) (42). PCR primers were synthesized by Eurogentec (Liege, Belgium) as follow (5'-3'): for LRP1: GCTATCGACGCCCCTAAGAC and CGCCAGCCCTTTGAGATACA; for DDR1: TGCTCTCCAATCCAGCCTAC and ATTATGCCGAGGCTGACATT; for RS18: GCAGAATCCACGCCAGTACAA and GCCAGTGGTCTTGGTGTGCT; for RPL32: CATTGGTTATGGAAGCAACAAA and TTCTTGGAGGAAACATTGTGAG.

Total protein extraction and immunoblotting

Cells were seeded in 3D type I collagen matrix for 5 days, then were harvested from digested matrices using collagenase P (2 mg/ml), washed twice with PBS, and lysed. The cells were then pelleted by centrifuging at 1000 rpm for 5 min. Whole-cell extracts were lysed in RIPA buffer (Thermofisher), sonicated and then incubated on ice. Cell lysates were collected after a centrifugation at 14000 rpm and 4°C for 15 min. Protein concentration was quantified by BCA assay (Thermofisher). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% skimmed milk (m/v) in Tris buffered saline (0.02 M Tris-HCl, 0.137 M NaCl, pH 7.4), supplemented with 1% Tween 20 (v/v) at RT for 1 hour. Blocked membranes were incubated with antibodies against LRP-1 β-chain (EPR3724), DDR1 (D1G6) and GAPDH (14C10) overnight at 4°C under gentle agitation. Finally, membranes were incubated with corresponding peroxidase conjugated secondary antibody at RT. Chemiluminescent reactions were revealed by using ECL Prime Kit

(GE Healthcare, Orsay, France), signal was detected by the Odyssey-FC system (Licor, Lincoln, NE, USA).

Cell surface protein isolation

The cells were treated with or without 500 nM RAP for 1 hour, washed twice with PBS before suffering a biotinylation with 0.5 µg/mL of EZ-Link sulfo-NHS-LC-biotin (Thermofisher) in cold PBS. After 3 washes, biotinylated cells were incubated with 100 mM glycine at 4°C during 30 min to limit nonspecific binding. Cells were washed three times with PBS before protein extraction. Cells were scrapped in cold lysis buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 2 mM Na3VO4, 5 mM EDTA, 1% Triton X-100, supplemented with protease inhibitor cocktail), followed by a quick sonication on ice. Cell extracts were pelleted at 10,000 g (20 min, 4°C) before protein quantification. Solubilized biotinylated proteins (200 µg) were then affinity purified using 40 µL of streptavidin-agarose beads (GE Healthcare), overnight at 4°C under gentle agitation. After washes with lysis buffer, Laemmli buffer was added and samples were heated at 100°C for 5 min and resolved by SDS-PAGE followed by immunoblotting analysis.

Endocytosis assay

Endocytosis assays were adjusted from validated method previously described (12). Cell-surface proteins were labeled using 0.5 µg/mL of EZ-Link sulfo-NHS-LC-biotin (Thermofisher) in cold PBS at 4°C for 30 min. After washes with PBS, cells were incubated with 100 mM glycine at 4°C for 15 min. Nonspecific binding and free biotin were discarded by warm PBS washes before addition of warm medium supplemented with 10% FBS. Cells were treated with 500 nM RAP at 37°C for 1 hour to antagonize endocytosis function of LRP-1. Cells were then quickly placed on ice to block internalization activities. After 3 washes with PBS, cells were incubated with 50 mM glutathione in cold buffer (75 mM NaCl, 75 mM NaOH, 1 mM MgCl2, 0.1 mM CaCl2, 10 mM EDTA, pH 8.6) at 4°C for 30 min to remove remaining biotin at the cell surface. To evaluate the total amount of surface biotinylation, one culture dish was kept on ice after biotin labeling and preserved from glutathione treatment. Whole-cells extracts were prepared as described above. Internalized DDR1 was determined from 350 µg of cell lysate by adding 40 µL of streptavidin-agarose beads (GE Healthcare), incubating overnight at 4°C under gentle agitation and using DDR1 antibody through immunoblotting, as described above.

Immunoprecipitation

Whole cell extracts from HT-29$^{DDR1\text{-}GFP}$ were performed as described in a previous study (12). Whole cell lysates were subjected to immunoprecipitation using anti-LRP-1 (EPR3724), anti-DDR1 (D1D6) antibodies or nonspecific IgGs at 4°C for 12 h, bound to protein G sepharose beads (GE Healthcare) at 4°C for 2 h and finally washed 3 times with cold lysis buffer followed by a protein denaturation step at 100°C for 5 min. After that, the samples were centrifuged at 10000 rpm for 1 min, supernatants were then subjected to a western blot analysis using anti-LRP-1 β-chain (clone EFR3724), anti-DDR1 (D1D6) and anti-GFP antibodies.

DDR1 phosphorylation analysis

HT-29 and HT-29 overexpressing DDR1-GFP (HT-29$^{DDR1\text{-}GFP}$) cells were cultured in 3D type I collagen matrices with or without 50 nM nilotinib treatment for 16 h. Matrices were digested before undergoing a standard procedure for total protein extraction in 3D (40). Then, 300 µg of whole-cell extracts were immunoprecipitated with anti-DDR1 (D1D6), as described above. The proteins were separated by SDS-PAGE and the immunoprecipitates were blotted with anti-

phosphotyrosine antibody, clone 4G10 (Millipore, 05-321). The blots were then stripped using a stripping buffer (200 mM glycine, 1% SDS, 0.02% sodium azide, pH 2.5) and re-probed with anti-DDR1 antibody.

Cell cycle

Double thymidine block procedure was adapted from an established protocol (43). Specifically, HT-29 and HT-29$^{DDR1\text{-}GFP}$ cells were cultured in medium supplemented with 2 mM thymidine for 18 h then switched to thymidine-free medium for 9 h. After 2 washes with PBS, cells were cultured again in medium supplemented with 2 mM thymidine for 15 h. Cells were released by washing twice with PBS before trypsinization. The synchronized cells were then seeded into 3D type I collagen matrices with or without 1 µM RAP treatment for 24 h. Collagen matrices were further digested to harvest cultured cells. Lastly, cells were washed twice with PBS and stained with nuclear isolation medium-4,6-diamidino-2-phenylindole dihydrochloride named NIM-DAPI (NPE Systems, Pembroke Pines, FL) at RT for 5 min. The samples were analyzed with an Accuri-C6 Special Order Product (BD Bioscience) by acquisition of 20000 events. Analysis was performed with an excitation wavelength of 375 nm and fluorescence detection at 427 ± 10 nm.

Apoptosis assay

HT-29 and HT-29$^{DDR1\text{-}GFP}$ cells were cultured in 3D type I collagen matrices with or without 1 µM RAP treatment for 3 days. The culture medium was replaced every 2 days by fresh complete DMEM medium with or without 1 µM RAP. After 5 days, cells were harvested as described above. Harvested cells were washed with PBS before suffering a quick trypsinization. The single cells were then incubated with Annexin V-iFluor 647 Apoptosis solution (Abcam, UK), supplemented with propidium iodide (Sigma-Aldrich). The incubation was carried out at RT for 30 min. Apoptosis assays were performed using flow cytometer, FL4 channel (BD Biosciences, San Jose, CA, USA).

Immunofluorescence

HT-29$^{DDR1\text{-}GFP}$ cells were seeded onto collagen-coated glass slides for 48 h at 37°C and then fixed in PBS containing 4% paraformaldehyde for 15 min at RT. After three washes with PBS, cells were incubated for 30 min in PBS containing 1% bovine serum albumin and then incubated overnight at 4°C with GFP primary antibodies. Then, after 5 washes with PBS, cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 (1/1000) during 1 h at RT. DAPI was added during washes. Slides were incubated with mounting medium. Immunofluorescence-labeled cell preparations were analyzed using a Zeiss LSM 710 confocal laser scanning microscope with the 63×oil-immersion objective Zeiss operating system (Carl Zeiss MicroImaging GmbH, Deutschland).

Data analysis

All statistical results were analyzed from at least three independent experiments. Data were represented as the standard deviation (SD) using Graphpad Prism software. Student's t-test and ANOVA test were used for statistical analysis. Immunoblotting images were analyzed by ImageJ software.

Results

LRP-1 inhibition decreases colon carcinoma cell proliferation only in 3D collagen matrices.

To study the involvement of LRP-1 and DDR1 in the regulation of colon tumor cell proliferation by 3D collagen matrix, the endogenous expression level of LRP-1 and DDR1 was analyzed by both RT-qPCR and immunoblotting in LS174T and HT-29 cells (Fig. 1). Results showed that the expression of the two receptors at the mRNA and protein levels in HT-29 cells are higher than in LS174T cells.

We then examined the effect of LRP-1 inhibition on HT-29 and LS174T cell proliferation in 2D and in 3D collagen matrices. For this purpose, we compared the cell proliferation after 5 days of culture in the presence or absence of RAP (receptor associated protein), the LRP-1 antagonist, or its blocking antibody (R2629). As shown in Figs. 2A and 2B, in both cell lines, treatment by RAP or R2629 did not modify cell proliferation in 2D collagen coating. By contrast, 3D-cell proliferation was decreased by about 50% in each cell line when using RAP or R2629 treatment. To focus on the role of LRP-1 in the regulation of cell proliferation in 3D collagen matrices, an RNA interference strategy against LRP-1 was performed in HT-29 cells. Two different cell lines that stably overexpressed a specific shRNA for LRP-1 (shLRP-$1_{(a)}$ and shLRP-$1_{(b)}$) were selected, and a control cell line was established after infection with control shRNA (shCTRL). The endogenous level of LRP-1 was assessed by both RT-qPCR and immunoblotting (Fig. 2C, left panel). As expected, infection with lentiviruses expressing shCTRL had no effect on the LRP-1 expression level while LRP-1-specific shRNA was able to efficiently knock-down the expression of LRP-1 at the mRNA level (data not shown) as well as at the protein level by about 90%. The same inhibition was observed for both shLRP-1 cell lines (Fig. 2C, left panel). As shown in Fig. 2C (right panel), proliferation of LRP-1-silenced cancer cells was decreased by about 50% in 3D collagen matrices, whereas no effect of LRP-1 silencing was observed in 2D (data not shown). Taken together, these data indicate that LRP-1 sustains colon cancer cell proliferation, and that this process occurs only in a 3D collagen environment.

LRP-1 and DDR1 coexist within the same molecular complexes.

Since LRP-1 had a positive effect on cell proliferation only in 3D collagen environment by LRP-1, we hypothesized that LRP-1 could interact with DDR1, one of the key collagen membrane receptors, to induce its endocytosis. To validate this hypothesis, we first evaluated whether LRP-1 may influence the DDR1 amount at the plasma membrane of HT-29 cells. After cell-surface protein labeling with the membrane-impermeable biotinylation reagent sulfo-NHS-LC-biotin, biotinylated proteins were selectively recovered from cell extracts by streptavidin affinity precipitation, and DDR1 was detected in the affinity precipitates by immunoblot analysis. After RAP treatment, DDR1 was found to accumulate at the plasma membrane fraction (Fig. 3A), suggesting that LRP-1 mediates DDR1 internalization. Thus, we investigated DDR1 uptake by using a previously validated endocytosis assay (12). This method requires labeling of cell surface proteins using the non-membrane permeating sulfo-NHS-LC-biotin at 4°C, then moving to a permissive temperature for endocytosis (37°C). Cell surface protein biotinylation as well as efficiency of biotin stripping with glutathione were controlled (Fig. 3B, left panel). As shown in Fig. 3B (right panel), DDR1 internalization was decreased by about 40% when LRP-1-mediated endocytosis was antagonized by RAP treatment. To test whether LRP-1 and DDR1 may participate in a common biomolecular complex, coimmunoprecipitation experiments were carried out in DDR1 overexpressing HT-29 cells (HT-$29^{DDR1\text{-}GFP}$). As shown in Fig. 3C, HT-$29^{DDR1\text{-}GFP}$ expressed a high level of recombinant DDR1-GFP. Our data clearly demonstrated that DDR1 was coimmunoprecipitated with LRP-1 (Fig. 3D). Reverse immunoprecipitation experiments with anti-DDR1 were also performed using the same cell lysates. The data presented in Fig. 3E confirmed that LRP-1 and DDR1 were detected in the same molecular complexes in colon carcinomas.

LRP-1 promotes HT-29 proliferation in a DDR1 dependent fashion.

Previous reports demonstrated that cancer cell growth was downregulated by 3D type I collagen matrix in epithelial-like breast carcinoma cells and that this was dependent on activation of DDR1 by collagen (33, 34). Considering that LRP-1 induced HT29 cell proliferation in 3D collagen matrices (Fig. 2) and drives endocytosis of DDR1 (Fig. 3), we assume that the cell-surface expression level of DDR1 may constitute a key parameter to control growth and survival of colon cancer cells. To address this hypothesis, we compared the cell proliferation of HT-29$^{DDR1\text{-}GFP}$ and control counterparts. As expected, overexpression of DDR1 led to decreased cell proliferation in collagen 3D matrices by about 40%, compared to control cells (Fig. 4A). Furthermore, cell proliferation in collagen 3D matrices was decreased by about 60% under RAP or R2629 antibody treatments in HT-29 cells overexpressing DDR1-GFP (Fig. 4B). Interestingly, the proliferative inhibition under LRP-1 antagonization was more important when DDR1 was overexpressed.

DDR1 activity is necessary to induce cell proliferation in HT-29$^{DDR1\text{-}GFP}$ cells.

We then evaluated the effect of the inhibition of DDR1 kinase activity on cell proliferation using the pharmacological inhibitor nilotinib, in both control (Fig. 4C) and HT-29$^{DDR1\text{-}GFP}$ (Fig. 4D) cells. Nilotinib treatment had no effect on cell proliferation in control cells (Fig. 4C, right panel) whereas carcinoma cell proliferation in DDR1-overexpressing cells was increased after nilotinib treatment (Fig. 4D, right panel). Consistently, DDR1 phosphorylation was drastically inhibited upon nilotinib treatment in HT-29$^{DDR1\text{-}GFP}$ cells (Fig. 4D, left panel).

LRP-1 inhibition induces cells cycle arrest in the G0/G1 phase.

To further characterize the role of LRP-1 in the regulation of cancer cell proliferation, we investigated whether RAP treatment affects the cell cycle of HT-29 colon carcinomas. First, HT-29 and HT-29$^{DDR1\text{-}GFP}$ cells were synchronized in G0/G1-phase by double thymidine blocking. The cells were then seeded in 3D collagen matrix to allow their re-entry into the cell cycle. The results of flow cytometric analysis revealed that HT-29 cells treated by RAP displayed an increased cell proportion in G1-phase (35% vs 19%) and a decreased cell proportion in (S+G2-M)-phase (60% vs 75%), compared to non-treated cells (Fig. 5A). Moreover, the effect of RAP treatment on G1 and (S+G2-M)-phases was higher in HT-29$^{DDR1\text{-}GFP}$ (54% and 48%, respectively) (Fig. 5B). To confirm whether LRP-1 inhibition affects the G1/S transition, HT-29 and HT-29$^{DDR1\text{-}GFP}$ were treated with the R2629 blocking antibody (Figs. 5C and 5D). R2629 treatment has confirmed the obtained data wherein cells were treated with RAP. In fact, R2629-treated cells displayed also an increase in the proportion of cells in G1-phase and a decrease in S-phase cell population, compared to non-treated cells (Figs. 5C and 5D).

LRP-1 counteracts the DDR1-dependant promotion of apoptosis in colon carcinomas.

The inhibition of breast cancer cell growth induced by type I collagen 3D matrices has been previously attributed to a strong DDR1-dependent induced apoptosis (33, 34). To evaluate whether type I collagen/DDR1 axis can induce apoptosis in colon carcinoma, the apoptosis assay was performed using Annexin V staining and flow cytometry. As shown in Figs. 6A and 6B, LRP-1 antagonization by RAP resulted in an increase in the proportion of apoptotic and necrotic cells in 3D collagen environment. Interestingly, this effect was higher in HT-29$^{DDR1\text{-}GFP}$ cells (15,0% of apoptotic cells), compared to HT-29 cells (5,9% of apoptotic cells). The ability of DDR1 to increase apoptosis of colon carcinomas was confirmed in Fig. 6C. These results were corroborated by immunofluorescence experiments. As shown in Fig. 6D, the assay

consistently shown the increased presence of nuclear condensation and DNA fragmentation upon LRP-1 inhibition.

Discussion

The findings of this study have highlighted the first ever molecular association between LRP-1 and DDR1 in colon carcinoma. Indeed, we showed that the endocytic receptor LRP-1 established tight molecular connections with DDR1 at the plasma membrane of colon cancer cells. In this tumor context, we provide evidence that LRP-1 promotes cell proliferation through regulating the levels of membrane DDR1 in 3D collagen matrices. The LRP-1 mediated endocytosis of DDR1 supports colon carcinoma cell proliferation by promoting the entry of cell cycle to the S phase and decreasing apoptosis.

LRP-1 is considered as a key integrator of signals from the ECM and a multifunctional regulator of cancer-related events. Its overall function remains nevertheless extremely complex to decipher especially because the deregulation degree of its expression is highly variable depending on the type of tumors and the stage of cancer progression. In malignant diseases, the current trend seems to correlate LRP-1 overexpression with poor prognostic, increased cell proliferation, invasiveness and tumor recurrence (14, 44, 45). To date, few studies have examined the contribution of LRP-1 in the field of CRC despite obvious clinical interest. We have recently highlighted that low LRP-1 immunohistochemistry score in malignant colon adenocarcinoma cells is a strong prognosis marker (19). We especially reported that in patients with metastases, LRP-1 expression predicts a shorter overall survival, especially when patients were treated by anti-VEGF therapies. The lower expression of LRP-1 in malignant cells is partly explained by LRP-1 gene mutation through the hypermutator type of CRC. In the present study conducted using relevant 3D collagen matrices, we showed in a surprising way that LRP-1 inhibition decreased colon carcinoma cell proliferation. Although these results seem to be conflicting with the previous data (19), it could be explained by the fact that the studied cell lines in this work are non-invasive cells. Although it is well documented that LRP-1 may activate crucial downstream signaling pathways such as Ras, c-Myc, MAPK, and Akt/PI3K, which are widely known as oncogenic pathways, especially in cell proliferation and survival processes (46), very few data have previously involved LRP-1 during cancer cell proliferation steps. Salama and collaborators reported the involvement of LRP-1:tPA pathway in promoting melanoma cell migration and proliferation (10). Their results, using loss- and gain-of-function strategy demonstrated a model wherein LRP-1 drives melanoma growth and metastases by enhancing ERK activation resulting in increased proteolytic events and in changing the cellular content within the tumor. Data from Beaujouin and colleagues also revealed that secreted pro-cath-D binds to LRP-1 promoting human mammary fibroblast outgrowth (15).

Interestingly, our findings stressed that LRP-1 displays a pro-proliferative effect on colon cancer cells only in 3D type I collagen matrices. During tumor progression, especially after degradation of the basement membrane, type I collagen is a key component of the stroma at the invasion front of human colorectal cancer (21). In addition to its properties as a scaffold protein, type I collagen can induce different cellular signaling pathways, which regulate several functions of tumor cells (47). Accumulating evidence suggest that DDR plays a key role in cancer progression by regulating the interactions of cells with the stromal collagen (36, 48-50). Data obtained on HT-29 cells demonstrated that inhibition of LRP-1-dependent endocytosis by either RAP or R2629 antibodies led to membrane DDR1 accumulation in the same extent. We then demonstrated that LRP-1 and DDR1 are tightly associated in the same biomolecular complexes at the plasma membrane of colon carcinoma to constitute a new endocytosis complex. These results are even more interesting, as so far, little information is available

concerning the regulation of DDR1 expression at the cell membrane. It is nevertheless known that activated DDR1 undergoes aggregation followed by cytoplasmic internalization and incorporation into early endosomes (51). In mouse fibroblasts, DDR1 was reported to be internalized alone or complexed with other receptor tyrosine kinases (RTKs). Indeed, IGF-I receptor can phosphorylate DDR1 in breast carcinoma thus induce co-internalization of the receptors and incorporation into early endosomes (31). Internalized RTKs can recycle back to the plasma membranes, be degraded, or undergo an endosome/Golgi/endoplasmic reticulum retrograde pathway. Interestingly, a novel mechanism whereby activated DDR1 plays a role of transcription factor has been demonstrated in injured human and mouse kidney proximal tubules (52).

Our findings showed that LRP-1 exerts its proliferative effects by down-regulating the amount of DDR1 at the plasma membrane. Indeed, by inducing the endocytosis of DDR1, LRP-1 counteracts the negative effect of DDR1 on cancer cell proliferation. Antagonization of LRP-1 by RAP or blocking antibodies indeed induced a significant cell cycle arrest in G1 phase, and this is magnified under DDR1 overexpression. Moreover, inhibition of LRP-1 by RAP treatment increases apoptosis of wild-type HT-29 cells and more importantly of HT-29$^{DDR1\text{-}GFP}$. In a coherent way, overexpression of DDR1 in HT-29 cells favors cell cycle arrest and apoptosis of colon carcinoma in 3D environment. These data are consistent with those previously obtained by Erik Maquoi's group demonstrating that MCF-7 and ZR-75-1 breast carcinoma cell growth was reduced in 3D type I collagen gels, but not when the cells were plated on a 2D matrix (33, 53). Moreover, type I collagen was able to induce apoptosis in these cells. In fact, type I collagen can activate DDR1 to induce the expression of BIK, a pro-apoptotic member of the BCL-2 protein family, thereby triggering apoptotic cell death in these breast cancer cell lines (33). In addition, our group already demonstrated that young collagen inhibited cell proliferation and induced apoptosis when compared to the old one, due to a higher level of DDR1 phosphorylation (33, 34). Furthermore, DDR2 is able to inhibit proliferation of human melanoma and fibrosarcoma cells by inducing a growth arrest in the G0/G1 phase of the cell cycle when the cells were plated on fibrillar collagen. This process was shown to be induced through p15INK4b cyclin-dependent kinase inhibitor, suggesting that this protein could be a downstream target of DDR2 signaling (54-56). Moreover, DDR2, upon activation by 3D collagen, was able to target the cell cycle by increasing the expression of the cyclin-dependent kinase inhibitor p21^{CIP1} and thus inhibiting cell proliferation in a fibrosarcoma model (40). In contrast, DDR1 activation can also induce pro-survival signals (57). In colon carcinoma cells, DDR1 regulates the cleavage of Notch 1by a γ-secretase and the subsequent release and translocation of its intracellular domain to the nucleus to stimulate pro-survival genes (58). The collective findings suggest that DDR1 can induce survival as well as apoptosis, highly depending on experimental settings.

Finally, we identified a new molecular way that controls the cell-surface expression of DDR1 and suggested an additional role of LRP-1 as a key sensor of the tumor microenvironment.

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Author contributions

C.C.L. was responsible for the execution of experiments, data analysis and preparation of the paper. C.H., G. C., A. B. and V. L. supported the experimental work and data analysis. A.B. contributed to the interpretation of the results and the writing of the paper. A. A.-C., H. M. and S. D. designed, supervised the study and wrote the paper. All authors critically commented on and approved the final submitted version of the paper.

Additional informations

Conflict of interest: The authors declare no competing interests.

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Figures for Original Article

Fig. 1.
Molecular characterization of colorectal carcinoma cell lines. **a** Transcriptional level of LRP-1 and DDR1 were assessed using RTqPCR. LRP-1 and DDR1 mRNA expression levels in HT-29 (black boxes) and LS174T (grey boxes) were normalized with both RPL32 and RS18 mRNA expression. **b** Whole cell extracts from HT-29 and LS174T cells were analyzed by SDS PAGE followed by western blotting using anti-DDR1, anti-LRP-1 and anti-GAPDH antibodies. Graphical representations of LRP-1 (**c**) and DDR1 (**d**) expression at protein level as normalized with GAPDH. All experiments were performed in three biological replicates. Plots are presented as the mean SD, **: $p < 0.01$; ****: $p < 0.0001$, n=3, two sample t-test.

Fig. 2.
Effect of LRP-1 antagonists and LRP-1 knockdown on colorectal cancer cell proliferation. LS174T (**a**) and HT-29 (**b**) cells were cultured in 2D type I collagen coating (left panels) or 3D type I collagen matrices (right panels) without (black boxes) or with RAP (500 nM, light grey boxes) or R2629 (2.5 µg/mL, dark grey boxes) treatment. After 5 days of culture, cell growth indices were assessed using at least three separate sets of culture, all conditions were repeated at least three times. (**c)** HT-29 cells were transduced with lentivirus encoding non-silencing shRNA (shCTRL) or shRNA targeting LRP-1 ($shLRP1_{(a)}$ and $shLRP1_{(b)}$) (right panel). Whole-cell extracts from each clonal cell were submitted to immunoblot analysis using anti-LRP-1 antibody (5A6). GAPDH expression level served as a loading control. shCTRL (black boxes) and $shLRP\text{-}1_{(a)}$ or $shLRP\text{-}1_{(b)}$ (grey boxes) HT-29 cells were seeded in 3D type I collagen matrix (left panel) during 5 days with or without RAP and R2629 treatment. Cell growth was evaluated by at least three separate experiments, each done in triplicate. The data are presented as the mean SD. ***: $p < 0.001$; ****: $p = 0.0001$; ns: not significant, One-way ANOVA test using Dunnett's multiple comparisons.

Fig. 3.
RAP treatment inhibits DDR1 endocytosis and led to its accumulation at the plasma membrane. **a** Plasma membrane extracts from cell surface biotinylated proteins were obtained from HT-29 cells treated or not with RAP (1µM, 1 h). Immunoblot analysis was performed using anti-DDR1 antibodies. Expression level of GAPDH in the intracellular fraction served as a loading control and for normalization. Three independent experiments were conducted, the data is represented as the mean SD. **: $p < 0.005$, two sample t-test. **b** HT-29 cells were treated with/without RAP (1µM) for 1 h. Plasma membrane proteins were biotinylated and endocytosis assay was carried out as reported in the experimental procedure section. DDR1 internalization was quantified by immunoblotting using DDR1 antibody (right panels including graph, ****: $p < 0.0001$, two sample t-test). Left panel (4°C) serves to control DDR1 binding to the cell surface (-Glut, without glutathione) and glutathione efficacy for biotin stripping (+ glut, with glutathione). **c** Whole-cell extracts were obtained from HT-29 cells overexpressing GFP (control) or DDR1-GFP. Immunoblot analysis was performed using anti-DDR1 and anti-GFP antibodies and GAPDH served as a loading control. LRP-1 (**d**) or DDR1 (**e**) containing complexes were immunoprecipitated (IP) from DDR1-GFP overexpressing HT-29 cells whole-cell extracts by using anti-LRP-1 (clone EPR3724) or anti-DDR1 (D1G6) monoclonal antibody, respectively. Immunocomplexes were then subjected to SDS-PAGE and immunoblotted (IB) by using specific antibodies for LRP-1, DDR1 and GFP.

Fig. 4.
DDR1 down-regulates colorectal cancer cell proliferation in 3D collagen matrix. **a** Wild-type HT-29 were cultured in 3D type I collagen matrix for 5 days, then cell proliferation was

evaluated by three independent experiments. The data are represented as the mean SD, **** $p < 0.0001$, two sample t-test. **b** HT-29$^{DDR1\text{-}GFP}$ cells were seeded in 3D type I collagen matrix during 5 days with/without RAP or LRP-1 blocking antibodies (R2629). Cell proliferation was then evaluated by at least 3 separate sets of culture, the data are presented as the mean SD and compared to untreated cells. ****: $p = 0.0001$, One-way ANOVA test using Dunnett's multiple comparisons. HT-29 (**c**) and HT-29$^{DDR1\text{-}GFP}$ cells (**d**) were seeded in 3D type I collagen matrix and cultured with 50 nM nilotinib or DMSO (that served as a control) for 5 days. Left panels: DDR1 containing complexes were immunoprecipitated (IP) whole-cell extracts by using an anti-DDR1 monoclonal antibody (D1G6). Immunocomplexes were then subjected to SDS-PAGE and immunoblotted (IB) by using anti-DDR1 (D1G6) and anti-phospho-DDR1 (Tyr792, 4G10). The bottom panel indicates the expression of DDR1 and GAPDH in whole cell lysates and served as a control. Right panels: cell proliferation was evaluated by three independent experiments, the data are presented as the mean SD. **: $p < 0.005$; ns: not significant, two sample t-test.

Fig. 5.
Inhibition of LRP-1-mediated endocytosis induces cell cycle arrest at G1 phase. HT-29 (**a** and **c**) and HT-29$^{DDR1\text{-}GFP}$ (**b** and **d**) cells were grown on plastic surface and synchronized by double thymidine block. Synchronized cells were then seeded in 3D type I collagen matrix with or without 1µM RAP (**a** and **b**) or LRP-1-blocking antibodies (R2629, 30 µg/mL) (**c** and **d**) for 24 hours, followed by a cell cycle analysis. After nuclear staining with DAPI, 20.000 events were acquired and analyzed by flow cytometry. On the left colored panels, cell cycle distributions of HT-29 (**a** and **c**) and HT-29$^{DDR1\text{-}GFP}$ (**b** and **d**) cells treated with or without RAP or R2629 for 24 hours are shown as histogram plots of the FL3 fluorescence channel. On the right panels, histograms represent the percentage of interphase stages (G1, S+G2/M) and the relative (S+G2-M)/G1 ratio of HT-29 (**a** and **c**) or HT-29$^{DDR1\text{-}GFP}$ (**b** and **d**) cells treated with (grey boxes) or without (black boxes) RAP or R2629. The data are presented as the mean SD. *: $p < 0.05$; **: $p = 0.01$, two samples t-test. Cell cycle assays were performed in four separate biological experiments for RAP treatment (**a**-**b**) and two separate experiments, each conducted in double triplicates for R2629 treatment (**c**-**d**).

Fig. 6.
Inhibition of LRP-1 results in an increase in apoptosis. HT-29 (**a**) and, grey boxes HT-29$^{DDR1\text{-}GFP}$ cells (**b**) were seeded in 3D type I collagen matrix and were treated without (black boxes) or with RAP (1µM) for 3 days. The cells were then collected from digested matrix and suffered a rapid trypsinization before underwent an apoptotic assay. Apoptotic cells were stained with Annexin V and histogram (left panel), showed the percent of apoptotic. The values of treated samples were normalized to their controls, the data are represented as the mean SD, *: $p < 0.05$, two sample t-test. (**c**) The plot represents the apoptotic indices of wild-type HT-29 overexpressing GFP (black boxes) and HT-29$^{DDR1\text{-}GFP}$ (grey boxes) cells seeded in 3D collagen matrix. The apoptosis assays were performed in two distinct experiments, each done in double triplicates. (**d)** Immunostaining of recombinant DDR1 (green) in untreated (left picture) or RAP-treated (right picture) HT-29$^{DDR1\text{-}GFP}$ cells. DNA is stained with DAPI. Scale bar: 5 µm.

Figure 1. Cuong *et al.*

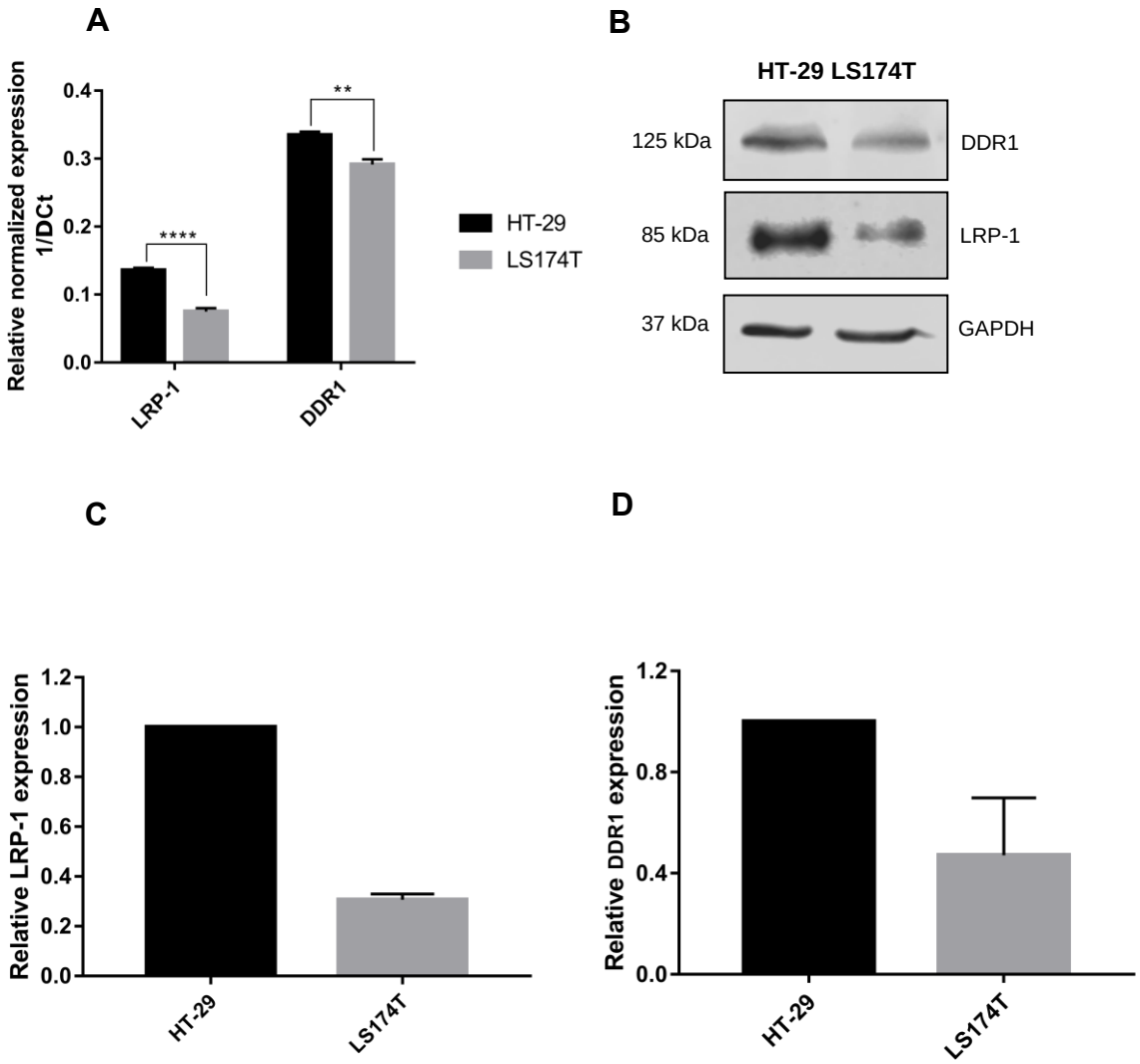


Figure 2. Cuong *et al.*

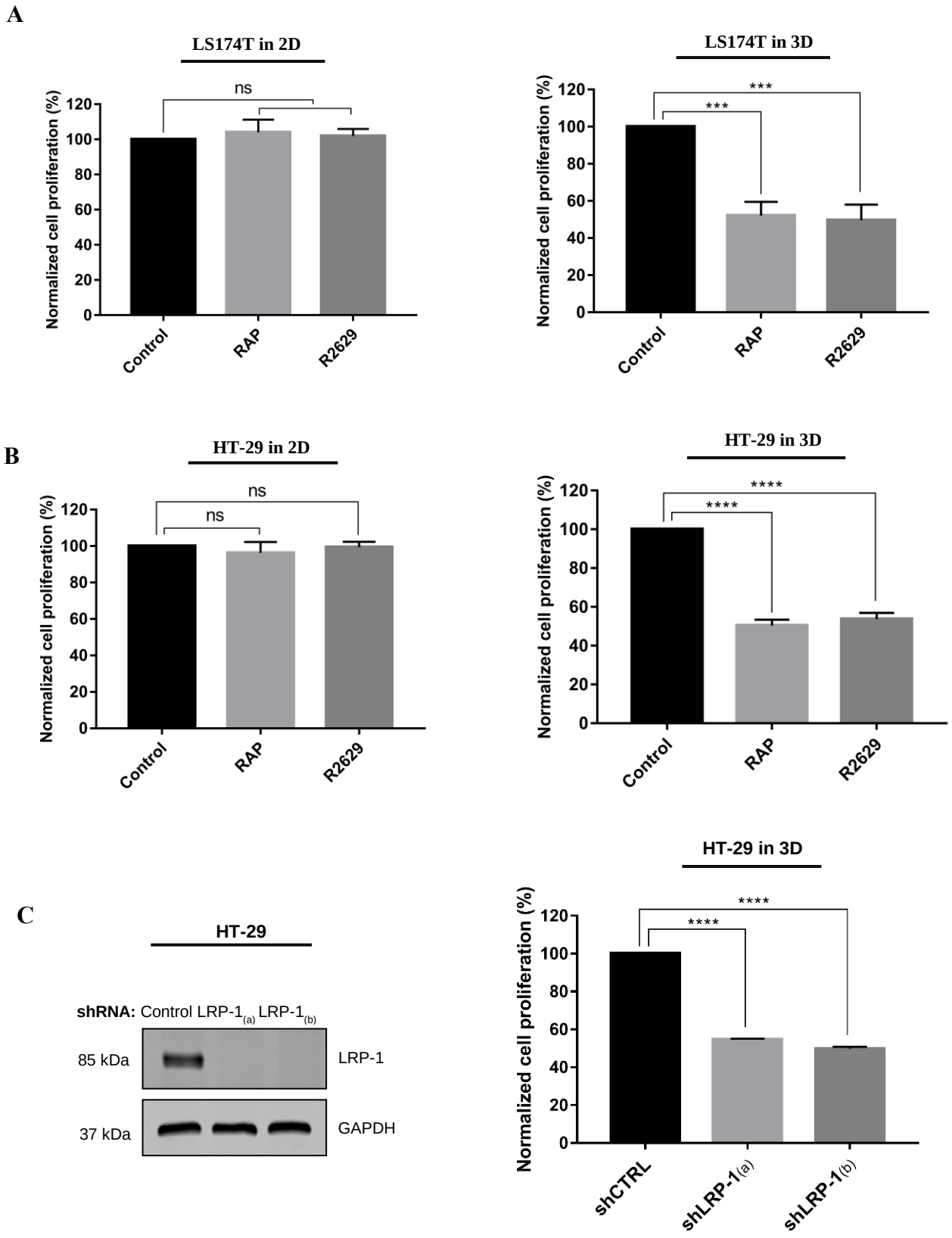
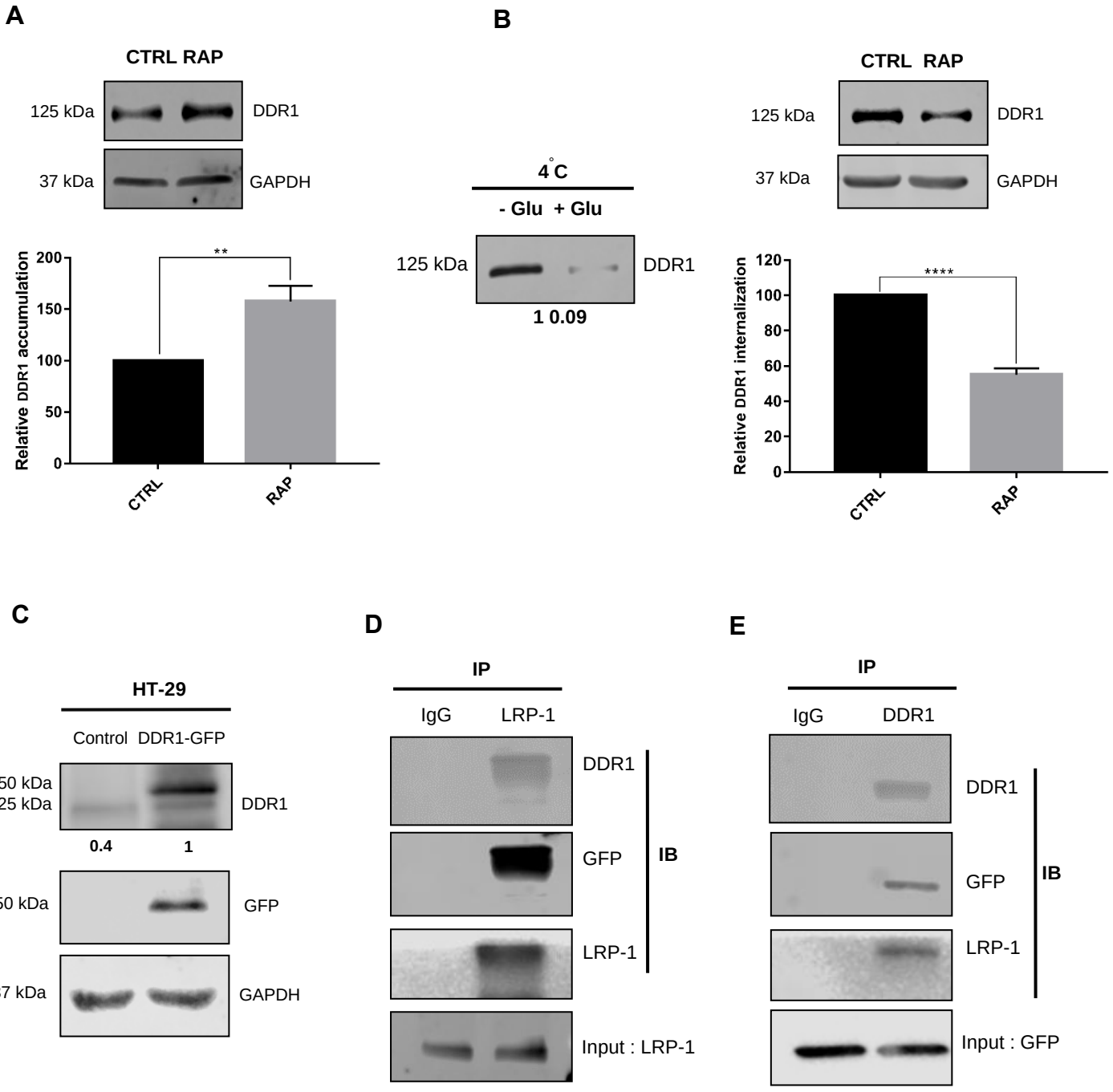


Figure 3. Cuong *et al.*



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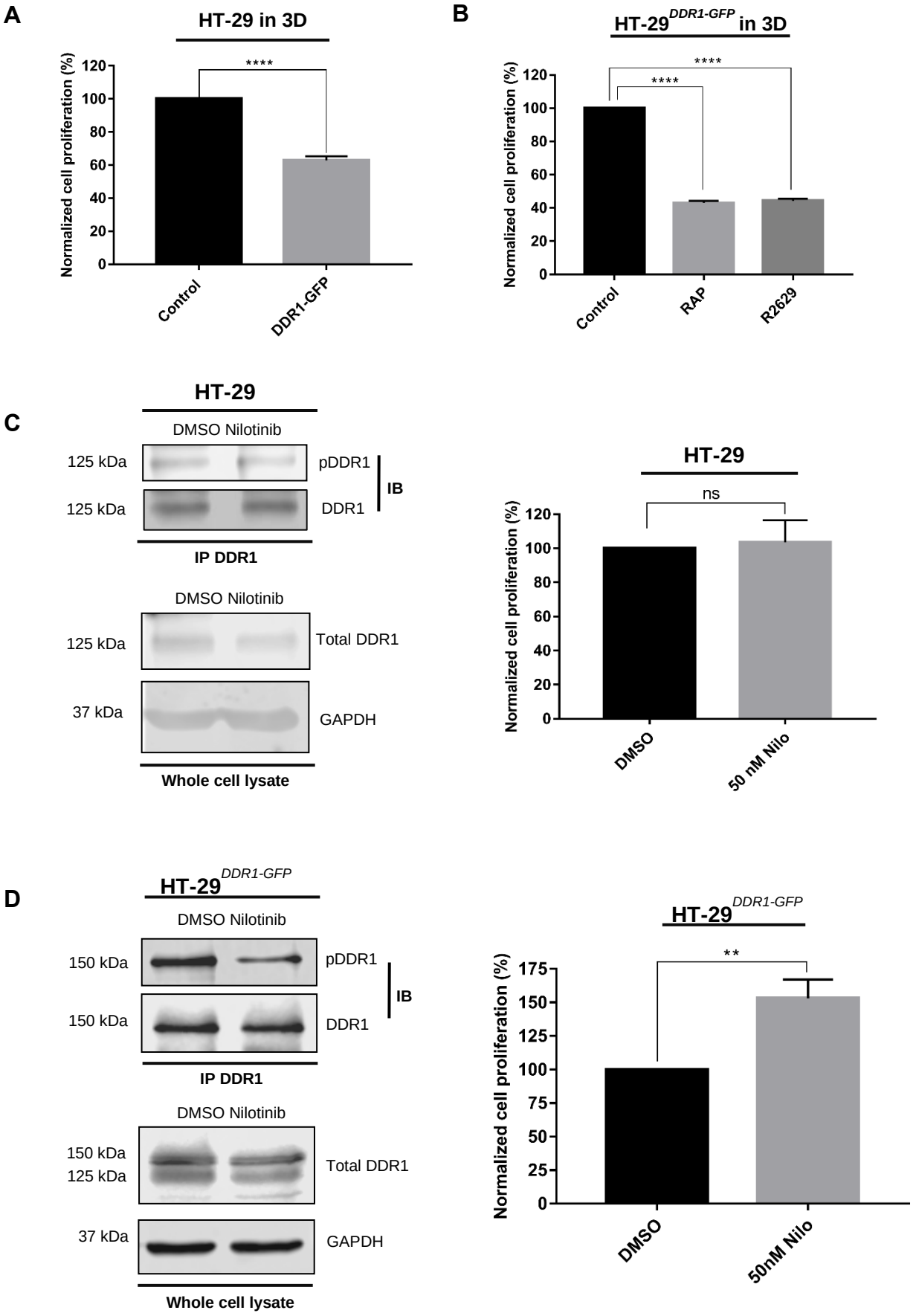
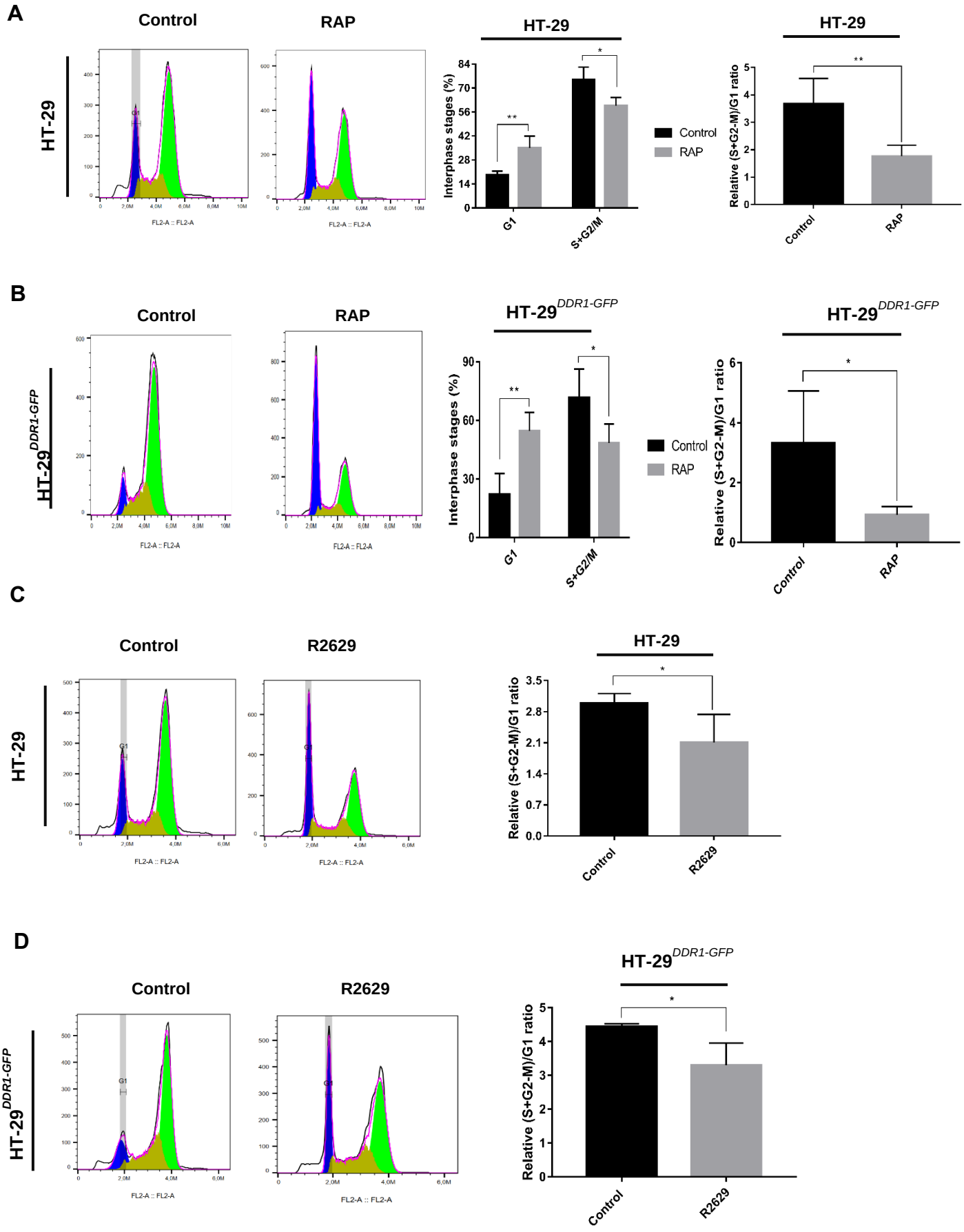


Figure 5. Cuong *et al.*



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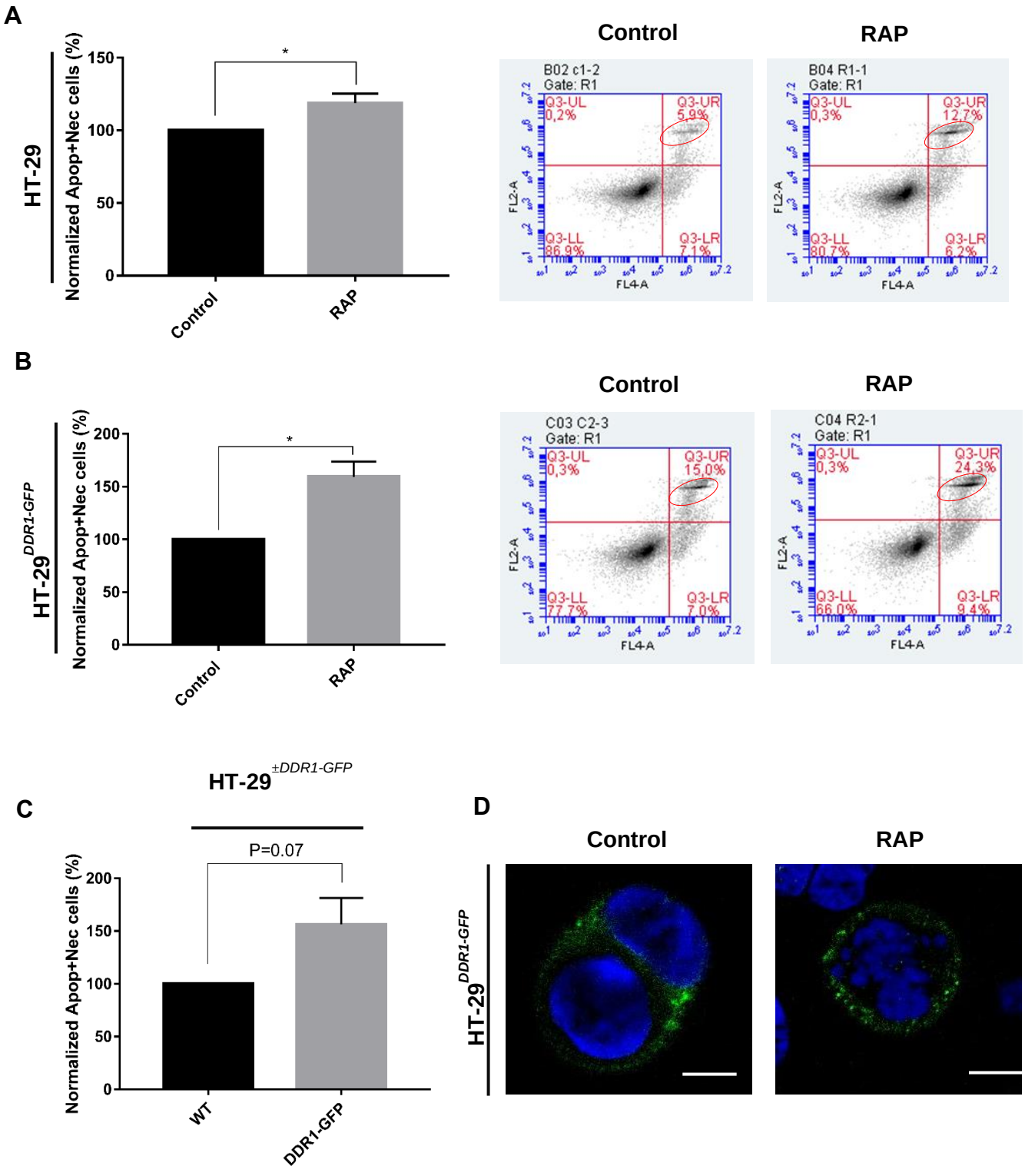

Revue

Interaction fonctionnelle entre le réseau de collagène et les cellules du microenvironnement tumoral dans le cancer colorectal

Review manuscript (summary in French)

Le cancer colorectal est le deuxième cancer le plus fréquent chez les hommes et le troisième chez les femmes dans le monde. Au cours des dernières décennies, plusieurs travaux ont souligné l'importance du microenvironnement dans la progression du cancer du côlon [169]. Dans le microenvironnement tumoral, la MEC joue un rôle clé dans ce processus. L'organisation des composants de la MEC intervient non seulement en tant que soutien structural des cellules mais permet également le contrôle de nombreuses fonctions cellulaires, y compris la prolifération, la migration, la différenciation et la survie et permet ainsi de réguler l'homéostasie et la morphogenèse. Les modifications de la composition de la MEC et de ses propriétés mécaniques pendant la cancérogenèse sont essentielles pour l'initiation et la progression de la tumeur. Le matrisome tumoral est constitué de cinq classes de macromolécules que sont les collagènes, les laminines, la fibronectine, les protéoglycanes et les hyaluronanes. Dans la plupart des tissus, le collagène fibrillaire est le principal composant de la MEC. Les cellules au sein de ce collagène fibrillaire interagissent avec lui par l'intermédiaire de leurs récepteurs de surface, tels que les intégrines et les récepteurs à domaine discoïdine (DDRs). D'une part, les cellules intègrent des signaux provenant de la MEC ce qui modifient leurs fonctions et leurs comportements. D'autre part, toutes les cellules de l'environnement tumoral (cellules cancéreuses, fibroblastes associés au cancer, cellules endothéliales, cellules immunitaires) synthétisent et sécrètent des macromolécules de la matrice sous le contrôle de signaux extracellulaires multiples. Ce dialogue cellule-MEC participe de manière dynamique à la formation et aux propriétés biophysiques et biochimiques de la MEC. Certaines études récentes basées sur des approches transcriptomiques ou protéomiques globales apportent un éclairage nouveau sur les marqueurs spécifiques qui sont dérégulés au cours des premières étapes de la carcinogenèse du côlon, mais aussi dans le cancer colorectal localement avancé ou métastatique (CRC) [501-503]. De manière intéressante, l'analyse protéomique des fractions insolubles de tumeurs primaires du côlon et de métastases hépatiques dérivées, comparées aux tissus non tumoraux adjacents, a montré l'enrichissement spécifique de certaines protéines du matrisome et de plusieurs enzymes modifiant le collagène comme les MMPs, ADAMs et LOXL1 [501]. La desmoplasie et le dépôt de collagènes constituent des caractéristiques du cancer colorectal et divers collagènes, dont les types I, VI, VII, VIII, X, XI et XVIII, sont accumulés dans les échantillons de cancer colorectal [504-510]. Une étude récente a montré que la quantité de collagène de type I est plus élevée dans les tissus tumoraux que dans les tissus normaux [423]. De plus, il a été démontré que l'ARNm du collagène de type

I est également plus élevé dans le sang des patients atteints de cancer colorectal que dans celui des individus sains [511, 512].

Les récepteurs du collagène de type I les plus étudiés sont les intégrines α1β1, α2β1, α10β1 et α11β1 [229]. La reconnaissance de la triple hélice de collagène par les intégrines se fait au niveau de la séquence GFOGER du collagène de type I [513]. Une étude menée sur des patients a montré que parmi les hétérodimères cités ci-dessus, l'intégrine β1 était le récepteur le plus exprimé dans le carcinome du côlon [237]. Un niveau d'expression élevé de l'intégrine β1 dans les tumeurs est corrélée à une réduction de la survie globale et à un taux de survie sans récidive plus faible chez un grand nombre de patients atteints de cancer colorectal [423]. L'intégrine β1 est notamment présente dans le sérum de patients atteints de cancer colorectal et son niveau d'expression semble corrélé au stade de la tumeur, au potentiel invasif et à la présence de micrométastases [514]. La surexpression de l'intégrine β1 est également associée à la progression du cancer colorectal et du cancer colorectal métastasés au foie [514, 515]. Cependant, bien que l'intégrine β1 semble contribuer au développement des métastases, il a été démontré que le traitement ciblant cette protéine n'est pas efficace dans la prise en charge du cancer colorectal. En fait, l'inhibition simultanée de l'intégrine β1 et de l'EGFR dans le cancer colorectal n'améliore pas l'efficacité de la radiothérapie [504].

De plus en plus d'études s'intéressent aux récepteurs à domaine discoïdine DDR1 et DDR2 qui interagissent également avec le collagène de type I [516] et jouent un rôle dans la progression tumorale [300]. Ces récepteurs, qui ont une activité tyrosine kinase, reconnaissent la séquence GVMGFO du collagène de type I [271] et présentent une activation relativement tardive et prolongée [258]. Des études ont montré que DDR1 est exprimé dans le carcinome du côlon et favorise les métastases dans le carcinome invasif du côlon [12, 13, 517]. En ce qui concerne DDR2, son expression élevée est associée à un taux plus important de métastases ganglionnaires et à un mauvais pronostic, ce qui suggère que l'expression de DDR2 pourrait être une cible thérapeutique efficace [518].

L'ensemble de ces données démontre le rôle du collagène et de ces partenaires en tant que biomarqueurs du cancer colorectal, ce qui suggère leur importance dans les processus de tumorigénèse, y compris la croissance cellulaire, la différenciation et la diffusion métastatique. Cette revue s'intéresse aux données actuelles décrivant les interactions fonctionnelles qui s'établissent entre les cellules du microenvironnement tumoral et le réseau de collagène dans le développement du cancer colorectal. Dans ce cadre, nous y décrivons notamment l'importance des relations qui s'établissent entre les principaux types de cellules qui composent le microenvironnement tumoral, comme les cellules cancéreuses, les fibroblastes associés au

cancer et les cellules endothéliales. Enfin, nous faisons également un point technique sur les systèmes d'études, notamment tridimensionnels, permettant d'appréhender au mieux ces interactions et sur l'intérêt de la microscopie de génération de seconde harmonique (SHG) pour étudier la structure et l'état du collagène lors des processus de cancérisation.

Review manuscript

Functional interplay between collagen network and cell behavior within tumor microenvironment in colorectal cancer

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Colorectal cancer is the second most common cancer diagnosed in men and the third most commonly occurring in women worldwide. Interactions between cells and the surrounding extracellular matrix (ECM) are involved in the development and progression of tumors in many types of cancer. The organization of the ECM molecules provides not only physical scaffoldings and dynamic network into which cells are embedded but also allows the control of many cellular behaviors including proliferation, migration, differentiation and survival to regulate homeostasis, and morphogenesis. Modifications of ECM composition and mechanical properties during carcinogenesis are critical for tumor initiation and progression. The core matrisome is constituted of five classes of macromolecules which are collagens, laminins, fibronectin, proteoglycans, and hyaluronans. In most tissues, fibrillar collagen is the major component of ECM. Cells embedded into fibrillar collagen interact with it through their surface receptors, such as integrins and discoidin domain receptors (DDRs). On the one hand, cells incorporate signals from ECM that modify their functionalities and behaviors. On the other hand, all cells within tumor environment (cancer cells, cancer-associated fibroblasts, endothelial cells, immune cells) synthesize and secrete matrix macromolecules under the control of multiple extracellular signals. This cell-ECM dialog participates in a dynamic way in the formation and the biophysical and biochemical properties of ECM. Here we will review the functional interplay of cells from the tumor microenvironment and collagen network during colorectal cancer progression.

Keywords: Colorectal cancer, collagen, cancer-associated fibroblast, tumor cell, endothelial cell, in vitro model

Collagen and colorectal cancer: state of play.

In recent decades, several works have underlined the importance of the microenvironment in colon cancer progression [1]. In the tumor microenvironment (TME), extracellular matrix (ECM) plays a key role in this process. Among ECM adhesives components, type I collagen is one of important factors regulating cancer-related events at different tumorigenesis stages [2]. After effacement of the basement membrane, paracrine signals from the nascent tumor lead to profound reorganizations of submucosal ECM that include deposition of fibrillar collagens together with growth factors and ECM modifying enzymes which stimulate active vascular remodeling. Some recent studies based on global transcriptomic or proteomic approaches shed new light on the specific markers that are dysregulated during early steps of colon carcinogenesis, but also in locally advanced or metastatic colorectal cancer (CRC) [3-5]. Interestingly, proteomic analysis of detergent insoluble fractions of paired primary colon tumors and liver metastasis compared with adjacent non tumorous tissues, illustrated the pathological samples specific enrichment in core matrisome and several collagen modifying enzymes such as MMPs, ADAMs and LOXL1 [5]. Desmoplasia and collagens deposition constitute a hallmark of CRC and various collagens including type I, VI, VII, VIII, X, XI and XVIII were found accumulated in CRC samples [6-12]. A recent study showed an increase of type I collagen in tumor tissues compared to normal tissue [13]. Moreover, type I collagen mRNA were also reported as increased in blood of CRC patients compared to healthy individuals [13, 14]. Consistently, second harmonic generation imaging of fibrillar collagens contents have shown clinical efficacy stratify high grade tumors and relevance to predict CRC patient outcome [7, 15].

The most studied type I collagen receptors are integrins α1β1, α2β1, α10β1 and α11β1 [16]. For their activation, these receptors recognize GFOGER sequence of type I collagen [17]. α1β1 dimer was considered as the most expressed receptor in colon carcinoma [18]. β1-integrin expression in tumors was correlated with reduced overall survival and reduced disease-free survival in a large cohort of CRC patients [19]. Notably, β1 integrin is detected in CRC patients serum and its level of expression appears to correlate with aggressiveness and presence of micrometastasis [20]. β1 integrin overexpression is also associated with CRC progression and colorectal liver metastasis [20, 21]. However, although β1 integrin seems to contribute to metastasis development, β1 integrin targeted therapy is not successful in CRC management. In

fact, simultaneous inhibition of β1 integrin and EGFR in CRC does not improve radiotherapy efficiency [22].

Collagen also signals to cells through the receptor tyrosine kinases discoidin domain receptors DDR1 and DDR2, both of them have also been reported to interact with type I collagen [23] and to play a role in tumor progression [24]. These receptors, which harbor a tyrosine kinase activity, recognize GVMGFO sequence of type I collagen [24] and exhibit a relatively late and prolonged activation [25]. DDR1 is expressed in colon carcinoma and promote metastasis in invasive colon carcinoma [26-28]. Concerning DDR2, a high expression was associated with higher frequencies of T4, lymph node metastasis, peritoneal spread, and worse prognosis, suggesting that DDR2 expression might be an effective therapeutic target [29].

This growing data set supports a key role of collagens and their partners during tumorigenesis processes and as potential biomarkers of CRC. The following parts aim to highlight current evidence regarding the functional interplay between cells within the TME and collagen network during CRC progression. The main data are presented in Figure 1.

The relationship between collagen and cancer cells

Analysis of ECM signatures in patients colon tumors has revealed that type I collagen is highly expressed [5]. Accordingly, high density of type I collagen constitutes a poor prognosis factor in colon carcinoma and type I collagen-rich environment is able to induce mesenchymal gene expression and invasion [30]. Beside the density, collagen topology (fiber alignment) and elasticity (stiffness) appear to be also associated to colon tumorigenesis. Brauchle and co-workers have demonstrated that the alignment of collagen fibers is increased in colon carcinoma tissues when compared to normal tissues,and associated with increased stiffness [31]. Biophysical investigations have also shown different molecular fingerprints for collagen fibers in colon carcinoma tissues when compared to normal tissues [31]. Another study has shown that density and collagen fiber alignment were higher in tumor invasion front than in primary tumor and normal tissue [32]. Of note, hypoxia, that is associated to collagen density and organization, has an impact on colon cancer carcinoma migration and invasion through promotion of epithelial to mesenchymal transition [33].

At the functional level, the homeobox transcription factor Cdx2 has been reported to play a role as a tumor suppressor and be down-regulated in colon carcinoma [34]. Furthermore, Cdx2 expression was significantly lower in colon carcinoma with the highest grades [35]. Interestingly, type I collagen was previously considered to promote tumorigenesis by

downregulating Cdx2 expression [36]. Brummer's group has demonstrated few years ago a close correlation between BRAF mutation and low level of Cdx2 expression in colon carcinoma. Type I collagen at high density has been also reported to suppress HNF4α when inducing mesenchymal gene expression in vitro and in patient-derived colon tumors [30] Consistently, invalidation or inhibition of HNF4α promotes colon carcinogenesis, whereas its enforced expression is able to inhibit cell growth in colon carcinoma [37, 38].

Concerning the role of type I collagen receptors in tumor progression, Roche's group has elegantly recently shown that DDR1 plays a crucial role in the invasion function of metastatic colon carcinoma [26, 27]. They have particularly demonstrated that pharmacological inhibition of DDR1-BCR signaling axis decreased invasion and metastatic processes in colon carcinoma, suggesting that DDR1 targeting could be an efficient co-treatment strategy in colon carcinoma [26, 27]. More recently, NSD2 circular RNA has been shown to promote DDR1 expression and colorectal cancer metastasis by targeting miR-199b-5p [39]. For integrins, Wu and co-workers have lately reported that type I collagen is able to support colon carcinoma cell stemness, invasion and metastasis through activation of α2β1 integrin heterodimer and PI3K/AKT/Snail signaling pathway [40].

Regarding the role of type I collagen in the cancer cell sensitivity to targeted therapies, a recent study has demonstrated that 3D type I collagen may protect colon carcinoma against the anti-EGFR cetuximab therapy by increasing tyrosine phosphorylation of MET and RON [30]. The effect of 3D type I collagen on the sensitivity to vemurafenib of colon carcinoma, carrying the $BRAF^{V600E}$ mutation has also been investigated. At the opposite of the general concept describing type I collagen as a shield of colon carcinoma cells against therapies, authors have demonstrated that cells seeded in 3D type I collagen were 10-fold more sensitive to the vemurafenib targeted drug. On the contrary, 3D matrix was able to protect tumor cells against the cytotoxic effect of the fluorouracil chemotherapeutic agent [41].

A recent study has shown that 3D type I collagen was also able to increase sensitivity of colon carcinoma to chemotherapy by regulating the expression of ABCB1 gene which encodes P-glycoprotein [42]. Interestingly, the expression of ABCB1/P-glycoprotein appears to be associated with high Cdx2 expression in normal colon tissue and basal/low Cdx2 expression in colon carcinoma [43].

The relationship between collagen and cancer-associated fibroblasts

The most abundant cell type in TME are cancer-associated fibroblasts (CAFs), an activated type of fibroblasts that play a major role in tumorigenesis and metastatic processes [44]. CAFs demonstrate a functional heterogeneity in colorectal cancer (CRC) that may arise from different cellular origins and can affect the clinical course of colon cancer patients [45]. In CRC, an abundance of CAFs in the TME has been associated with poor outcomes and transcriptomic studies linked CAF signature with poor-prognosis and highly aggressive CRC molecular subtypes. CAFs are not only associated with advanced CRC but also found in early stages [46]. Several studies identified CAFs as potential prognosis and recurrence markers in patients with colon cancer [47-50]. Histologic evaluations of CRC patient samples and organotypic 3D co-culture models demonstrated that CAFs are the primary drivers of collagen synthesis and remodeling in the highly desmoplastic environment found in CRC [51, 52]. Interestingly, a significant heterogeneity was observed within CAF population related to collagen remodeling [53]. Transcriptome and Proteome profiling identified CRC CAFs as the main source for connective tissue components of the ECM, such as collagens, thus altering the molecular composition of the matrix by increasing the deposition of new matrix components [54, 55]. Another way for CAFs to remodel ECM is to degrade it by using MMPs and formation of degradative protrusions. Genes induced in CRC CAFs, compared to normal colonic fibroblasts, include several tumor-promoting MMPs and TGF-ß1 [54-56]. In CAFs, invadopodia are formed upon Twist1 translocation into the nucleus, which upregulates the expression of the actin-binding protein paladin (isoform 4). Interestingly, Twist1 and paladin are overexpressed in purified colon CAFs as compared with their normal counterparts and associate with poor prognosis in CRC [57]. In addition to MMPs, CAFs also express other proteases such as the fibroblast activation protein (FAP), a collagenase and gelatinase [58]. Stromal FAP expression in human colon cancer samples is a marker of early stage in cancer development and correlated with poor patient outcome [59]. FAPα activity has a strong impact on fibroblasts secretome composition, including matrix processing enzymes, and influence morphology and collagen contraction capacity of immortalized CRC CAFs. Recent studies established a direct link between CAFs and the modifications of ECM organization and stiffness described in colon cancer. LOXL2, a collagen cross-linker was reported as highly expressed in CAFs and is associated with poor CRC survival [60]. Hic-5, a non-enzymatic adaptor protein, was described as a novel factor responsible for the development of CRC, by promoting in CAFs the production of collagen I and LOX that lead to stiffness of cancer tissues [61]. More recently, in a collagen gel co-culture system, with fibroblasts and CRC cells, Delaine-Smith's group demonstrated that fibroblast-derived TG2 (transglutaminase-2), a protein cross-linking

enzyme, induced gel stiffening by formation of thicker collagen fibers and proposed a regulatory link between TG2 and LOX. In addition, stiffness is further increased by fibroblast/CRC crosstalk and a potential role for extracellular vesicles in mediating this tumor-driven fibroblast response is suggested by authors [62]. Another study reported that fibroblasts activated by late-stage CRC cell-derived exosomes, became specialized in type I collagen and physical remodeling of ECM through cytoskeletal re-organization, membrane protrusion formation and secretion of matrix-remodeling proteins [63].

The relationship between collagen and endothelial cells

Angiogenesis exerts crucial functions during major steps of CRC progression [1, 3, 64]. Stimulation of CRC cells by oncogenic drivers such as EGF or stabilization of hypoxia inducible factors (HIFs) were involved in the secretion of angiogenic diffusible factors and ECM structural compounds in the TME [65]. Moreover, collagen supports nascent vascular structures during intussusceptive angiogenesis in CRC [66]. A nine genes signature including collagen I, X and XI was specifically enriched in angiogenic and hypoxic CRC genesets [4]. Another study identified a matrisomal signature of 110 genes induced during the angiogenic switch of the standard RIP1-Tag2 murine model of tumor angiogenesis [3]. The expression of this set of genes, which includes collagens I, VI, VIII, X and various ECM regulators was positively correlated with that of endothelial cell markers and increased with CRC progression. This signature was also specifically induced in hepatic metastasis suggesting a functional contribution to both early events and metastatic cascade. It is now well-established that tumor and stromal cells synergize to activate pro-angiogenic signals in the TME [3, 59, 64, 67, 68]. CAFs and tumor-associated macrophages (TAM) are both involved in TGF-β signaling activation during the angiogenic switch [3]. Stromal activation of this pathway promotes both tumor initiation and early metastatic events [64] and was specifically associated to consensus molecular subtypes CMS4 of CRC that express various angiogenesis markers and present the worst overall survival [69]. Several reports illustrated the contributions of tumor resident or infiltrated stromal cells to ECM modifying events that accommodate endothelial cells fitness and provide angiogenic cues [59, 67, 70]. Although a clear scenario is sometimes difficult to draw on the angiogenic consequences of collagen deposition, emerging angio-active parameters include types of collagens (network, fibrils-anchoring or fibrillar collagens that convey different angiogenic signals), topology and stiffness. Post-translational modifications such as proteolytic degradation or cross-linking can modulate the biophysical properties of collagen rich scaffolds [11, 68, 70, 71]. FAP-α expression and activity was linked to the

secretion of pro-angiogenic factors such as angiopoietin-1 and VEGF-C by colon patient-derived CAFs [59]. Gain and loss of function experiments illustrated that FAP-α-dependent CAF secretome can stimulate 3D endothelial spheroids sprouting. In vivo, targeting of FAP-α into an immune competent murine model of colon cancer decreased blood vessel density and induced fibrillar collagen accumulation [67]. The activity of SNAI1 and PDGFBB contributes to CAF ability to assemble aligned collagen fibers that promote endothelial cell proliferation and morphogenesis in a 3D model of CAF-derived matrices [70]. SNAI1 expression by fibroblasts was also associated with the abundance of CD34 positive endothelial cells in an in vivo model of CRC. Co-culture of TAM with CRC cells can potentiate the production of tumor-derived MMP2 and MMP9 [72]. Recruitment of collagenolytic enzymes-expressing immune cells in the CRC TME might influence the bioavailability of ECM-immobilized angiogenic factors such as VEGF, as reported in other tumor context [73, 74]. Collagen-enriched niches emerge as biomarkers of desmoplastic and angiogenic CRC microenvironment [75, 76]. High expression of collagens I and IV, with tumor endothelial marker-1 (TEM-1, endosialin), especially when distributed around tumor vessels, allows stratification of CRC patients according to their poor prognosis [75]. Collagen-enriched niches might also account for the adaptive response of the TME to anti-angiogenic therapies [76, 77]. Collagen IV empty sleeves resulting from tumor vessel pruning triggered by VEGFR2 can promote a rapid vascular regrowth after treatment withdrawal [77]. Although, VEGFR2 blocking in a CRC model normalized tumor vessels, decreasing diameter while ameliorating collagen IV perivascular coverage [76]. Endostatin, a collagen XVIII-derived fragment, is an inflammatory marker detected around blood vessels and in the plasma of advanced CRC patients [12]. This molecule, efficient to inhibit both lymphangiogenesis and hemangiogenesis [78], is considered as a valuable tool to control metastatic CRC growth since several studies reported its moderate toxicity without observing the increased metastatic dissemination encountered in response to the anti-VEGF antibody bevacizumab [28].

In vitro tumor microenvironment models using collagen

Two-dimensional (2D) collagen-coated systems routine use has largely shown their limitations to summarize the complexity of tumor initiation and progression processes. It is absolutely necessary to include some major extracellular components to mimic properties of the TME such as the spatial configuration [79] and the addition of supporting materials with mechanical properties close to the ECM encountered during disease progression [80]. The use of in vitro 3D models should fill the gap between traditional 2D cell culture and animal models, by

mimicking the cancer micro- and macro-environment potentially able to integrate multiple cell types in a controlled environment and should allow to better characterize CRC drivers and develop new therapeutic strategies in constantly upgraded models of growing complexity. One possible approach is to develop spheroids of cancer cells seeded on low-attachment tissue culture plates. Whereas this type of culture allows cancer cells to communicate with one another and to release low levels of intrinsic collagen [81], substantial aspects of TME are still missing. More complex models using biological scaffolds such as collagen are therefore added to create an ECM to obtain biomimicry and study cancer progression by recreating the tumor microenvironment. Patient-derived xenograft models are an important tool for preclinical and clinical research, especially when orthotopically transplanted. However, in this model, the principal limit is that TME cannot be properly reconstituted owing to important stromal cells such as cancer-associated fibroblasts and endothelial cells which are not derived from the tumor samples and can be late recruited [82]. New 3D models of cancer using a collagen matrix can promote the crosstalk between cancer and stromal cell. Co-cultures of different colorectal cancer cell lines with fibroblasts and endothelial cells in 3D spheroids have been elaborated to test drug dose-response and compared with results in 2D and homotypic 3D cultures. The results suggest that 3D co-cultures are more relevant, providing a higher level of translational information that should help to define patient-specific treatment options [83].

Pape and colleagues developed a CRC model using high-density type I monomeric collagen, termed as tumoroids [84]. This model consists of a central cancer mass containing either the highly invasive HCT116 or less invasive HT29 cells embedded in collagen type I hydrogels to mimic the TME in situ [85]. The stromal compartment in this model is easily manipulated and ECM components and stromal cell types can be added accordingly. Furthermore, on-chip biomimetic microenvironments using microfluidic technologies are being developed to better reproduce the complexity of in vivo restrictions. In this model, human colonic microvascular endothelial cells cultivated in a 3D vessel-mimetic device are attached to the wall of the lateral channels of the microfluidic chip whereas HCT-116 cells are embedded in collagen IV-enriched Matrigel in the central chamber [86].

Considering the feature of tumor heterogeneity, the main limitation of these models is the presence of a single CRC cell type exhibiting a unique genetical pattern. The development of more realistic preclinical models is absolutely required and is a major challenge for the coming years, especially for improving drug screening. The use of patients-derived 3D tumor models may provide a solution to overcome the oversimplified 2D cell cultures and the limitations of

in vivo models [87]. These new designs are not intended to completely supplant but rather complete and expand the currently available techniques.

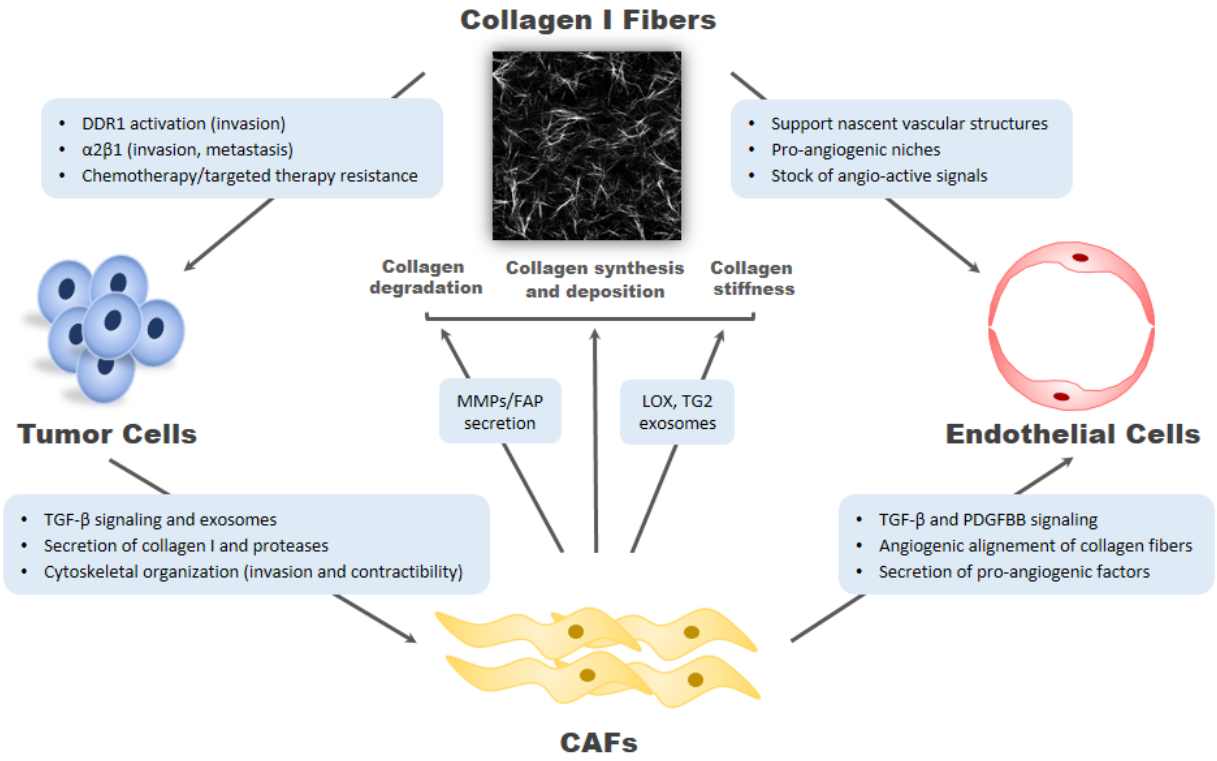

Figure 1. Relationship between type I collagen network and cells in tumor microenvironment. On the one hand, cancer cells, cancer-associated fibroblasts and endothelial cells influence collagen structure and composition. On the other hand, type I collagen participates to tumor progression. DDR1: discoïdin domain receptor I, FAP: fibroblast activation protein, LOX: lysyl oxidase, MMP: metalloproteinase, PDGFBB: Platelet-derived growth factor BB, TG2: Transglutaminase 2, TGF-β: Transforming growth factor-β.

Author Contributions

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

Conflict of Interest

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.

Abbreviations

CAF: cancer-associated fibroblast, CRC: colorectal cancer, DDR: discoïdin domain receptor, ECM: extracellular matrix, FAP: fibroblast activation protein, SHG: second harmonic generation, TME: tumor microenvironment

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Discussion and Conclusion

Discussion et Conclusion (summary in French)

L'ensemble de nos données a permis de mettre en évidence une association moléculaire fonctionnelle entre LRP-1 et DDR1 dans des cellules de cancer colorectal. En effet, nous avons montré que LRP-1 est capable d'endocyter et de réguler l'expression de DDR1 à la membrane plasmique de carcinomes colorectaux. Cette association permet de réguler la prolifération des cellules de cancer colorectal dans une matrice 3D de collagène de type I en favorisant l'entrée du cycle cellulaire en phase S et en diminuant l'apoptose.

De nombreux travaux ont montré que LRP-1 peut être considéré non seulement comme un intégrateur des signaux issus du microenvironnement tumoral mais aussi comme un récepteur multifonctionnel régulant de nombreux processus tels que la migration et la survie des cellules cancéreuses. Sa fonction globale reste néanmoins extrêmement complexe à interpréter d'autant plus que le niveau de son expression est très variable selon le type de tumeurs et le stade d'évolution du cancer. Cependant, dans différents types de cancer, la tendance semble montrer une corrélation entre la surexpression de LRP-1 avec un mauvais pronostic [440, 519], une prolifération cellulaire accrue, un pouvoir invasif plus important et une récidive tumorale [29, 444, 489]. Jusqu'à présent, peu d'études ont recherché la contribution de LRP-1 dans la pathologie du CCR malgré un intérêt clinique évident. Nous avons récemment démontré que la perte d'expression de LRP-1 est associée à un mauvais pronostic dans les cancers du côlon [29]. De plus, chez les patients au stade métastatique, l'expression de LRP-1 est associée à une survie globale plus courte. Dans ce travail, nous avons montré que l'inhibition de l'expression de LRP-1 diminue la prolifération des cellules du carcinome du colon et ce uniquement en matrice 3D de collagène de type I. Bien que de nombreux travaux aient prouvé que LRP-1 peut activer des voies de signalisation telles que Ras, c-Myc, MAPK et Akt/PI3K, largement connues comme des voies oncogéniques impliquées dans les processus de prolifération et de survie cellulaire [392], très peu de données ont montré que LRP-1 jouait un rôle dans la prolifération cellulaire. Salama et ses collaborateurs ont décrit récemment l'implication de la voie LRP-1:tPA dans la migration et la prolifération des cellules de mélanome [461]. Leurs résultats ont permis de proposer un modèle selon lequel LRP-1 augmente la prolifération des cellules de mélanome et le nombre de métastases de manière dépendante de la voie de signalisation ERK. Les données de Beaujouin *et al*. ont également montré que la liaison de la pro-cath-D à LRP-1 favorise la croissance des fibroblastes dans le cancer mammaire [490].

Il est intéressant de noter que nos résultats montrent que LRP-1 régule la prolifération des cellules cancéreuses du côlon uniquement lorsque ces cellules sont cultivées en matrice 3D de

collagène de type I. Au cours de la progression tumorale, après le passage de la membrane basale, les cellules cancéreuses vont migrer au sein d'un stroma principalement composé de collagène de type I [494]. En plus de ses propriétés en tant que protéine de structure, le collagène de type I peut activer différentes voies de signalisation cellulaire responsables de plusieurs fonctions des cellules tumorales [260]. De plus en plus d'études suggèrent que les récepteurs DDR jouent un rôle clé dans la progression tumorale en régulant les interactions des cellules avec le collagène [12, 257, 327, 520]. Nos données montrent que l'inhibition de la fonction d''endocytose de LRP-1 induit une accumulation de DDR1 à la surface cellulaire. Nous avons ensuite montré que les récepteurs LRP-1 et DDR1 sont étroitement associés au sein d'un même complexe à membrane plasmique des cellules HT-29 et que LRP-1 constitue un récepteur d'endocytose de DDR1. Ces résultats sont d'autant plus intéressants que jusqu'à présent, peu d'informations sont disponibles sur la régulation de l'expression de DDR1 au niveau de la membrane plasmique. En présence de LRP-1, le récepteur DDR1 pourrait être endocyté dans des endosomes, menant soit à sa dégradation au niveau du lysosome, soit à son recyclage au niveau de la membrane plasmique [273]. Dans des fibroblastes de souris, DDR1 est internalisé seul ou complexé avec d'autres récepteurs à activité tyrosine kinase (RTK). En effet, dans le carcinome mammaire, le récepteur de l'IGF-I peut phosphoryler DDR1 induisant ainsi leur internalisation et leur incorporation dans les endosomes précoces [305]. Les RTK internalisés peuvent être recyclés à la membrane plasmique, être dégradés ou subir une voie rétrograde (endosome/Golgi/réticulum endoplasmique). De récents résultats montrent que DDR1, suite à son activation, est internalisé dans le noyau pour jouer le rôle de facteur de transcription dans des cellules rénales [279].

Nos résultats ont montré que LRP-1 exerce ses effets prolifératifs en régulant de façon négative la présence de DDR1 au niveau de la membrane plasmique. En effet, en induisant l'endocytose de DDR1, LRP-1 abolit l'effet inhibiteur de DDR1sur la prolifération cellulaire. L'inhibition de LRP-1 induit un arrêt du cycle cellulaire en phase G1, et ce de manière plus importante lorsque DDR1 est surexprimé. De plus, l'inhibition de LRP-1 induit une augmentation de l'apoptose des cellules HT-29 et de façon plus importante quand celles-ci surexpriment DDR1. Ces données concordent avec celles obtenues par le groupe d'Erik Maquoi qui a montré que la prolifération cellulaire des cellules de carcinome mammaire de type épithélial et non invasives (MCF-7 et ZR-75-1) était diminuée uniquement dans des matrices 3D de collagène de type I [9, 257]. Dans ces études, DDR1 semble induire l'apoptose *via* l'induction de l'expression de la protéine pro-apototique BIK [260]. D'autre part, des résultats précédents obtenus par notre groupe ont montré que lorsque des cellules de carcinome

mammaire MCF-7 et ZR-75-1 sont cultivées en matrice 3D de collagène de type I âgé, l'activation du récepteur DDR1 est diminuée. Cela induit par conséquent une protection de ces cellules contre le pouvoir suppresseur de la prolifération cellulaire de DDR1, la surexpression de BIK et par conséquent l'apoptose induite par celui-ci [9, 310]. D'autres études ont montré que DDR2 pouvait inhiber la prolifération des cellules de mélanome humain et de fibrosarcome en induisant un arrêt en G0 / G1 du cycle cellulaire. Il a été démontré que ce processus était induit par p15INK4b, un inhibiteur de protéine-kinase dépendante des cyclines (CDK), suggérant que cette protéine pourrait être une cible en aval de la signalisation DDR2 [10, 11, 521]. Saby et ses collaborateurs ont également montré que le vieillissement du collagène de type I induit une diminution de l'activation de DDR2 engendrant une augmentation de la prolifération des cellules de fibrosarcome en matrice 3D. Dans cette étude, le collagène de type I jeune active fortement DDR2 induisant par conséquent une augmentation de l'expression de $p21^{CIP1}$ et une diminution de la prolifération cellulaire. Cependant, en présence du collagène de type I âgé, ils ont observé une diminution de l'activation de DDR2 accompagnée par une diminution de l'expression de $p21^{CIP1}$ et une augmentation de la prolifération cellulaire [15].
Pour conclure, mon travail de thèse nous a permis d'identifier non seulement un nouveau mécanisme moléculaire contrôlant l'expression de DDR1 à la surface des cellules mais aussi un rôle supplémentaire de LRP-1 en tant qu'intégrateur des signaux issus du microenvironnement tumoral.

Comme mentionné précédemment, les résultats de cette étude ont mis en évidence une nouvelle association fonctionnelle entre LRP-1 et DDR1 jouant un rôle dans la régulation de la prolifération cellulaire et l'apoptose des cellules de cancer du côlon. Cette étude a été réalisée dans un modèle de culture en matrice 3D de collagène de type I. Dans cette étude, ce système de culture est décisif puisque les mêmes effets n'ont pas été observés dans un modèle classique de culture en 2D sur *coating* de collagène de type I.

Cependant, dans ce modèle, la principale limite est l'absence des cellules stromales, composantes essentielles du microenvironnement tumoral, comme les fibroblastes associés au cancer et les cellules endothéliales. De nouveaux modèles 3D de cancer utilisant une matrice de collagène peuvent inclure ces composants pour permettre la communication entre les cellules cancéreuses et stromales, comblant ainsi cette lacune des modèles traditionnels de cancer. Des méthodologies récentes ont été développées pour discriminer différentes populations cellulaires dans les modèles de co-culture et pour analyser leurs interactions et leurs phénotypes [522-525]. L'utilisation de la GFP (*Green Fluorescent Protein*) et d'enzymes tels que la luciférase / luciférine dans les co-cultures a été largement décrite et utilisée [523].

D'autres composants de la MEC devraient également être pris en compte. En effet, une étude récente a montré que par rapport à un tissu normal, la MEC tumorale du cancer colorectal contient aussi spécifiquement d'autres composants protéiques (fibronectine, collagène de type VI, ténascine...) qui doivent être pris en compte [526]. Cependant, la mise en œuvre de ces systèmes multi-cellulaires est extrêmement difficile et prend beaucoup de temps, et les résultats techniques se limitent souvent à combiner au mieux deux types de cellules différents, et rarement plus. De plus, en raison des exigences de culture extrêmement complexes et parfois antagonistes entre les types de cellules, il est difficile d'utiliser ces systèmes pour de longues observations, alors que nous avons pu le faire pendant plus de 5 jours avec notre système 3D.

En ce qui concerne nos données, il apparaît essentiel d'analyser les effets du complexe LRP-1/DDR1 sur la prolifération des cellules CRC dans des contextes cellulaires distincts en utilisant des co-cultures avec des cellules stromales et/ou des études *in vivo*. Par exemple, les expériences menées par notre équipe sur le rôle de LRP-1 dans les CAF devraient permettre d'accroître nos connaissances moléculaires sur ce sujet et d'envisager dans les prochains mois des modèles de co-culture 3D pertinents incluant les CAF.

De plus, l'impact pronostique et clinique de l'expression du DDR1 dans les cancers colorectaux et son association potentielle avec un profil morphologique et/ou moléculaire spécifique doit être étudié avec précision. Une étude clinique menée par notre groupe est actuellement en cours afin d'élargir nos connaissances actuelles sur l'expression de DDR1 dans les cancers colorectaux et son association avec l'expression de LRP-1. Ces travaux sont actuellement réalisés à partir d'une cohorte de patients de l'hôpital de Reims préalablement caractérisée [29]. Les premiers résultats de ce travail récemment initié ont montré que DDR1 est très fortement exprimé dans les tissus de cancer colorectal dans tous les cas, alors qu'il ne l'est que de manière modérée à forte dans 93 % des cas dans les tissus sains. Ces résultats sont très prometteurs et doivent être confirmés et étendus. Nous chercherons notamment à comparer nos résultats concernant l'expression de DDR1 avec ceux déjà obtenus sur LRP-1 et à les corréler avec les données de survie ou de récidive des patients.

En ce qui concerne les données obtenues dans cette étude, bien que nous ayons observé un effet de l'inhibition de LRP-1 sur le cycle cellulaire, la régulation du cycle cellulaire doit être étudiée plus en détails. Une meilleure compréhension des mécanismes moléculaires impliqués dans les effets médiés par l'axe LRP-1/DDR1, notamment en ce qui concerne les voies de signalisation et les cibles en aval, permettrait de mieux comprendre les mécanismes de régulation du cycle cellulaire et de l'apoptose [527, 528]. Par exemple, le facteur de transcription c-Myc favorise la progression du cycle cellulaire en induisant la transcription des

gènes impliqués dans l'activation du cycle cellulaire et en inhibant celle impliqué dans la régulation négative du cycle cellulaire. Les suppresseurs de tumeurs tels que p53 et RB inhibent la progression du cycle cellulaire et induisent l'apoptose [527-531]. Ce sont là des exemples de cibles potentielles qui devront être évaluées.

De nombreuses études ont montré que les interactions entre les cellules et la MEC sont à l'origine de plusieurs fonctions cellulaires dont la prolifération, la migration et la différenciation des cellules. Le remodelage de la MEC se produit naturellement pendant le développement, l'homéostasie tissulaire et les maladies [214]. Comme mentionné précédemment, le collagène de type I est l'une des protéines les plus abondantes dans tout l'organisme et est le principal composant de la MEC. Il joue un rôle crucial dans les conditions physiologiques et pathologiques [532-534]. Le remodelage du collagène de type I est associé à une agressivité de la tumeur et à un mauvais pronostic chez les patients [535-537]. Dans le cancer colorectal, le collagène de type I est impliqué dans la tumorigénèse, la transition épithélio-mésenchymateuse, le caractère invasif et les métastases [30, 511, 538-540]. En effet, le remodelage du collagène favorise la progression du cancer [214, 541]. Le collagène de type I présente un taux de renouvellement excessif lors développement du cancer colorectal [538]. De plus, LRP-1 est bien connu pour être impliqué dans l'endocytose de plusieurs constituants de la MEC (fibronectine, décorine, métalloprotéases, facteur de croissance...) et joue un rôle crucial dans l'assemblage, le renouvellement et le contrôle de l'organisation de la matrice. Afin d'étudier (*i*) si les cellules de cancer colorectal peuvent remodeler la matrice de collagène de type I dans des conditions *in vitro* et (*ii*) si LRP-1 intervient dans ce processus, nous nous proposons d'analyser finement l'organisation des fibrilles de collagène dans un système de matrice 3D. Dans nos expériences préliminaires, les cellules de carcinome colorectal ont été ensemencées dans des matrices de collagène 3D de type I en présence ou non de RAP. Les cellules et les fibres de collagène ont ensuite été analysées par SHG (**Figure 27**, page **156**). De manière intéressante, nos résultats ont montré que l'inhibition de LRP-1 entraîne une diminution significative du diamètre des fibres de collagène (**Figure 28C-D,** page **157**). Le traitement RAP n'a toutefois pas induit de changements significatifs sur la longueur et la rectitude des fibres de collagène (**Figure 28E-H,** page **157**). Ces données préliminaires suggèrent pour la première fois que les processus induits par LRP-1 pourraient être directement impliqués dans le remodelage du collagène.

Nous chercherons à renforcer nos résultats préliminaires en utilisant l'imagerie vibrationnelle (imagerie Raman) pour mieux caractériser les effets de l'endocytose médiée par LRP-1 sur l'organisation 3D des fibres de collagène. Afin de continuer cette étude, nous

proposons notamment d'utiliser plusieurs modèles cellulaires de cancer colorectal présentant différents niveaux d'agressivité et des niveaux d'expression des protéases extracellulaires distincts, pour évaluer les effets de LRP-1 sur les propriétés du collagène de type I. Ces recherches seront complétées par des analyses biochimiques de la dégradation du collagène de type I.

Discussion and Conclusion

CRC treatments are nowadays often based on conventional methods such as surgery, chemotherapy, radiotherapy or a combination of some of them [34, 43, 60]. The therapeutic outcomes are highly dependent on the stages at diagnostic. In general, the tumor recurrence rate is still very high in most stages [48]. For instance, in stage III CRC patients, 33% of them will have recurrence after surgery [542], and the percentage is 15 to 50% after adjuvant therapy [43]. Recently, targeted therapies and immunotherapy are emerging and promising approaches for CRC treatment [137, 143]. However, the efficacy of these therapies remains controversial. Immunotherapy targeting PD1, PDL1, CTLA4, and TCR has been proposed recently and clinical trials are under investigation [149, 543]. Although several studies have defined some common genetic and epigenetic alterations in CRC [149], there is no fully comprehensive understanding of biological and clinical distinct subsets of CRC. Some somatic mutations are commonly found in advanced stages of CRC. However, the frequency of these mutations is variable, suggesting that the known genetic alterations may partly contribute to colorectal progression [34, 79]. Thus, other potential markers of multistep carcinogenesis in CRC progression need to be identified.

Only a few studies have assessed the expression of LRP-1 in CRC [29, 492]. In the present work, we investigated whether LRP-1 may contribute to CRC development and focused on its putative role in tumor cell proliferation. In fact, sustaining proliferation is one of the most important hallmark features of cancer [80]. By using 3D collagen matrix model, we have identified DDR1 as a potential target of LRP-1 in the negative regulation of CRC proliferation. In fact, we demonstrate that LRP-1, by inducing DDR1 endocytosis, decreases its expression at the cell surface and thus inhibits its function as cell proliferation suppressor.

Recent studies indicated the involvement of LRP-1 in cell proliferation in both physiological and pathological contexts. In fact, LRP-1 works either as a regulator or as a mediator to control key proliferative signaling pathways such as PDGF, ERK/MAPK, and AKT/PI3K [392-395, 397]. In addition, upregulation of LRP-1 has been associated with pathogenesis. As an example, upregulation of cell proliferation by LRP-1 results in renal fibrosis [544, 545]. In contrast, LRP-1 was also shown to protect vascular wall integrity and to act against atherosclerosis [393, 546] wherein LRP-1 induces both proliferation and anti-proliferation [24, 547]. Very few studies have reported the role of LRP-1 in regulating the proliferation of cancer cells and some of the published data described a role of LRP-1 as an inhibitor of cancer cell proliferation [23, 29, 435-437, 441-443]. However, a very recent study

indicated that LRP-1:tPA pathway promotes melanoma cell migration and proliferation [461]. Other studies have shown that LRP-1 overexpression seems to correlate with poor prognosis, increased cell proliferation, invasiveness and tumor recurrence [440, 444, 489, 519]. In CRC, little is known about the contribution of LRP-1 in CRC proliferation. Recent studies in our team demonstrated that low LRP-1 expression in colorectal tumors is correlated with unfavorable clinical outcomes [29]. In an apparently contradictory way, in the present work, we demonstrated that LRP-1 up-regulates colorectal adenocarcinoma cell proliferation in 3D collagen matrices (**Figure 2**, Results part). In fact, LRP-1 knockdown using shLRP-1 results in restoration of DDR1 amount at the cell surface and its activation by collagen, thus inducing an inhibition of CRC proliferation (**Figure 3**, Results part). As usual when working on LRP-1, it is somewhat difficult to associate the conclusion from the data of these studies. Indeed, while the first clinical study conducted in the team highlights a role of LRP-1 as tumor suppressor in tumors from patients [12], we showed using our *in vitro* 3D matrix that LRP-1 plays a positive role in CRC tumorigenesis through promotion of proliferation. However, it is important to note that while we investigated the role of LRP-1 in CRC proliferation (tumorigenesis), especially in the non-invasive HT-29 cells, the clinical study cited above has investigated the statute of LRP-1 based on the outcome of patients associated to the risk of aggressiveness and metastasis [12]. This clearly feeds our reflection on the fact that this receptor can play distinct, and sometimes contradictory, roles depending on the cells within the microenvironment and especially on the temporality of tumor development. Concerning DDR1, its role seems to be also controversial. In fact, we described its role as a proliferation (or tumorigenesis) restrictor. However, in the recently published data by Serge Roche's group, DDR1 has been described as a promotor of CRC invasion and metastasis [12, 13]. In this case also, it is quite difficult to associate the conclusions of the two studies since the role of DDR1 has been addressed on two different cellular functions, cell proliferation and invasion, respectively.

As LRP-1 is a multifunctional endocytotic receptor, LRP-1 inhibition results in the accumulation of its ligands or effectors at/around the plasma membrane. In this study, we have clarified that LRP-1 inhibition leads to increase in membrane DDR1 accumulation (**Figure 4A**, Results part), that consequently reduces CRC proliferation. Since LRP1-upregulated cell proliferation was only observed in 3D collagen matrices (**Figure 2**, Results part), we supposed that the density of collagen fibers in 2D models was not sufficient and optimized to induce cell proliferation through LRP1-induced DDR1 endocytic pathway. Although the down-regulated effects of DDR1 on cell proliferation have been well discussed in the Results part of the manuscript (see the original article), some questions concerning other type I collagen receptors

need to be clarified. β1-integrin heterodimers (α1β1, α2β1, α10β1, and α11β1) have been the first studied and the most studied type I collagen receptors [229, 230, 548, 549]. Glycoprotein VI (GPVI) and leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) have been described also as receptors of type I collagen [260]. In the case of integrins, recent studies in our team demonstrated that LRP-1 induces β1-integrin internalization and recycling back to plasma membrane in tumor cells [25]. However, β1-integrin has been reported to negatively regulate cell proliferation in a wide range of cellular environment [549-552]. Recent studies in our team demonstrated that β1-integrin is not involved in the regulation of cell proliferation in epithelial-like breast carcinomas and fibrosarcoma cells in collagen 3D matrix. Indeed, DDR1 and DDR2 have been identified to play a role of cell proliferation suppressors in these two cancer cell models [9, 15, 16, 310]. More importantly, both α1β1 and α2β1 have been reported to promote CRC progression, cell proliferation and survival [553-556]. Little is known about the effects of α10β1 and α11β1 on cell proliferation. Lu and collaborators have shown that α11-integrin is essential for non-small cell lung adenocarcinoma A549 cell proliferation [242]. In the agreement with this study, a very recent work also indicated that α11-integrin mediates 3D cell invasion and ERK-mediated cell proliferation [557]. Similarly, α10β1 was recently shown to promote glioblastoma cell proliferation [558].

To date, a comprehensive understanding of type I collagen's contribution in CRC progression has not yet fully covered. However, recent studies have suggested a role for type I collagen in CRC. Type I collagen has been reported to promote CRC progression through the activation of integrin/PI3K/AKT/Snail signaling pathway [30, 511, 538-540]. In our study, the effects of LRP-1 inhibition was only observed in 3D type I collagen environment (**Figures 2 and 3**, Results part), underlining the fact that a 3D matrix model is the most physiologically relevant to mimic the natural tumor microenvironment and investigate the phenotype of cancer cells *in vitro*.

In this study, we have shown that LRP-1 inhibition by using both RAP and blocking antibodies leads to cell cycle arrest at G1 phase (**Figure 6**, Results part). These data suggested a role for DDR1 in the regulation of cell cycle. Others studies have demonstrated that DDR2 also inhibit cell proliferation by regulating proteins involved in the cell cycle [10, 11, 521].

Our results also indicated that LRP-1 inhibition leads to increase in apoptotic index (**Figure 7**, Results part).

Little is known about a direct involvement of LRP-1 in driving apoptosis. Nevertheless, numerous studies have reported the contribution of LRP-1 to cell survival. For example, early studies showed that LRP-1 deletion leads to mouse embryonic lethality [352]. Wang and

collaborators recently reported that LRP-1 is able to reduce the effects of oxidative stress and neuronal apoptosis in mice [559]. *In vitro*, LRP-1 has been shown to trigger the activation of AKT pathway to promote cell survival [384, 389]. Furthermore, LRP-1 was reported to inhibit apoptosis by modulating JNK signaling pathway [390], and anoikis through TSP1/calreticulin axis signaling [391]. LRP-1 knockdown or inhibition has also been shown to lead to a decrease in AKT activation and to an increase in pro-apoptotic Caspase-3 activation [386-388, 560]. Interestingly, our findings suggested that the accumulation of membrane-anchored DDR1 upon LRP-1 inhibition may mainly contribute to apoptosis in a non-direct manner through the tumor suppression function of DDR1. Consistently, overexpression of DDR1-GFP induced an increase in CRC apoptosis (**Figure 7C**, Results part). Indeed, DDR1 was reported to induce BIK expression, a pro-apoptotic member of the BCL-2 protein family that triggers apoptotic pathway [9, 310]. However, it has been demonstrated that DDR1 promotes cell survival [6, 303, 561-563]. Moreover, DDR1 has been shown to be necessary for the formation of linear invadosomes at the level of collagen fibers for the invasion process of basal-like breast carcinoma cells without requiring the kinase function of the receptor [313].

In conclusion, we identified that LRP-1-mediated internalization of DDR1 promotes non-invasive CRC proliferation. This is probably just one of several features supported by the LRP-1/DDR1 interaction in the CRC context. This new mechanical insight is proposed in the graphical abstract below (**Figure 26**, page 152).

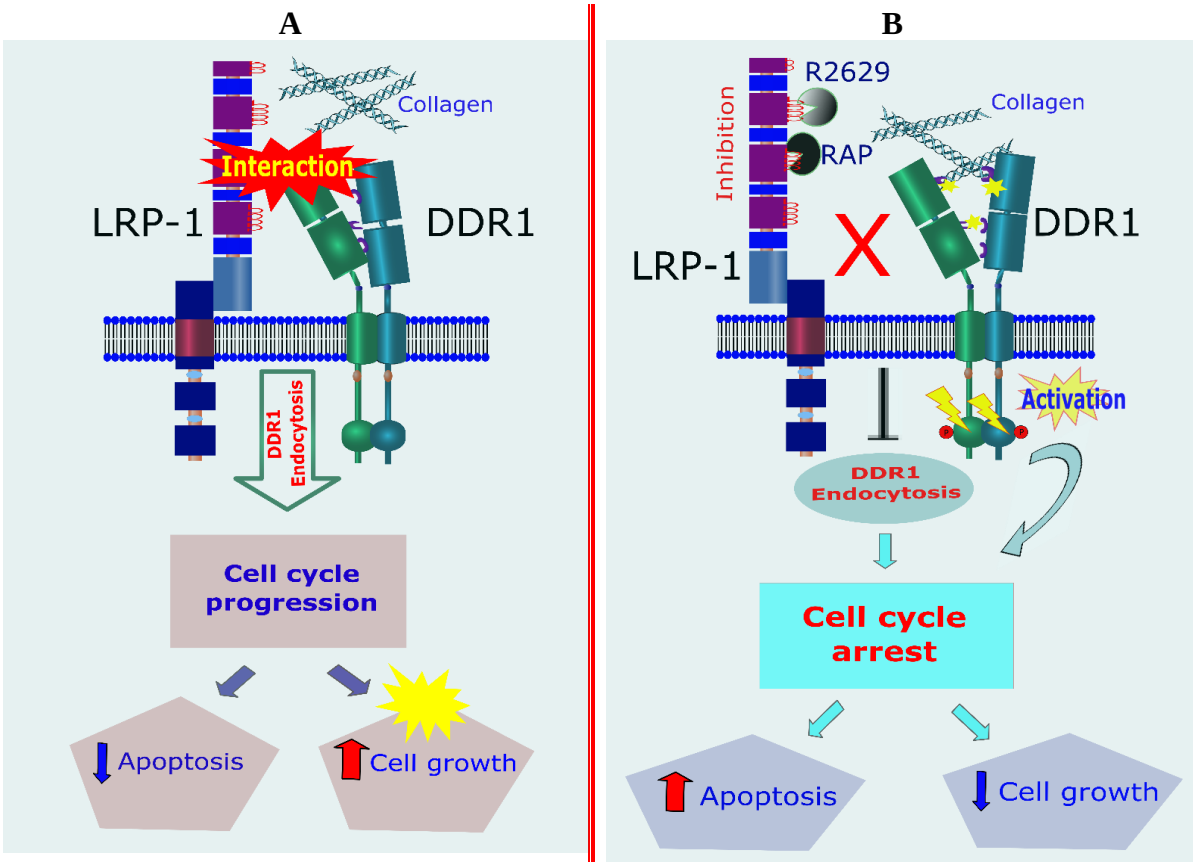

Figure 26. A proposed model for LRP-1/DDR1 pathway in non-invasive CRC. *(**A**) In basal conditions, LRP-1 induces DDR1 internalization, sustaining cell cycle progression and leading to decreased cell apoptosis and increased cell proliferation. (**B**) Upon LRP-1 antagonization or inhibition by either RAP or R2629, LRP-1 becomes unable to interact with cell-surface DDR1, thus decreasing DDR1 endocytosis and increasing the amount of activated DDR1 at the cell surface. This leads to cell cycle arrest and subsequent down-regulation of cell proliferation and up-regulation of apoptosis.*

Perspectives

As mentioned previously, the findings of this study highlighted a new functional association between LRP-1 and DDR1 playing a role in the regulation of cell proliferation and apoptosis of colon carcinomas. This study was carried out in 3D type I collagen matrix culture system. This culture system was critical since 2D collagen coating conditions did not lead to the same effects.

However, in this model, the principal limit was that the tumor microenvironment cannot be reconstituted nor mimicked owing to important stromal cells, such as cancer-associated fibroblasts and endothelial cells. New 3D models of cancer using a collagen matrix can include these components to allow the cancer-stromal cell crosstalk, therefore being able to fill this gap in the biomimicry of cancer disease models. Recent methodologies have been developed to discriminate different cell populations in co-culture models and to analyze their interactions and phenotypes [522-525]. The use of GFP and luciferase/luciferin reporters in co-cultures have been largely described and used [523]. Other components of the ECM should be also considered. In fact, a recent study has shown that compared to normal tissue, tumor ECM of CRC contains also specifically other adhesive components (fibronectin, type VI collagen, tenascin …) that should be taken into account, notably in the right ratios [526]. However, the implementation of these multi-cellular systems is extremely difficult and time-consuming to implement, and the technical deliverables are often limited to combining two different cell types at the best, and rarely more. In addition, due to the extremely complex and sometimes antagonistic culture requirements between cell types, it is difficult to use these systems for long observations, when we were able to do by for more than 5 days using our 3D system.

Regarding our data, it appears essential to elucidate the effects of the LRP-1/DDR1 complex on CRC cell proliferation in separate cellular contexts using co-cultures with stroma cells and/or *in vivo* assays. For instance, the experiments being carried out by the team on the role of LRP-1 in CAFs should make it possible to increase our molecular knowledge on this topic and to consider relevant 3D co-culture models including CAFs in the coming months.
Moreover, prognostic and clinical impact of DDR1 expression in colorectal cancers and its potential association with a specific morphologic and/or a molecular profile needs to be accurately addressed. A clinical study conducted by our group is currently underway to expand our current knowledge about the expression of DDR1 in colorectal cancers and its association with LRP-1 expression. This work is carried out using a previously validated and annotated cohort of patients from the Reims Hospital [29]. The first results of this recently initiated work showed that DDR1 is very strongly expressed in colorectal cancer tissues in all cases, whereas it is only expressed in a moderate to strong manner in 93% of cases in normal tissues. These

results are very promising and needs to be confirmed and extended. We will notably seek to merge and reconcile our results regarding DDR1 expression with those already obtained on LRP-1 using the same cohort and explore correlations with patient survival or recurrence data.

Although we have observed changes in cell cycle upon LRP-1 inhibition, the regulation of cell cycle needs to be studied in more detail. A better understanding of the molecular mechanisms involved in the LRP-1/DDR1-mediated pathway, especially regarding signaling pathways and the downstream targets, would allow to better establish how the cell cycle and the apoptosis are regulated [527, 528]. For instance, the transcription factor c-Myc promotes cell cycle progression by inducing transcription of genes involved in the cell cycle activation and by suppressing the expression of genes that are involved in the negative regulation of cell cycle. Tumor suppressors such as p53 and RB inhibit cell cycle progression and induce apoptosis [527-531]. These are examples of potential targets that will need to be assessed.

It is well documented that interactions between cells and ECM drive several processes including cell proliferation, migration, and differentiation. ECM remodeling naturally occur during development, tissue homeostasis and diseases [214]. As mentioned previously, type I collagen is one of the most abundant proteins throughout the body and is the main component of ECM, which plays crucial roles in both physiological and pathological conditions [532-534]. Type I collagen remodeling has been *associated* with *tumor aggressiveness* and poor prognosis in *patients [21, 25, 26]*. In CRC, type I collagen has been reported to be involved in tumorigenesis, EMT, invasiveness, and metastasis [30, 511, 538-540]. In fact, it has been well-documented that collagen remodeling promotes cancer progression [214, 541]. Excessive type I collagen turnover during CRC progression has been reported [538]. Moreover, LRP-1 is well-known to be involved in endocytosis of several constituents of the ECM (fibronectin, decorin, metalloproteases, growth factor…) and plays a crucial role in the assembly, the turnover and the control of matrix network organization. To know whether CRC cells can remodel type I collagen matrix under *in vitro* conditions and whether LRP-1 could play a role in this process, we propose to finely analyze collagen fibril organization in 3D matrix system. In our preliminary experiments, the colorectal carcinoma cells were seeded in 3D type I collagen matrices in the presence or not of the LRP-1 antagonist RAP. Then, cells and collagen fibers were analyzed by SHG (**Figure 27**, page 156). Excitingly, our results showed that LRP-1 inhibition results in a significant decrease in collagen fiber diameter (**Figure 28C-D** page 157). RAP treatment did not however induced significant changes on the collagen fiber length and straightness (**Figure 28E-H**, page 157). These preliminary data suggest for the first time that LRP-1-engaged processes could be directly involved in collagen remodeling.

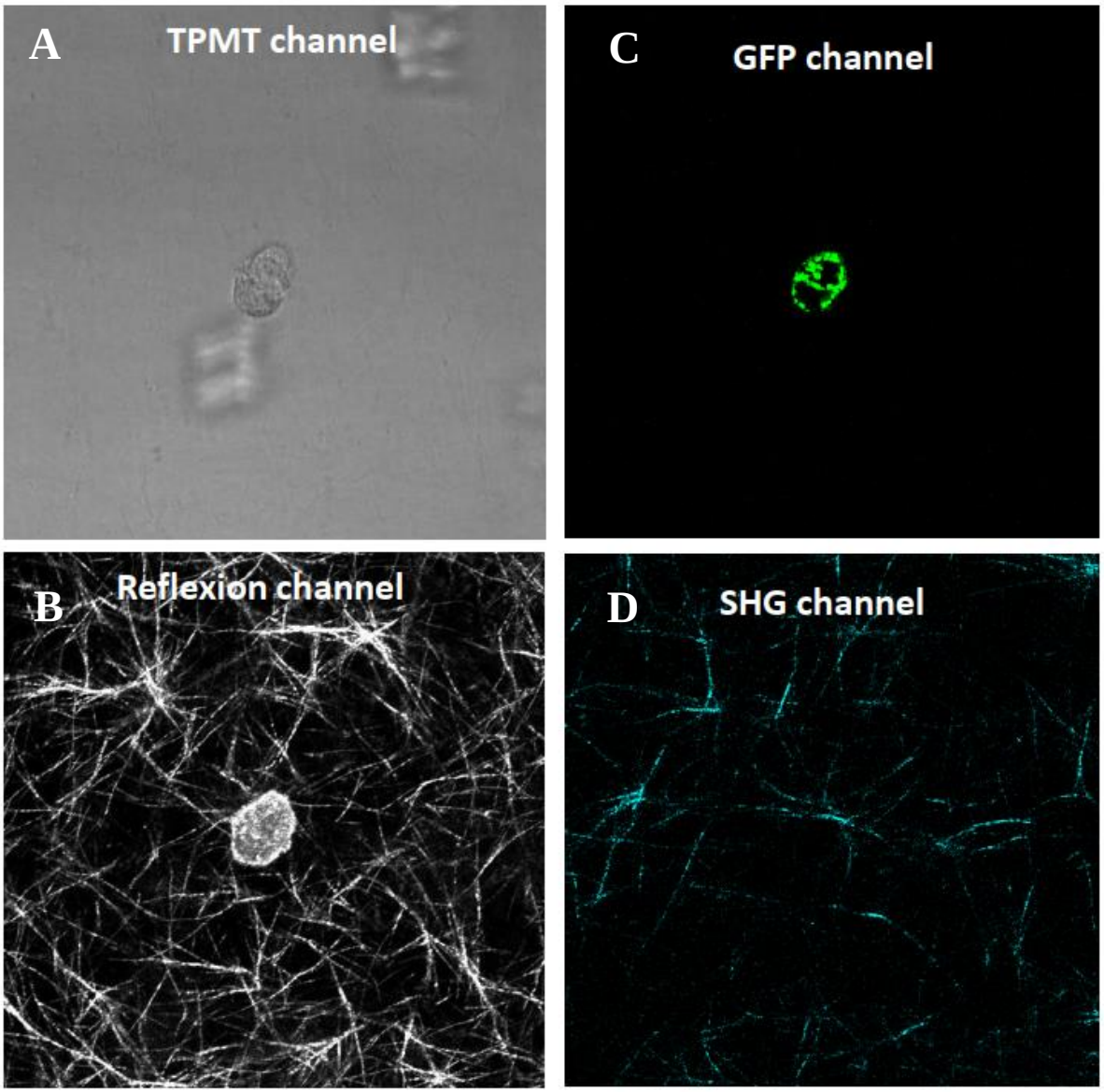

Figure 27. Second Harmonic Generation imaging. *HT-29$^{DDR1\text{-}GFP}$cells were seeded in 3D type I collagen matrix with/without RAP treatments (500 nM) for 3 days. SHG images were performed using Zeiss LSM 710-NLO confocal microscopic system. Laser excitation was set at 860 nm using a CHAMELEON femtosecond Titanium-Sapphire laser (Coherent, Courtaboeuf, France). Laser power was adjusted up to 20 mW.* The emitted SHG signal, *after spectral* filtering using a *420–440 nm band-pass filter, is shown in blue. Backward pSHG images (425 µm × 425 µm) were acquired using ZEN imaging software. (**A**) Pixel by pixel detection of transmission-photomultiplier tube. (**B**) The organization of collagen fibers and cells using reflection channel (**C**). Detection of HT-29$^{DDR1\text{-}GFP}$cells with GFP channel. (**D**) Detection of collagen fibers with SHG channel.*

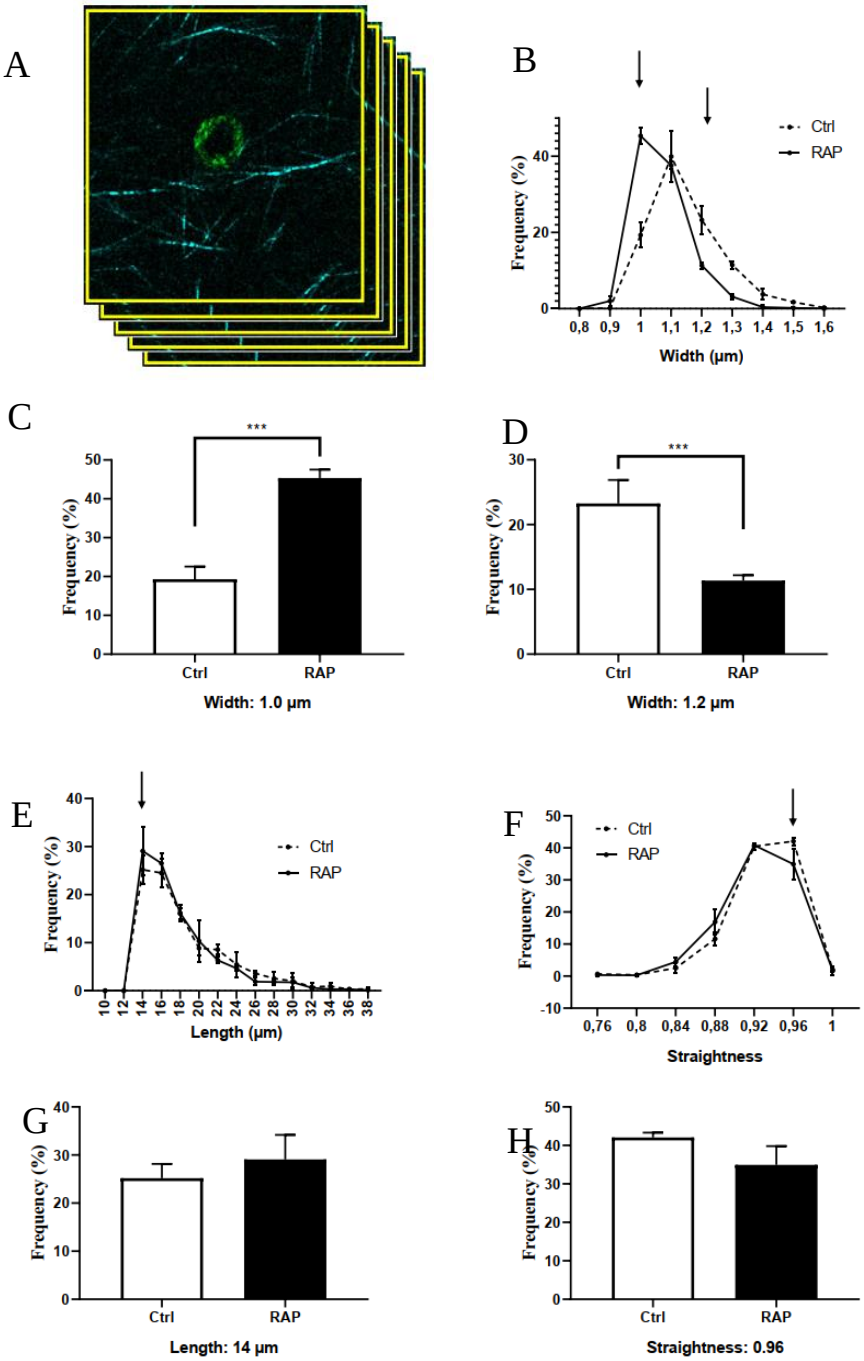

Figure 28. Analysis of SHG signal. *(**A**) The model of frame-by-frame analysis for studying fibrillar type I collagen remodeling. (**B**) The graph represents the distribution of width values of all fibers in control and RAP treated conditions. (**C, D**) RAP treatment results in a significant decrease in the width of collagen fibers. The graphs E and F represent the distribution of length (**E**) and straightness (**F**) respectively of the fibers. The quantitative analysis of fiber length and straightness is presented in **G** and **H** respectively.*

Although the mechanisms of ECM remodeling by tumor cells have not yet well been elucidated, recent studies have shown a cross-talk between tumor cells and ECM which involves a role of factors secreted by tumor cells in the remodeling and/or degradation of ECM components, particularly type I collagen. It is known that type I collagen degradation are processed by multiple proteases. *Metalloproteinases (*MMP) family is among the most well-known and characterized proteases involved in ECM remodeling, especially in the degradation of type I collagen [564-566]. For example, MMP-9 was reported to degrade type I collagen [567]. Karagiannis and Popel have reported that MMP-2 is likewise involved in type I collagen degradation [568] while serum levels of type I collagen degradation products have been associated with poor prognosis in lung cancer [569]. It should be noted that MMP-1 and MMP-14/MT1-MMP are the most widely studied proteases regarding type I collagen degradation. MT1-MMP plays also an important role in MMP-2 activation [570-572], underlining the intricacy of the networks for activating these processes. Furthermore, urokinase-type plasminogen activator (uPA) also has been shown to promote type I collagen degradation through inducing MMP activation [459, 460]. In CRC, the expression of MMP-1, MMP-2, MMP-9 and MMP-13 have been demonstrated to correlate with tumor invasiveness, metastasis, and poor prognosis in patients with CRC [227]. More importantly, MMP-9 expression level has been reported as an indicator of advanced CRC stages [573-575]. As previously mentioned, LRP-1 regulates through endocytosis the pericellular levels of several extracellular proteases, especially MMP-2, MMP-9, MMP-13 and uPA among those mentioned above [55-58], suggesting that LRP-1 indirectly contributes by this way to ECM remodeling.

Our data may partly explain how LRP-1 inhibition leads to collagen fiber remodeling and further investigations should be necessary to confirm this hypothesis. We will seek to reinforce our preliminary findings by using adapted vibrational imaging (Raman imaging) to better characterize the effects of LRP-1-mediated endocytosis on the 3D organization of the collagen fibers. To go further on that way, we notably propose in the forthcoming weeks to use several CRC cell models exhibiting different invasiveness levels and distinct patterns of expression for extracellular proteases, to evaluate the effects of LRP-1 on the type I collagen properties. These investigations will be completed by biochemical analyzes of type I collagen degradation.

Scientific activities and achievements

International publications

Le Cao Cuong, Amar Bennasroune, Guillaume Collin, Cathy Hachet, Véronique Lehrter, Damien Rioult, Stéphane Dedieu, Hamid Morjani, Aline Appert-Collin. LRP-1 promotes colon cancer cell proliferation in 3D collagen matrices by mediating DDR1 endocytosis. *British Journal of Cancer (submitted).*

Le Cao Cuong, Amar Bennasroune, Benoit Langlois, Stéphanie Salesse, Camille Boulagnon-Rombi, Hamid Morjani, Stéphane Dedieu & Aline Appert-Collin. The Impact of Tumor Extracellular Matrix Cross-Talk on Cancer Hallmarks. *Frontiers in Oncology (submitted).*

Oral presentations

Cao-Cuong Le, Amar Bennasroune, Guillaume Collin, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune. Role of LRP-1 in Colon Cancer Cell Proliferation in 3D Culture Systems. Cancéropôles Grand Est-11th Forum, ***November 15-16, 2018***, Centre des congrès, Reims, France.

Cao-Cuong Le, Amar Bennasroune, Guillaume Collin, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune LRP-1 increases colon cancer cell proliferation in 3D culture systems by mediating DDR1 endocytosis. First International Conference on Discoidin Domain Receptor Biology *(1st DDRs Meeting)*, ***May 22-24, 2019***, Bordeaux, France.

Cao-Cuong Le, Amar Bennasroune, Guillaume Collin, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune. LRP-1 mediates colon cancer cell proliferation in 3D culture systems by mediating DDR1 endocytosis. Journée Rémoise Des Jeunes Chercheurs en Santé 2019, ***October 17, 2019***, *Faculty of Medicine (URCA), Reims, France.*

Poster presentations

Cao-Cuong Le, Amar Bennasroune, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune. 1ère Journee Role of LRP-1 in colon cancer cell proliferation in 3D culture systems**.** Doctorale Transfrontaliere, ***March 7th, 2018***, University of Reims Champagne-Ardenne, Reims, France.

Cao-Cuong Le, Amar Bennasroune, Guillaume Collin, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune. Role of LRP-1 in colon cancer cell proliferation in 3D culture systems. Journée. Rémoise Des Jeunes Chercheurs en Santé 2018***, October 18, 2018****, Faculty of Medicine (URCA), Reims, France.*

Cao-Cuong Le, Amar Bennasroune, Guillaume Collin, Stéphane Dedieu, Hamid Morjani, Aline Bennasroune. LRP-1 increases colon cancer cell proliferation in 3D culture systems by mediating DDR1 endocytosis. Annual Meeting of the French Society For Extracellular Matrix Biology, ***May 15-17, 2019***, Faculty of Medicine (URCA), Reims, France.

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Additional courses

Catégorie : Autres dispositions permettant l'octroi des crédits

☛ JREDs - Journées de Rentrée des Ecoles doctorales STS et SHS de l'URCA (09 janvier 2018) Campus Croix rouge - Amphi Recherche
16 heures Note : 16 Crédit : 2

Total du nombre d'heures pour la catégorie Autres dispositions permettant l'octroi des crédits : *16 h*
Total du nombre de crédits pour la catégorie Autres dispositions permettant l'octroi des crédits : *2*

Catégorie : Connaissance du Milieu Professionnel

☛ UE 32c2 - Dispositifs internationaux en matière de recherche et innovation (21 juin 2018) Ecoles Doctorales - Bâtiment 13 - R320 - Campus Croix-Rouge
3 heures Crédit : 1

Total du nombre d'heures pour la catégorie Connaissance du Milieu Professionnel : *3 h*
Total du nombre de crédits pour la catégorie Connaissance du Milieu Professionnel : *1*

Catégorie : Diffusion De La Recherche

☛ Communication en anglais dans un colloque international : 1st DDRs Meeting, université Bordeaux Universite' de Bordeaux
48 heures Crédit : 4

Total du nombre d'heures pour la catégorie Diffusion De La Recherche : *48 h*
Total du nombre de crédits pour la catégorie Diffusion De La Recherche : *4*

Catégorie : Langues et Techniques de communication

☛ UE 01 - Anglais - Entrainement conversation niveau avancé (B2 et+) - Interculturalité (07 février 2019) Animation doctorale - Bâtiment 13 - 1er étage aile gauche - Salle R320
16 heures Crédit : 4

☛ UE 01 - Anglais - Entrainement conversation niveau avancé (B2 et+) - Interculturalité (08 février 2018)
14 heures

☛ UE 02b - Anglais - Rédaction en anglais STS (26 avril 2017) Campus Sciences Moulin de la Housse - Salle 3R80
16 heures Crédit : 4

Total du nombre d'heures pour la catégorie Langues et Techniques de communication : *46 h*
Total du nombre de crédits pour la catégorie Langues et Techniques de communication : *8*

Catégorie : Méthodologie et outils de la thèse

☛ UE 12a - Introduction à la recherche de brevets (04 avril 2019) BU Moulin de la housse
2 heures Crédit : 1

☛ UE 12c - Seminaire BU : You and the library : an overview – Panorama pour étudiants non-francophones (Présentation) (08 mars 2018) Campus Moulin de la Housse, BU
2 heures Crédit : 1

☛ UE 12d - Journée d'étude Science Ouverte (26 mars 2019) Campus Croix Rouge - Amphi 10
4 heures Crédit : 2

☛ UE 12d - Seminaire BU Reims - atelier Scopus (23 avril 2019) Facultés de Médecine et de Pharmacie, pôle Santé de Reims, amphithéâtre n° 5
2 heures Crédit : 1

Total du nombre d'heures pour la catégorie *: 10 h*
Total du nombre de crédits pour la catégorie *: 5*

Total participation : 123 heures / 10 modules

Total des Crédits de Thèse : 20

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Rôle de LRP-1 dans la prolifération des cellules issues de cancer du côlon en matrice tridimensionnelle de collagène de type I

Le récepteur *low-density lipoprotein receptor-related protein-1* (LRP-1) est un récepteur d'endocytose multifonctionnel impliqué dans de nombreux processus physiologiques et pathologiques. Plusieurs études ont montré que LRP-1 joue un rôle crucial lors des processus de tumorigenèse et durant la progression tumorale, notamment en régulant l'expression de protéines membranaires. Des études antérieures ont montré l'implication des récepteurs du collagène de type I de la famille des *Discoidin Domain Receptors* (DDRs) dans la régulation de la prolifération des cellules cancéreuses en 3D. Le but de ce travail est d'étudier s'il existe une interaction fonctionnelle entre LRP-1 et DDR1 et si celle-ci pourrait moduler la prolifération des cellules de cancer colorectal (CRC) cultivées dans une matrice 3D de collagène de type I.

Nos résultats ont permis de montrer qu'une invalidation de LRP-1 ou une inhibition de son activité par l'utilisation d'antagonistes sélectifs (RAP, anticorps bloquants) altère la prolifération des cellules de CRC LS174T et HT-29, uniquement lorsque qu'elles sont intégrées dans une matrice 3D de collagène de type I. De plus, la surexpression de DDR1-GFP dans les cellules HT-29 (HT-29$^{DDR\text{-}GFP}$) diminue leur taux de croissance, tandis que l'inhibition de LRP-1 par RAP induit un arrêt du cycle cellulaire et une augmentation de l'apoptose dans les cellules HT-29 et HT-29$^{DDR\text{-}GFP}$. Nous avons montré que la quantité de DDR1 à la surface cellulaire était augmentée et que l'endocytose de DDR1 était réduite de moitié lors du traitement par RAP, mettant ainsi en évidence une nouvelle voie d'internalisation pour DDR1. De plus, LRP-1 et DDR1 co-immunoprécipitent ensemble indiquant que ces récepteurs sont fortement associés au sein d'un même complexe moléculaire dans les cellules de CRC.

Nos résultats mettent en évidence l'existence d'une interface fonctionnelle entre LRP-1 et DDR1 I soutenant la prolifération des cellules de CRC dans une matrice 3D de collagène.

LRP-1, DDR1, cancer colorectal, prolifération, matrice 3D de collagène de type I

Role of LRP-1 in colon cancer cell proliferation in three-dimensional culture systems

Low-density lipoprotein receptor related protein-1 (LRP-1) is a multifunctional endocytic receptor mediating the clearance of various molecules from the extracellular matrix, including metalloproteases and various glycoproteins. Several studies have shown that LRP-1 plays crucial roles in tumorigenesis and during tumor progression. LRP-1 also functions as a main regulator of signaling pathway by interacting with other cell-surface receptors. Previous studies have highlighted the involvement of Discoidin Domain Receptors (DDRs), type I collagen receptors with tyrosine kinase activity, in the regulation of cancer cell proliferation in 3D experimental models. The aim of this work is to study the potential functional interplay between LRP-1 and DDR1 in order to investigate whether this interaction may modulate the proliferation of colorectal cancer (CRC) cells in highly relevant 3D type I collagen matrices.

In this study, we demonstrated that inhibition of LRP-1-mediated endocytosis using RNA interference or selective antagonists (RAP and R2629 blocking antibodies) impaired LS174T and HT-29 carcinoma cell proliferation, but only when embedded in a 3D collagen matrix. Using 3D cultures, DDR1-GFP overexpressing HT-29 (HT-29DDR-GFP) reduced the colorectal carcinoma cell growth rate, whereas RAP treatment led to cell cycle arrest and induced apoptosis in both HT-29 and HT-29DDR-GFP. By streptavidin/biotin-based immunoassays, we demonstrated that membrane-anchored DDR1 amount was increased upon RAP treatment while DDR1 uptake was reduced by a half upon LRP-1 inhibition, highlighting a new way for DDR1 internalization and dynamics. Consistently, co-immunoprecipitations confirmed the existence of a LRP1:DDR1 biomolecular complex at the cell surface of CRC cells.

Our results suggest a role for LRP-1 in promoting CRC cell proliferation in 3D collagen environment by mediating DDR1 endocytosis.

LRP-1, DDR1, colon cancer cell, proliferation, 3D collagen matrix

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