

Université de Reims Champagne-Ardenne

UFR Pharmacie



Année 2012

N° :

Thèse présentée pour l'obtention du grade de

DOCTEUR DE L'UNIVERSITE DE REIMS CHAMPAGNE-ARDENNE

Mention : Sciences de la Vie

Spécialité : Biologie Cellulaire et Moléculaire

Présentée et soutenue publiquement le 7 décembre 2012 par

Mathilde POPLINEAU

Née le 23 mai 1984 à Villers-Semeuse (08)

Rôles des mécanismes épigénétiques dans la régulation de l'expression de gènes impliqués dans l'invasion de cellules tumorales

Unité MEDyC, CNRS FRE 3481, SFR Cap Santé, UFR Pharmacie

Membres du jury

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Rapporteurs : Dr Robert DANTE (CRCL, Lyon)
Pr Patrick HENRIET (UCL, Bruxelles)

Examineur : Dr Michael SCHNEKENBURGER (LBMCC, Luxembourg)

Directeurs de thèse : Pr Jean DUFER (MEDyC, Reims)
Dr Aurélie TRUSSARDI-REGNIER (MEDyC, Reims)

Remerciements

A Monsieur le Professeur Patrick Henriet

Je vous remercie d'avoir accepté de juger ce travail de thèse et d'en être rapporteur scientifique. Veuillez trouver ici l'expression de ma reconnaissance et soyez assuré de mon profond respect.

A Monsieur le Docteur Robert Dante

Robert, je vous adresse mes remerciements les plus respectueux. Je ne saurais à quel point vous dire merci pour vos encouragements, vos conseils et critiques qui ont toujours été constructifs durant ma thèse. Je vous remercie pour l'intérêt que vous avez apporté à mes travaux en tant que rapporteur scientifique.

A Monsieur le Professeur Frank Antonicelli

Je suis très honorée de vous compter parmi les membres de mon Jury et je vous remercie pour votre encadrement durant mon cursus universitaire. Merci d'avoir cru en mon savoir scientifique et de m'avoir toujours poussée (à bout) pour tirer le meilleur de mes compétences.

A Monsieur le Docteur Marc Diederich

Je vous remercie très sincèrement de m'avoir accueillie au sein de votre laboratoire. Ce séjour m'a donné l'opportunité d'évoluer dans une équipe très dynamique et m'a permis d'acquérir des compétences certaines dans le domaine de l'épigénétique. Je vous remercie pour la confiance et le soutien que vous avez su m'accorder. Veuillez accepter toute ma gratitude et soyez assuré de tout mon respect.

A Monsieur le Docteur Michael Schnekenburger

Michael, je ne saurais à quel point te remercier pour m'avoir également encadrée durant ma thèse. Tes conseils, remarques et critiques ont toujours été très constructifs. Je tiens à t'adresser toute ma gratitude pour l'appui scientifique que tu m'as apporté, pour le temps que tu m'as consacré, ainsi que pour ton soutien amical et pour la confiance que tu as su m'accorder.

Remerciements

A monsieur le Professeur Jean Dufer

Monsieur, je vous suis très reconnaissante de m'avoir encadrée et de m'avoir guidée dans ce travail. Je vous remercie pour votre disponibilité et votre efficacité mais également pour votre gentillesse et votre bonne humeur. Merci de m'avoir accordé votre confiance dans mon approche du sujet (...et d'avoir toléré ma forte personnalité). Je vous remercie également de m'avoir donné la chance de participer à de nombreux congrès nationaux et internationaux.

A Madame le Docteur Aurélie Trussardi-Régnier

Aurélie, je te remercie sincèrement pour ton investissement dans ce travail de thèse. Tu as toujours été à mes côtés et tu as toujours su défendre les intérêts de ton étudiante. Mon fort caractère et ma détermination n'ont pas toujours été évidents à gérer pour toi et je te remercie d'avoir accepté ces traits de personnalité. Je te remercie également pour ta gentillesse et ta bonne humeur permanente et je n'oublierai jamais les bons moments que nous avons pu passer ensemble. Merci de m'avoir transmis ton savoir et de m'avoir enrichie par tes conseils.

Je tiens également à remercier :

Monsieur le Docteur Olivier Piot, pour m'avoir initiée aux spectroscopies vibrationnelles. Je vous remercie pour votre soutien scientifique et moral lors de la rédaction de notre papier paru dans *Epigenomics*.

Monsieur le Docteur William Hornebeck et Madame le Docteur Sylvie Brassart-Pasco, pour leur disponibilité et leurs conseils scientifiques.

Madame le Docteur Hélène Bobichon, pour m'avoir initiée aux microscopies optiques. Merci pour votre disponibilité et votre rigueur scientifique.

Laurence Van-Gulick, pour sa gentillesse et ses compétences techniques. Merci de m'avoir guidée dans certaines de mes expériences.

Ma grande amie, **Marie Gilbert**. Durant ces années de travail, tu as été le moteur de ma réussite. Tu m'as toujours soutenue et je ne te remercierai jamais assez. Nos pauses café et nos grandes conversations scientifiques vont dès à présent me manquer mais tu resteras toujours une de mes plus fidèles amies.

Remerciements

*Tous mes autres amis doctorants et post-doctorants de la « team MÉDIAN »: **Aurélie, Caroline, David, Georges, Hadrien, Jaya, Mathieu, Marwa, Nathalie, Teddy, The Thuong et Vincent**.... Sans oublier mes amis de Regentis : **Joan et Julien**. Un grand merci également à **Fanny** pour tes conseils en biologie moléculaire.*

Monsieur le Professeur Michel Manfait et Monsieur le Professeur Pierre Jeannesson.

Christine, Cyril, Ganesh, Hamid, Irène, Jennifer et Nathalie.

***Madame Pisani, Madame Cousina et Céline,** pour leur gentillesse, leur amabilité et leur hospitalité.*

*Mes **collègues** et **amis du LBMCC** qui m'ont accueillie et intégrée dans leur équipe de recherche.*

*Enfin, je remercie toute ma **famille** et mes **proches** pour leur soutien et leurs encouragements.*

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Résumé & Abstract

Les propriétés invasives des cellules cancéreuses sont liées à des modulations importantes de l'expression de gènes. Des protéases doivent être exprimées afin de permettre la dégradation de la matrice extracellulaire (MEC), l'activation protéolytique de protéines matricielles et la libération de facteurs de croissance, de cytokines, de récepteurs et de molécules d'adhérence. Parmi ces protéases, les métalloprotéinases matricielles (MMPs) jouent un rôle crucial dans la dégradation de la MEC et dans le remodelage tissulaire observé au cours de l'invasion tumorale. L'émergence de thérapeutiques anticancéreuses basées sur des stratégies épigénétiques nécessitent d'évaluer leurs effets sur les propriétés des cellules tumorales. Ce travail a pour objectif d'analyser les effets de modulateurs épigénétiques (un agent hypométhylant de l'ADN et des inhibiteurs d'histone désacétylases (inhibiteurs d'HDACs ou HDIs)) sur l'expression des MMP-1, -2 et -9 dans la lignée cellulaire de fibrosarcome humain HT1080. Dans un premier temps, il apparaît que l'agent hypométhylant de l'ADN, la 5-aza-2'déoxycytidine (5-azadC), augmente l'expression génique et protéique des MMP-1, -2 et -9. Ces modifications de l'expression sont associées à (i) une déméthylation globale de l'ADN et (ii) des modifications de la supra-organisation chromatinienne correspondant globalement à une chromatine moins condensée. De plus, la 5-azadC est capable d'accroître les propriétés invasives des cellules par l'intermédiaire, notamment, d'une augmentation de l'expression de la MMP-1 par un mécanisme transcriptionnel. Cette augmentation de la transcription implique le recrutement du facteur Sp1 et un remodelage chromatinien au niveau du promoteur du gène de la MMP-1. Néanmoins, une déméthylation totale de ce promoteur n'est pas nécessaire à cette induction. De manière complémentaire, le traitement des cellules HT1080 par différents HDIs révèle le rôle potentiel d'HDACs dans la régulation de l'expression de la MMP-1. Un HDI à large spectre, la trichostatine A (TSA), est capable de moduler l'expression de la MMP-1 et la texture nucléaire, mais uniquement après déméthylation préalable de l'ADN par la 5-azadC. Par contre, l'HDI spécifique des HDACs de classe I, le MS-275, est capable d'induire, à lui seul, l'expression génique et protéique de la MMP-1. Cette expression génique requiert un remodelage de la chromatine et le recrutement de l'histone acétyltransférase p300 au niveau du promoteur du gène de la MMP-1. L'ensemble de ces résultats suggèrent que des mécanismes épigénétiques jouent un rôle crucial dans le contrôle de l'expression de la MMP-1 dans les cellules HT1080, influençant ainsi les propriétés invasives de ces cellules.

Invasive properties of cancer cells require critical changes in gene expression. Proteases must be expressed for the degradation of the extracellular matrix (ECM), the proteolytic activation of matrix proteins and the release of bioactive molecules such as growth factors, cytokines, receptors and adhesion molecules. Among these proteases, the matrix metalloproteinase (MMP) family members play a crucial role in the ECM breakdown and remodeling of tissues during tumor invasion. The introduction of epigenetic strategies in the therapeutic arsenal against cancer led to the need to evaluate the effects of such therapeutic approaches on cell behavior. Here we focused our attention on the effects of epigenetic modulators, a DNA hypomethylating agent and histone deacetylase inhibitors (HDAC inhibitors or HDI), on the expressions of MMP-1,-2, and -9 in the human HT1080 fibrosarcoma cell line. First, we showed that the DNA hypomethylating drug 5-aza-2'-deoxycytidine (5-azadC) increases MMP-1, -2, -9 expressions both at the mRNA and protein levels. These changes in gene expression are associated with (i) a global DNA demethylation and with (ii) modifications in chromatin supra-organization which globally correspond to a more decondensed chromatin. Moreover, 5-azadC is able to increase the invasive properties capability of the HT1080 cells mainly *via* MMP-1 transcription-dependent expression. This enhancement of transcription occurs through (i) Sp1 recruitment, (ii) chromatin remodeling and (iii) in absence of full demethylation on the MMP-1 gene promoter. Using different HDIs reveals that HDACs could potentially play a role in MMP-1 expression. The pan-HDI trichostatin A (TSA) act in synergy with 5-azadC and is able to modulate MMP-1 expression and nuclear texture, but only after DNA demethylation. In contrast, the HDAC class I inhibitor, MS-275, which display additive effect with 5-azadC, is able to induce, alone, MMP-1 gene expression through chromatin remodeling and p300 recruitment to its promoter. These data suggest that epigenetic mechanisms play a crucial role in MMP-1 expression control in HT1080 cells thus influencing the invasive potential of these cells.

Abréviations

5-azaC	5-azacytidine
5-azadC	5-aza-2'-désoxycytidine
5mC	5-méthylcytosine
ADAM	<i>A disintegrin and metalloproteinase domain-containing protein</i>
ADP	Adénosine diphosphate
AMM	Autorisation de mise sur le marché
AP-1	<i>Activator protein-1</i>
AP-2	<i>Activator protein-2</i>
ARN pol II	ARN polymérase II
ATP	Adénosine triphosphate
ATU	Autorisation temporaire d'utilisation
AUF1	<i>AU-rich element RNA-binding protein 1</i>
BCL3	<i>B-cell chronic lymphocytic leukemia/lymphoma 3</i>
BET	<i>Bromodomain and extra-terminal</i>
BRCA1	<i>Breast-cancer susceptibility gene 1</i>
C	Cytosine
CARM1	<i>Co-activator-associated arginine methyltransferase 1</i>
CBD	<i>Collagen binding domain</i>
CBP	<i>CREB-binding protein</i>
CEBP-β	<i>CCAAT/enhancer binding protein-β</i>
ChIP	<i>Chromatin immunoprecipitation</i>
CoA	Coenzyme A
CoBRA	<i>Combined bisulfite restriction analysis</i>
Col	Collagène
CpGi	Ilôts CpG
CREB	<i>cAMP responsive element-binding protein</i>
CXCL	<i>Chemokine (C-X-C motif) ligand</i>
Cys	Cystéine
DNMT	<i>DNA methyltransferase</i>
EGF	<i>Epidermal growth factor</i>
ERK	<i>Extracellular signal-regulated kinase</i>

Ets	<i>Erythromatosis twenty-six</i>
FADD	<i>Fas-associated protein with death domain</i>
FasL	<i>Fas ligand</i>
FDA	<i>Food and drug administration</i>
GNAT	<i>GCN5-related N-acetyltransferase</i>
GPI	<i>Glycophosphatidylinositol</i>
HAT	<i>Histone acétyltransférase</i>
HBS	<i>HIF-binding site</i>
HDAC	<i>Histone désacétylase</i>
HDI	<i>HDAC inhibitor</i>
HDM	<i>Histone déméthylase</i>
HIF	<i>Hypoxia-inducible factor</i>
HMT	<i>Histone méthyltransférase</i>
Hsp90	<i>Heat shock protein 90</i>
HuR	<i>Human antigen R</i>
Ig	<i>Immunoglobuline</i>
IGF2	<i>Insulin-like growth factor 2</i>
IL-1β	<i>Interleukin-1β</i>
IPSS	<i>International prognostic scoring system</i>
JAK	<i>Janus kinase</i>
JmJc	<i>Jumonji C</i>
Kb	<i>Kilobase</i>
Kit-L	<i>Kit-Ligand</i>
KRE	<i>Keratinocyte differentiation factor-1 responsive element</i>
LAM	<i>Leucémie aiguë myéloblastique</i>
LMC	<i>Leucémie myélomonocytaire chronique</i>
LRP1	<i>Low density lipoprotein receptor-related-protein 1</i>
LSD1	<i>Lysine-specific histone demethylase 1</i>
LSD2	<i>Lysine-specific histone demethylase 2</i>
MAPK	<i>Mitogen activated protein kinase</i>
MBD	<i>Methyl-CpG-binding domain</i>

MBD2 AS	<i>MBD2 antisens</i>
MBT repeat	<i>Malignant brain tumor repeat</i>
MCP-3	<i>Monocyte Chemotactic Protein-3</i>
me1	Monométhylé
me2	Diméthylé
me3	Triméthylé
me2s	Diméthylé symétriquement
me2a	Diméthylé asymétriquement
MEC	Matrice extracellulaire
MeCP2	<i>Methyl CpG binding protein 2</i>
Mi2/NuRD	<i>Mi-2/nucleosome remodeling and deacetylase</i>
MLL2	<i>Histone-lysine N-methyltransferase</i>
MMP	Métalloprotéinase matricielle
MT-MMP	<i>Membrane-type MMP</i>
MSK1	<i>Mitogen- and stress-activated protein kinase 1</i>
MSP	<i>Methylation-specific PCR</i>
MSRA	<i>Methylation-sensitive restriction assay</i>
NAD⁺	Nicotinamide adénine dinucléotide
NF1	<i>Nuclear factor 1</i>
NFκB	<i>Nuclear factor kappa B</i>
NURF	<i>Nucleosome remodeling factor</i>
OMS	Organisation mondiale de la santé
OSE-2	<i>Osteoblast-specific element-2</i>
PAR-1	<i>Proteinase-activated receptor-1</i>
pb	Paire de bases
PCAF	<i>p300/CBP associated-factor</i>
PCR	<i>Polymerase chain reaction</i>
PDGF	<i>Platelet-derived growth factor</i>
PDKA	Protéine kinase A
PEA3	<i>Polyoma enhancer-A binding protein 3</i>
PGP	Proline-glycine-proline

PHD	<i>Plant homeo domain</i>
Plg	Plasminogène
Poly(A)	Polyadénylé
PP1	Protéine phosphatase 1
PRMT	<i>Protein arginine methyltransferase</i>
RANKL	<i>Receptor activator of nuclear factor kappa-β ligand</i>
RARE	<i>Retinoic acid responsive element</i>
RSK2	<i>Ribosomal S6-kinase2</i>
SAHA	<i>Suberoylanilide hydroxamic acid</i>
SAF-1	<i>Serum amyloid A activating factor-1</i>
SAM	S-adénosylméthionine
SBE	<i>STAT-binding element</i>
sc	<i>Saccharomyces cerevisiae</i>
SET	<i>Su(var)39, Enhancer of zest, Trithorax</i>
Si	<i>Silencer sequence</i>
SIRT	Sirtuine
SMD	Syndrome myélodysplasique
SNP	<i>Single nucleotide polymorphism</i>
SOX-like	<i>Sry-related HMG box-like</i>
Sp1	<i>Specificity protein 1</i>
SPRE	<i>Stromelysin-1 PGDF-responsive element</i>
STAT	<i>Signal transducer and activator of transcription</i>
SWI/SNF	<i>Switch/sucrose non fermentable</i>
TCF-4	<i>T-cell factor-4/β-catenin-binding site</i>
TESS	<i>Transcription element search system</i>
TGF-β	<i>Transforming growth factor-β</i>
TIE	<i>TGF-β inhibitory element</i>
TIMP	<i>Tissue inhibitors of MMPs</i>
TNF-α	<i>Tumor necrosis factor-α</i>
TRAIL	<i>TNF-related apoptosis inducing ligand</i>
TSA	Trichostatine A

Abréviations

TSS	<i>Transcription start site</i>
UTR	<i>Untranslated region</i>
UTX	<i>Ubiquitously transcribed tetratricopeptide repeat, X chromosome</i>
UV	Ultra-violets
VEGF	<i>Vascular endothelial growth factor</i>

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Avant-propos

Au cours de ces dernières années, il est apparu évident que les cancers ne peuvent se réduire uniquement à des aberrations génétiques, mais qu'un nouveau paramètre était à prendre en compte, l'épigénétique. Ce terme définit des modifications réversibles et transmissibles de l'expression des gènes, qui ne s'accompagnent pas de changements dans la séquence d'ADN. Un processus épigénétique largement étudié à l'heure actuelle, correspond à des modifications de configuration chromatinienne capables de moduler l'accès de la machinerie transcriptionnelle à l'ADN.

Au cours de cette thèse, nous nous sommes intéressés aux mécanismes épigénétiques impliqués dans la régulation de l'expression de gènes codant des protéines participant à l'invasion tumorale : les métalloprotéinases matricielles ou MMPs. Ces protéases sont de puissants régulateurs des interactions entre les cellules cancéreuses et leur environnement cellulaire, l'ensemble constituant ce qu'on appelle le « microenvironnement tumoral ». Leur action protéolytique participe au remodelage de la matrice extracellulaire au cours de processus tels que la migration cellulaire, l'inflammation, l'invasion tumorale et la formation de métastases. Dans le but de mieux comprendre les mécanismes de régulation de ces gènes (en particulier les gènes de MMP-1, -2 et -9), nous avons analysé les effets d'agents épimutagènes, c'est-à-dire susceptibles de modifier le profil épigénétique cellulaire, sur leur niveau d'expression et nous avons recherché quel pouvait être l'impact d'une telle régulation sur les propriétés invasives de cellules de fibrosarcome humain.

De plus, dans la mesure où les mécanismes épigénétiques sont susceptibles de remanier la structure chromatinienne, leur impact sur la texture nucléaire a été recherché. Cette texture nucléaire représente une des approches possibles de la supra-organisation chromatinienne dont des modifications pourraient refléter des changements du fonctionnement cellulaire, et éventuellement des modulations de l'expression de gènes. Ainsi, une partie du travail a consisté en l'analyse de la texture chromatinienne de cellules de fibrosarcome humain exposées à ces modulateurs épigénétiques. Cette analyse a eu pour objectif de tenter de corréler les modifications épigénétiques et les changements de l'architecture nucléaire, au profil d'expression de quelques gènes.

Avant de présenter les publications issues de ce travail de thèse, nous présenterons une synthèse sur les connaissances actuelles concernant d'une part, la régulation de l'expression des gènes des MMPs et leur rôle dans le cancer et d'autre part, les principaux mécanismes épigénétiques impliqués dans le contrôle de l'expression génique, en nous focalisant plus particulièrement sur ceux impliqués dans la régulation de l'expression des gènes des MMPs au cours du cancer.

***Aspects
bibliographiques***

I. Matrice extracellulaire et métalloprotéinases matricielles

A. Composition et rôles de la MEC

La Matrice Extracellulaire (MEC) est un réseau tridimensionnel de macromolécules (glycosaminoglycannes, glycoprotéines, élastines, collagènes) servant de support architectural pour les cellules et permettant la cohésion des tissus. Longtemps qualifiée de structure amorphe, elle est maintenant considérée comme une structure nourricière dynamique permettant la transduction des signaux vers des récepteurs présents à la surface des cellules. De ce fait, la MEC constitue un environnement instructif pour les cellules et régule diverses fonctions essentielles telles que l'adhésion, la migration, la prolifération, la différenciation et la survie cellulaires (Reilly, 2010; Schenk, 2003).

La MEC est en perpétuel renouvellement ce qui implique la mise en jeu de nombreux systèmes protéolytiques. Parmi ces systèmes, les métalloprotéinases matricielles (MMPs) jouent un rôle fondamental dans le remodelage de la MEC.

B. Caractéristiques générales des MMPs

Au cours des processus physiologiques et pathologiques, les interactions cellules-MEC sont modulées grâce à l'action enzymatique des MMPs. Physiologiquement, les MMPs sont impliquées dans le remodelage de la MEC lors de la reproduction, l'embryogénèse, la morphogénèse tissulaire ou encore la cicatrisation (Klein, 2011; Sternlicht, 2001). Les MMPs permettent également de moduler l'activité de facteurs de croissance et de cytokines liés à la MEC en induisant une rupture de leurs liaisons à la MEC. Une dérégulation de l'activité des MMPs et/ou de leurs inhibiteurs endogènes contribue à des processus pathologiques tels que l'inflammation, la progression et l'invasion tumorales (Klein, 2011; Sternlicht, 2001).

1. Classification structurale des MMPs

Chez les vertébrés, on dénombre 25 MMPs (dont 23 ont été retrouvés chez l'homme) regroupées en différentes classes en fonction de leur spécificité de substrat et en fonction de leur structure (Raffetto, 2008; Yan, 2007).

Les MMPs sont des endopeptidases à doigt de zinc qui partagent des domaines communs tels que (FIGURE 1):

- le **peptide signal** : responsable de l'adressage des MMPs à la membrane plasmique en vue de leur sécrétion.
- le **pro-domaine** : qui permet aux MMPs de rester sous forme latente. Le pro-domaine peut contenir une séquence consensus reconnue par des convertases. En fonction de cette séquence, le clivage protéolytique du pro-domaine est assuré, soit par des furines convertases dans le milieu intracellulaire, soit par des MMPs ou autres sérines protéases telles que la plasmine dans le milieu extracellulaire.
- le **domaine catalytique** : Zn^{2+} -dépendant, responsable de l'activité enzymatique des MMPs.
- le **domaine hemopexine** : relié au domaine catalytique par une courte région charnière.

En fonction de leur structure, on distingue (FIGURE 1):

- les **MMPs solubles** : sécrétées dans le milieu extracellulaire.
- les **MMPs membranaires** : elles sont soit ancrées à la membrane plasmique (MT-MMPs ou *membrane-type MMPs*) via un domaine transmembranaire ou un groupement glycoposphatidylinositol (ancre GPI) ou présentent un signal d'adressage à la membrane plasmique.

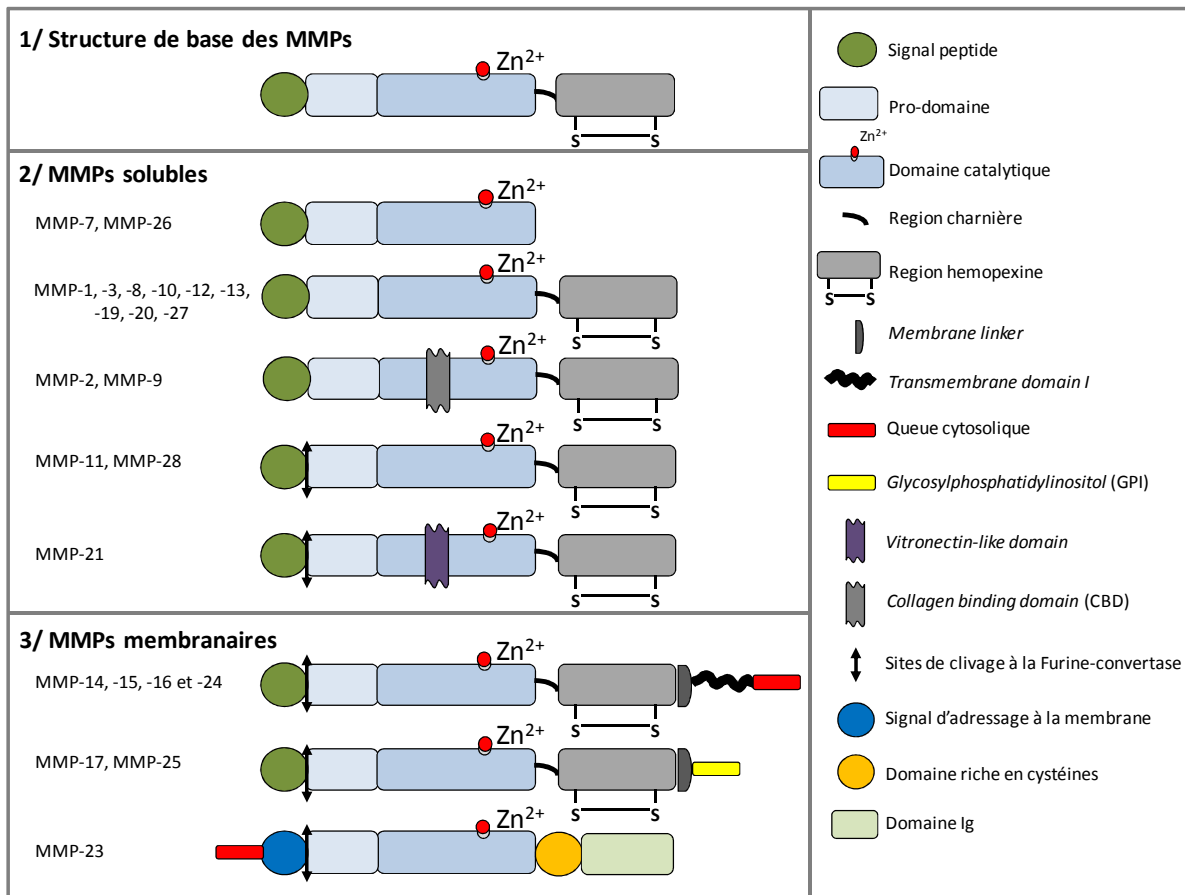


Figure 1 : Structure schématique des MMPs chez l'homme

(Ala-aho, 2005; Nagase, 2006; Raffetto, 2008)

2. Activation et activités enzymatiques des MMPs

Les MMPs sont synthétisées sous un état enzymatique inactif (pro-forme ou zymogène) dû à l'interaction d'un résidu cystéine du pro-domaine avec le site Zn^{2+} du domaine catalytique. Après protéolyse de cette interaction par un mécanisme communément appelé « cystéine switch », les MMPs deviennent enzymatiquement actives (FIGURE 2).

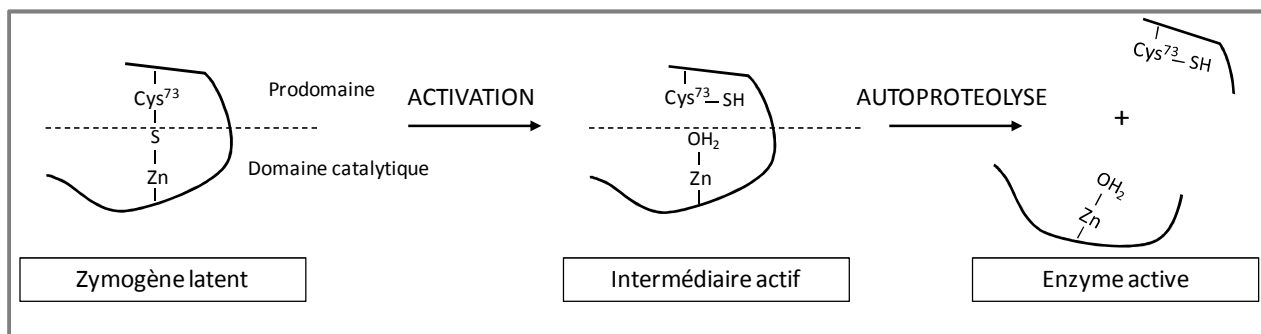


Figure 2 : Mécanisme d'activation des MMPs

(Chakraborti, 2003)

Les MMPs sont donc capables de dégrader de nombreux substrats matriciels et non matriciels (TABLEAU I). En fonction de cette spécificité de substrats et leur spécificité structurale, les MMPs sont classées en différentes catégories parmi lesquelles on retrouve les **collagénases**, les **gélatinases**, les **stromélysines**, les **MT-MMPs** et les **autres MMPs** (TABLEAU I).

Aspects bibliographiques

Enzymes	MMPs	Poids moléculaire (kDa)		Substrats collagéniques	Substrats non collagéniques
		Proforme	Active		
Collagénases					
Collagénase-1	MMP-1	55	45	I, II, III, VII, VIII, X	Aggrecan, gélatine, MMP-2, -9
Collagénase-2	MMP-8	75	58	I, II, III, V, VII, VIII, X	Aggrecan, élastine, fibronectine, gélatine, laminine
Collagénase-3	MMP-13	60	48	I, II, III, IV	Aggrecan, gélatine
Collagénase-4 (<i>Xenope</i>)	MMP-18	70	53	I	Gélatine
Gélatinases					
Gélatinase A	MMP-2	72	66	I, II, III, IV, V, VII, X, XI	Aggrecan, élastine, fibronectine, gélatine, laminine, proteoglycannes, MMP-9, -13
Gélatinase B	MMP-9	92	86	IV, V, VII, X, XIV	Aggrecan, élastine, fibronectine, gélatine
Stromélysines					
Stromélysine-1	MMP-3	57	45	II, III, IV, IX, X, XI	Aggrecan, élastine, fibronectine, gélatine, laminine, proteoglycannes, MMP-1, -7, -8, -13
Stromélysine-2	MMP-10	57	44	III, IV, V	Aggrecan, élastine, fibronectine, gélatine, laminine, MMP-1, -8
Stromélysine-3	MMP-11	51	44	∅	Aggrecan, fibronectine, laminine
Stromélysine-4	MMP-19	54	45	IV	Aggrecan, COMP, fibronectine, gélatine, laminine
Membrane-type MMPs					
<i>(1) Domaine transmembranaire</i>					
MT1-MMP	MMP-14	66	56	I, II, III	Aggrecan, élastine, fibronectine, gélatine, MMP-2, -13
MT2-MMP	MMP-15	72	50	I	Fibronectine, gélatine, laminine, MMP-2
MT3-MMP	MMP-16	64	52	I	MMP-2
MT5-MMP	MMP-24	57	53	Non identifié	Fibrine, gélatine
<i>(2) GPI anchor</i>					
MT4-MMP	MMP-17	57	53	Non identifié	Fibrine, gélatine
MT6-MMP	MMP-25	34	28	IV	Gélatine, fibrine, fibronectine, laminine
Autres MMPs					
Matrilysine-1	MMP-7	28	19	IV, X	Aggrecan, élastine, fibronectine, gélatine, laminine, proteoglycannes, MMP-1, -2, -9
Elastase macrophage	MMP-12	54	45/22	IV	Elastine, Fibronectine, gélatine, laminine
Enamélysine	MMP-20	54	22	V	
XMMP	MMP-21	62	49		α1-anti-trypsin
CMMP (<i>Poulet</i>)	MMP-22	51	/	Non identifié	Gélatine
Cysteine array MMP	MMP-23	28	19	Non identifié	Non identifié
Matrilysine-2	MMP-26	28	19	IV	Gélatine, fibronectine
/	MMP-27	/?	/?	Non identifié	Non identifié
Epilysine	MMP-28	56	45	Non identifié	Non identifié

Tableau 1 : Classification fonctionnelle des MMPs

(Nagase, 2006; Raffetto, 2008)

3. Inhibiteurs endogènes des MMPs

L'activité des MMPs est régulée en partie par deux types majeurs d'inhibiteurs endogènes : l' α_2 -macroglobuline et les TIMPs (*tissue inhibitors of MMPs*). L' α_2 -macroglobuline est une glycoprotéine plasmatique qui inhibe la plupart des protéinases en les piégeant au sein d'un complexe protéique qui est rapidement endocyté par des récepteurs tels que le récepteur LRP1 (*low density lipoprotein receptor-related-protein 1*) (Strickland, 1990). Les TIMPs, au nombre de quatre (TIMP-1, -2, -3, -4), inhibent l'activité des MMPs *via* leur domaine N-terminal qui interagit directement, de la même façon qu'un substrat, avec le site catalytique des MMPs.

II. Régulation de l'expression des gènes des MMPs

Dans ce sous-chapitre, nous nous intéresserons à la régulation transcriptionnelle des MMPs en focalisant plus particulièrement notre propos sur la régulation transcriptionnelle du gène de la MMP-1.

A. Régulation transcriptionnelle

1. Généralités

Les gènes des MMPs sont régulés au niveau transcriptionnel et présentent des niveaux d'expression cellulaire relativement faibles dans des conditions physiologiques normales. Leur expression est transitoirement augmentée lors du remodelage physiologique de la MEC au cours de processus tels que la cicatrisation, l'ovulation ou encore la résorption utérine après une grossesse (Brinckerhoff, 2002). La plupart des MMPs partagent des éléments *cis*-régulateurs communs au niveau du promoteur de leur gène, ce qui permet une régulation précise de leur expression. De plus, les gènes des MMPs sont considérés comme des gènes inductibles. Ils sont souvent co-exprimés ou co-réprimés en réponse à des *stimuli* tels que des cytokines inflammatoires, des facteurs de croissance, des glucocorticoïdes ou des rétinoïdes (Fanjul-Fernandez, 2010). Leur activation transcriptionnelle se déroule plusieurs heures après une exposition à un *stimulus* ce qui signifie que les gènes des MMPs sont des cibles situées en aval de voies signalisation de gènes à réponse rapide, qui eux sont

induits peu de temps après une stimulation cellulaire et en l'absence de synthèse protéique *de novo*. Ces gènes à réponse rapide codent des protéines de signalisation telles que les MAPKs (*Mitogen Activated Protein Kinases*) qui activent des facteurs de transcription et induisent leur fixation sur les promoteurs d'autres gènes. Parmi ces facteurs de transcription régulant l'expression des MMPs, on retrouve, par exemple : AP-1 et -2 (*activator protein -1, -2*), PEA3 (*polyoma enhancer-A binding protein 3*), NF- κ B (*nuclear factor kappa B*) et STAT (*signal transducer and activator of transcription*).

Les MMPs ont donc été classées en trois groupes qui se différencient par la présence ou non de sites régulateurs particuliers au sein des régions promotrices de leur gène (Yan, 2007). Les MMPs du **groupe I** contiennent une boîte TATA située aux environs de -30 bp avant le site d'initiation de la transcription (*transcription start site ou TSS*) et un site AP-1 dans la région proximale de leur promoteur (vers -70 bp). Ce groupe I comprend les MMPs -1, -3, -7, -9, -19, -12, -13, -19 et -26. La plupart de ces promoteurs contiennent également en amont, un site de fixation à PEA3 qui est souvent adjacent et coopératif avec un site de fixation AP-1. Les MMPs du **groupe II** (MMP-8, -11 et -21) possèdent une boîte TATA mais sont dépourvues de site AP-1 au niveau de leur promoteur tandis que les MMPs du **groupe III** (MMP-2, -14 et -28) ne possèdent ni boîte TATA, ni site AP-1. Les MMPs de ce dernier groupe sont souvent exprimées de façon constitutive, bien que leur expression tende à être augmentée au cours de nombreux processus pathologiques (Fingleton, 2006; Mancini, 2006) (FIGURE 3).

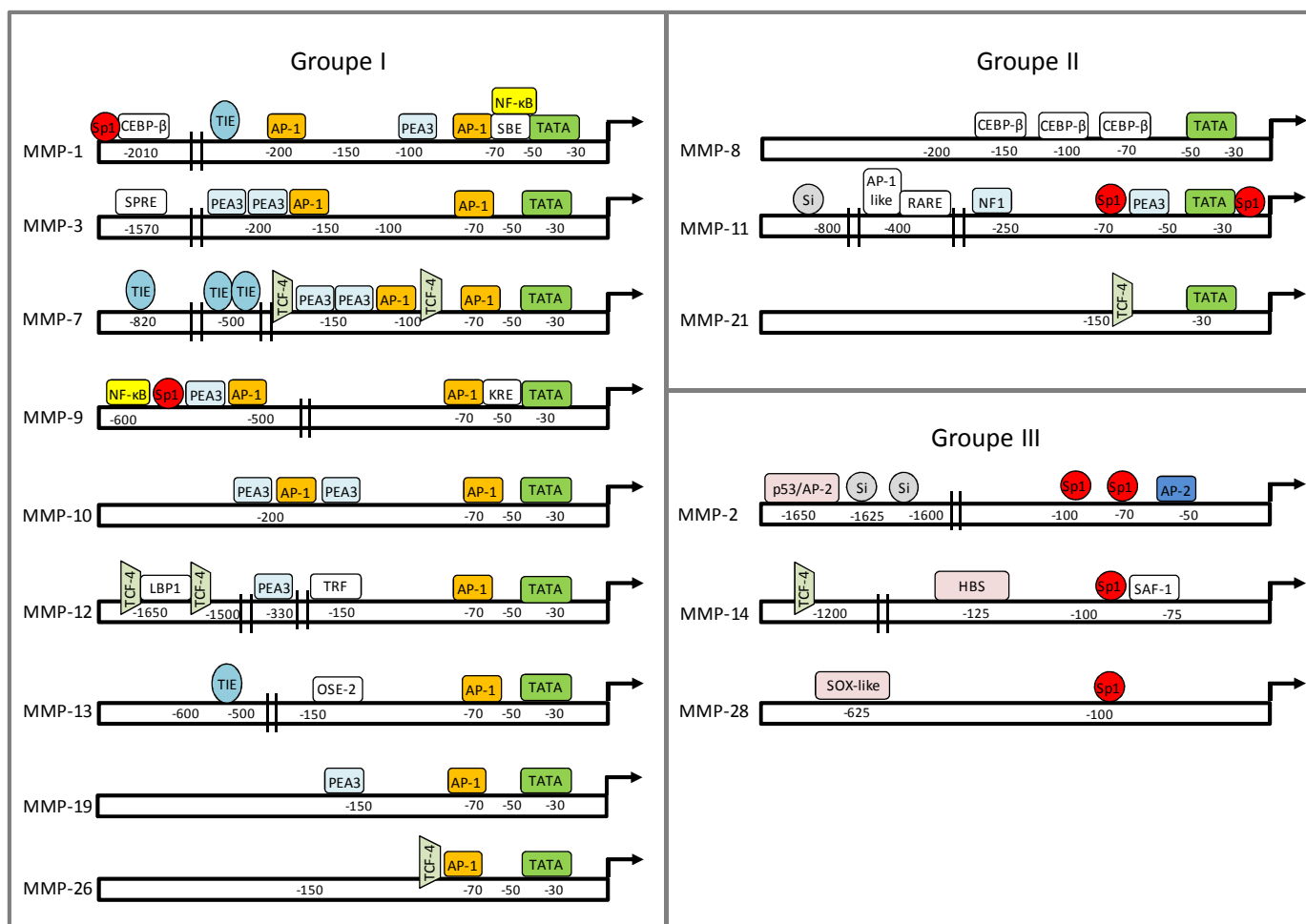


Figure 3 : Éléments cis-régulateurs au niveau du promoteur des gènes des MMPs

(Yan, 2007)

2. Élément de réponse AP-1 au niveau du promoteur du gène de la MMP-1

Le site AP-1 est l'un des éléments régulateurs majeurs de l'expression génique des MMPs. La plupart des promoteurs des gènes des MMPs, à l'exception des MMPs du groupe III, possèdent un ou plusieurs sites de fixation pour le facteur de transcription AP-1. Les complexes AP-1 sont des dimères de protéines appartenant à la famille de deux proto-oncogènes : *jun* et *fos*. La composition du complexe AP-1 ainsi que son positionnement sur le promoteur (juxtaposition par rapport à des sites liant d'autres facteurs de transcription), détermine la spécificité des gènes. Plusieurs études de co-transfection ont montré que la surexpression des protéines Jun et Fos augmentait l'activité du promoteur du gène de la MMP-1. De plus, il a été montré qu'une augmentation simultanée des niveaux d'ARNm de *c-jun* et *jun-B* précédait l'induction de l'expression du gène de la MMP-1 (Benbow, 1997;

Vincenti, 1996). c-Jun est décrit comme un activateur indépendant de l'expression du gène de la MMP-1 puisqu'il est capable d'induire une activité minimale de son promoteur en formant des homodimères Jun/Jun. A l'inverse, la *trans*-activation du promoteur du gène de la MMP-1 par Jun-B et par d'autres membres de la famille AP-1 est dépendante de leur interaction avec d'autres facteurs de transcription se liant à des éléments cis-régulateurs en 5' du gène (Chakraborti, 2003).

3. Élément de réponse PEA3 au niveau du promoteur du gène de la MMP-1

Le site PEA3, souvent adjacent au site AP-1, permet la fixation des facteurs de transcription de la famille Ets (*erythromatosis twenty-six*). Il a été montré que les membres de la famille Ets étaient capables de *trans*-activer des construits de promoteurs artificiels contenant uniquement l'élément de réponse PEA3. Cependant, ces facteurs Ets ne forment pas de dimères et ne sont pas capables de se lier seuls à l'ADN, mais forment préférentiellement des complexes avec d'autres facteurs de transcription tels que AP-1 qui agit donc comme un co-modulateur de la transcription (Buttice, 1996; Carrere, 1998; Kapila, 2009; Sharrocks, 1997). De plus, les sites PEA3 sont capables de fixer de nombreux membres Ets qui, en fonction de leur nature, sont responsables d'une régulation fine et spécifique. Par exemple, Ets1 augmente l'expression du gène de la MMP-1 par l'intermédiaire de son interaction avec c-Jun, tandis qu'ErgB induit son activation via Jun-B. A l'inverse la protéine Ets Pu1 réprime l'induction de MMP-1 en interagissant avec c-Jun et Jun-B (Chakraborti, 2003).

4. Élément de réponse NF- κ B au niveau du promoteur du gène de la MMP-1

La voie de signalisation NF- κ B est impliquée dans la régulation de diverses MMPs au cours de processus pathologiques tels que l'arthrite ou le cancer et après exposition à des facteurs de croissance et des cytokines. Les membres de la famille NF- κ B, incluant NF- κ B1, NF- κ B2, Rel A, Rel B et c-Rel, peuvent également interagir avec d'autres protéines en vue d'augmenter l'expression des MMPs. Par exemple, l'interleukine 1- β (IL-1 β) induit la formation d'homodimères NF- κ B et leur fixation sur BCL3 (*B-cell chronic lymphocytic leukemia /lymphoma 3*) permettant ainsi la transcription du gène de la MMP-1 (Elliott, 2002).

5. Élément de réponse STAT au niveau du promoteur du gène de la MMP-1

Les protéines de la famille STAT sont des facteurs de transcription activés après phosphorylation de résidus tyrosines par des Janus kinases (JAKs) et dimérisation. Les protéines STAT collaborent fréquemment avec différents facteurs de transcription en vue d'induire une expression spécifique de gènes. Une étude récente révèle qu'une activation aberrante de STAT3 et AP-1, ainsi que la formation de complexes entre ces deux facteurs de transcription dans des tissus de carcinomes colorectaux, entraîne une expression importante du gène de la MMP-1 (Zugowski, 2011). Il apparaît par ailleurs, que la stimulation de la voie STAT est un mécanisme additionnel permettant l'amplification de la transcription de MMP-1 au cours de conditions pathologiques telles que l'inflammation (Vincenti, 2007).

6. Élément de réponse Sp1 au niveau du promoteur du gène de la MMP-1

A l'heure actuelle peu d'études ont décrit la présence de sites consensus Sp1 au niveau du promoteur du gène de la MMP-1. Sp1 est capable de se fixer sur des boîtes GC dont la séquence minimale est 5'-GGGcGG-3' (Li, 2004). L'analyse du promoteur du gène de la MMP-1 révèle la présence d'un site similaire aux alentours de -2200 pb avant le TSS dont la séquence est 5'-GGcGGG-3' (Rutter, 1998). Par ailleurs, une analyse prédictive utilisant le logiciel TESS (*transcription element search system*) a permis de révéler la présence de sites de liaisons additionnels pour Sp1 au niveau du promoteur du gène de la MMP-1 (analyse réalisée sur une région promotrice comprise entre -4438 et 2844 pb avant le TSS) (Mercer, 2009). L'approfondissement de cette étude a montré que Sp1 joue un rôle essentiel dans l'induction de l'expression du gène de la MMP-1 lors d'une exposition à la fumée de cigarette en se fixant en -3987 pb et -3455 pb sur le promoteur de ce gène (Mercer, 2009; Wallace, 2012). En revanche, le site Sp1 en -2209 pb, qui est situé en dehors des éléments de réponse à la fumée de cigarette, présente des effets opposés puisque une exposition à la fumée entraîne une diminution du recrutement de Sp1 en cet endroit (Wallace, 2012).

7. Polymorphisme nucléotidique au niveau du promoteur du gène de la MMP-1

Des polymorphismes nucléotidiques, en l'occurrence des SNPs (*single nucleotide polymorphisms*) au niveau des promoteurs des gènes des MMPs, sont susceptibles de moduler l'expression de ces gènes (augmentation ou diminution de l'activité

transcriptionnelle) en altérant la fixation des facteurs de transcription sur leur site de liaison. Plusieurs polymorphismes fonctionnels ont été identifiés au niveau des promoteurs des gènes des MMPs. Un certain nombre d'entre eux sont associés au développement de différentes pathologies et peuvent permettre l'établissement de pronostic (Fanjul-Fernandez, 2010). Il existerait ainsi une corrélation entre la présence de SNPs au niveau des promoteurs des gènes des MMPs et la susceptibilité à développer un cancer (Fanjul-Fernandez, 2010). Au niveau du promoteur du gène de la MMP-1, le SNP décrit correspond à une insertion d'un résidu guanine en -1607 pb avant le TSS ce qui permet la création d'un site Ets additionnel adjacent à un site AP-1 localisé en -1602 pb (Rutter, 1998). La séquence de ce SNP peut donc être 5'-GGAA-3' (allèle 2G) ou 5'-GAA-3' (allèle 1G). L'allèle 2G augmente l'activité transcriptionnelle du promoteur dans de nombreuses tumeurs et est associé à une forte susceptibilité aux cancers pulmonaire et colorectal (Woo, 2007; Zhu, 2001). De plus, des études ont révélé une relation entre les génotypes 2G/2G et un mauvais pronostic chez des patients atteints d'un mélanome (Ye, 2001), d'un cancer du sein (Przybylowska, 2006) ou d'un carcinome ovarien (Six, 2006). D'autre part, le polymorphisme 1G/2G en -1607 pb est impliqué dans des pathologies non tumorales telles que la fibrose pulmonaire (Checa, 2008) et les cirrhoses (Okamoto, 2005).

B. Régulation post-transcriptionnelle

Le contrôle de l'expression des gènes peut faire intervenir des mécanismes post-transcriptionnels permettant de réguler la stabilité des ARNm (demi-vie des ARNm) dans le cytoplasme. Cette régulation nécessite la présence de motifs structuraux particuliers au niveau de l'ARNm tels que la coiffe en 5', la région non transcrite en 5' (région 5' UTR ou 5' *untranslated region*), la région codant la protéine, la région 3' UTR et la queue polyadénylée (queue poly(A)) en 3' (Guhaniyogi, 2001). Des facteurs *trans*-régulateurs tels que des protéines capables de se lier aux ARNm et des microARNs qui interagissent avec des éléments *cis*-régulateurs présents en différents endroits sur le brin d'ARNm, jouent également un rôle extrêmement important. Ainsi les facteurs *trans*-régulateurs peuvent stabiliser (*e-g* : HuR ou *human antigen R*) ou déstabiliser (*e-g* : AUF1 ou *AU-rich element RNA-binding protein 1*) les ARNm (Garneau, 2007).

La stabilisation des transcrits d'ARNm des MMPs représente un mécanisme additionnel de régulation de leur expression. L'ARNm de MMP-1 présente une faible stabilité dans des cellules non stimulées et, par conséquent, son niveau d'expression est peu ou pas détectable en dépit d'une activité basale transcriptionnelle élevée. Les régions 3' UTR des ARNm de la MMP-1 contiennent trois répétitions du motif AUUUA. Une mutation de ce motif permet la stabilisation des transcrits de MMP-1, ce qui suggère que ce motif est responsable du turnover rapide des ARNm de MMP-1 dans des cellules non stimulées. Néanmoins, en présence de cytokines ou facteurs de croissance, les effets déstabilisateurs de la région 3' UTR sont freinés et la stabilité de l'ARNm de MMP-1 est augmentée (Vincenti, 1996).

III. Mécanismes épigénétiques

A. Définition et généralités

Expression créée au 19^{ème} siècle et redéfinie par Conrad Waddington en 1942 dans son sens actuel, l'épigénétique se définit comme « l'étude des modifications de l'expression des gènes qui sont transmissibles au cours des divisions cellulaires et qui permettent de réguler des états fonctionnels au sein d'une cellule sans altérer la séquence d'ADN ». De plus, contrairement aux modifications génétiques, les modifications épigénétiques sont considérées comme dynamiques et réversibles.

Cette appréciation de l'importance de paramètres non codés génétiquement a pris un essor particulier avec les récents progrès réalisés dans la connaissance de l'organisation du génome humain. En effet, le génome au sein du noyau s'organise en une structure nucléoprotéique appelée chromatine qui, outre sa composante génétique, est riche en informations épigénétiques.

Le premier niveau d'organisation de la chromatine a été proposé par Kornberg *et al* en 1974. Il prédit que les molécules de chromatine, visibles en microscopie électronique, sont composées de disques octamériques (nucléosomes) empilés. Chaque nucléosome est constitué d'une particule cœur et d'une région internucléosomique. La particule cœur consiste en 146 paires de bases (146 pb) enroulées autour d'un octamère de petites

protéines basiques constitué chacun de 2 copies d'histones H2A, H2B, H3 et H4 (Luger, 2006). La région internucléosomique permet de relier les particules cœur adjacentes. Elle est formée d'une longueur variable d'ADN. Une histone de liaison (histone H1) permet la stabilisation des nucléosomes conduisant ainsi à la formation de fibres de chromatine et à la mise en place d'états chromatiniens de plus en plus condensés (FIGURE 4).

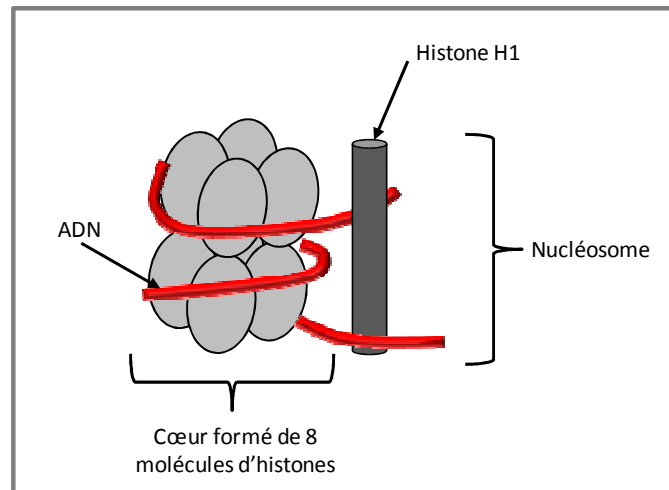


Figure 4 : Structure schématique du nucléosome

(Monneret, 2005)

En absence de division cellulaire, la chromatine s'organise en deux états fonctionnels : l'**euchromatine** et l'**hétérochromatine**, qui sont respectivement transcriptionnellement active ou inactive. L'euchromatine est la région d'ADN la plus accessible ; elle possède une conformation ouverte, conséquence d'une relaxation de l'état d'arrangement du nucléosome. Les régions génomiques de l'euchromatine sont plus flexibles, et contiennent des gènes dans des états actifs ou inactifs. Inversement, l'hétérochromatine correspond à des zones où la chromatine est fortement condensée ; l'ADN est donc très peu accessible à la machinerie transcriptionnelle. Les régions génomiques de l'hétérochromatine sont généralement des régions répétitives et contiennent des gènes éteints souvent associés à des processus de morphogénèse et de différenciation (*e-g* : inactivation du chromosome X). L'hétérochromatine est constituée de séquences génomiques possédant des fonctions essentielles dans le contrôle de la stabilité chromosomique et dans la prévention de mutations et de translocations (Muegge, 2005).

Le remodelage de la chromatine (passage d'un état condensé à un état décondensé, et inversement) fait intervenir différents acteurs. Ces acteurs peuvent affecter la structure de la chromatine en induisant des changements conformationnels au niveau des nucléosomes mais également au niveau des régions internucléosomiques. Ces acteurs sont de deux types : les premiers nécessitent de l'énergie sous forme d'ATP et sont généralement appelés **facteurs de remodelage de la chromatine**, et les seconds sont des enzymes assurant les **modifications post-traductionnelles des histones**. Les facteurs de remodelage sont des complexes multiprotéiques (familles SWI/SNF ou *switch/sucrose non fermentable*, Mi2/NuRD ou *Mi-2/nucleosome remodeling and deacetylase*) pourvus d'une activité ATPasique permettant de modifier la conformation chromatinienne grâce, en partie, à l'énergie libérée par l'hydrolyse d'ATP. L'étude des facteurs qui favorisent un espacement régulier des nucléosomes pendant l'assemblage de la chromatine a permis d'identifier plusieurs complexes capables *in vitro* de déplacer les nucléosomes le long de l'ADN (Alkhatib, 2011).

B. Modifications post-traductionnelles des histones

Les extrémités N-terminales des histones, et dans une moindre mesure leurs extrémités carboxyterminales, sont les cibles de modifications post-traductionnelles, parmi lesquelles l'acétylation, la méthylation, la phosphorylation sont les plus étudiées (Bannister, 2011). Cependant d'autres modifications des histones ont été décrites telles que ubiquitinylation, la SUMOylation, l'ADP-rybosylation, la biotinylation ou encore la glycosylation. Ces modifications affectent les interactions inter-nucléosomiques et modulent ainsi la structure de la chromatine. De ce fait, ces modifications influencent l'activité transcriptionnelle des gènes, mais aussi d'autres fonctions cellulaires liées à l'ADN telles que la réparation des dommages à l'ADN, la réplication ou encore la recombinaison des chromosomes. Nous nous intéresserons plus particulièrement aux processus d'acétylation, de phosphorylation et de méthylation des histones.

1. Acétylation des histones

À l'échelle cellulaire, le niveau d'acétylation des histones délimite topographiquement l'euchromatine et l'hétérochromatine au moyen d'un gradient d'acétylation et de désacétylation (Spotswood, 2002).

Le transfert de groupement acétyle au niveau de résidus lysine est assuré par l'action opposée de deux familles d'enzymes : les histones acétyltransférases (HATs) et les histones désacétylases (HDACs) (FIGURE 5).

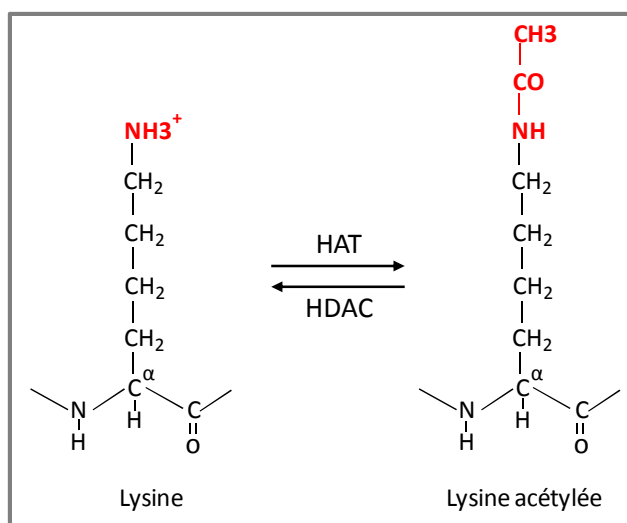


Figure 5 : Mécanismes d'acétylation et de désacétylation des histones

Les HATs catalysent le transfert de groupes acétyles sur un groupement amine ε d'une lysine (K) en utilisant l'acétyl-CoA comme cofacteur. Cette action enzymatique neutralise les charges positives des lysines et diminue l'affinité des histones pour l'ADN entraînant une décondensation de la chromatine et modulant ainsi l'activité transcriptionnelle des promoteurs (Di Mauro, 2002; Verdone, 2005). Les HATs sont regroupées en deux classes : les HATs de type A et les HATs de type B. Les HATs de type A sont subdivisées en trois groupes en fonction de leur homologie de séquence et de leur structure conformationnelle : les GNATs (*GCN5-related N-acetyltransferase*), MYST et CBP/p300 (*CREB-binding protein/p300*) (Hodawadekar, 2007). Les HATs de type B sont majoritairement cytoplasmiques et acétylent des histones nouvellement synthétisées favorisant ainsi leur incorporation au sein de la chromatine (Parthun, 2007). Les HDACs catalysent le retrait des groupes acétyles et restaurent la charge positive des résidus lysine.

Cette action enzymatique stabilise l'architecture chromatinienne et réprime généralement la transcription des gènes. Chez l'homme, les HDACs sont regroupées en quatre classes (Lin, 2006) :

- les **HDACs de Classes I** (HDAC1, 2, 3 et 8) : homologues de scRpd3 (sc pour *Saccharomyces cerevisiae*) présent chez la levure et exprimées de façon ubiquitaire, elles sont exclusivement nucléaires et agissent essentiellement comme corépresseurs transcriptionnels.
- Les **HDACs de Classe II** (HDAC4, 5, 6, 7, 9 et 10), homologues de scHda1 présent chez la levure, se déplacent entre le cytoplasme et le noyau laissant présager des fonctions extranucléaires en régulant l'acétylation de substrats non-histoniques. Par exemple, l'un des deux domaines catalytiques d'HDAC6 s'est révélé capable de désacétyler des substrats non-histoniques tels que l' α -tubuline ou la protéique de choc thermique Hsp90 (*heat shock protein 90*) (Glozak, 2005).
- L'**HDAC de Classe IV** représentée par l'HDAC11 est homologue de scRpd3. L'activité enzymatique de ces 3 classes d'HDACs est régie par la présence d'un site actif Zn^{2+} .
- Les **HDACs de Classe III** appelées **sirtuines** (SIRT 1 à 7), homologues de scSir2 présent chez la levure, utilisent NAD^+ comme cofacteur pour exercer leur activité catalytique contrairement aux HDACs de classe I, II, IV, qui utilisent un mécanisme catalytique Zn^{2+} -dépendant. Chez les mammifères, le rôle de ces enzymes semble essentiellement être lié à la désacétylation de protéines non-histoniques telles que le facteur de transcription p53 (Glozak, 2005).

De manière générale, les HDACs ont une faible spécificité de substrats et une seule enzyme est capable de désacétyler plusieurs sites au sein des histones. De plus, l'étude de la spécificité et du recrutement des HDACs s'avère délicate puisque ces enzymes sont généralement regroupées en différents complexes (Yang, 2008).

Les HDACs sont des régulateurs clés de plusieurs évènements cellulaires fondamentaux, tels que le contrôle du cycle cellulaire, la différenciation et l'apoptose. Une dérégulation de leur activité peut conduire à des états pathologiques. De ce fait, les HDACs constituent une cible thérapeutique d'intérêt et leur activité peut être réprimée par divers inhibiteurs. De nombreux inhibiteurs d'HDACs (HDIs ou *HDAC inhibitors*) ont été développés

au cours de ces dernières années, et un certain nombre d'entre eux sont actuellement en essai clinique pour le traitement de tumeurs solides et de leucémies (Florea, 2011). Les HDI, d'origine naturelle ou synthétique, sont divisés en quatre classes chimiques comme indiqué dans le tableau suivant (TABLEAU 2).

Classes chimiques	Nom	Cibles
Acides gras à chaîne courte	Butyrate de sodium	Pan-inhibiteur
	Phénylbutyrate de sodium*	Pan-inhibiteur
	Valproate de sodium*	HDAC Classe I/IIa
Acides hydroxamiques	TSA	Pan-inhibiteur
	Vorinostat (SAHA)*	Pan-inhibiteur
	Panobinostat (LBH589)*	Pan-inhibiteur
	Belinostat (PXD101)*	Pan-inhibiteur
Tétrapeptides cycliques	Depsipeptide (FK228)*	HDAC Classe I
Benzamides	Entinostat (MS-275)*	HDAC Classe I
	Mocetinostat (MGCD-0103)*	HDAC Classe I/IV

Table non exhaustive

TSA : Trichostatine A

SAHA : *suberoylanilide hydroxamic acid*

* Essai clinique dans le traitement des leucémies

Tableau 2 : Classification des inhibiteurs d'HDACs

(Florea, 2011)

2. Phosphorylation des histones

La phosphorylation des histones se déroule majoritairement au niveau de l'extrémité N-terminale de l'histone H3 sur les résidus serine (S), thréonine (T) et tyrosine (Y).

Le transfert de groupements phosphorylés, est assuré par des kinases (par exemple : MAPK1, Aurora B) et des phosphatases (par exemple : PP1 ou protéine phosphatase 1) (Oki, 2007). Les kinases phosphorylent les histones en transférant le groupe phosphorylé de l'ATP sur un groupe hydroxyle présent sur la chaîne d'acides aminés. Cette modification enrichit l'histone en charges négatives et modifie ainsi la structure de la chromatine. Des études antérieures ont montré que la phosphorylation de la sérine 10 et de la sérine 28 de l'histone H3 (H3S10 et H3S28) médiée par Aurora B joue un rôle important au cours de la mitose en favorisant la condensation et la ségrégation des chromosomes (Sawicka, 2012). La phosphorylation de H3S10 débute en fin de phase G2 au niveau de l'hétérochromatine péricentrique, puis s'étend le long du chromosome durant la transition G2-M et disparaît rapidement lors de l'anaphase (Henzel, 1997). Par ailleurs, la phosphorylation de H3S10 induite par la voie des MAPKs en réponse à des *stimuli* joue un rôle important dans l'activation transcriptionnelle. Différentes enzymes semblent être engagées dans cette voie d'activation telles PDKA (protéine kinase A), RSK2 (*ribosomal S6-kinase2*) ou MSK1 (*mitogen- and stress-activated protein kinase 1*) activées par p38 et/ou ERK (*extracellular signal-regulated kinase*) (Thomson, 1999).

A l'heure actuelle, peu d'informations sont connues sur le rôle des histones phosphatases. Il a été suggéré que l'activité des histones phosphatases devait être très élevée au sein du noyau pour permettre le « turnover » rapide de la phosphorylation des histones (Bannister, 2011).

3. Méthylation des histones

La méthylation des histones a lieu généralement au niveau des résidus lysine (K) et arginine (R). Contrairement aux processus d'acétylation et de phosphorylation, la méthylation n'altère pas la charge des histones. De plus, la méthylation des histones comporte un niveau additionnel de complexité puisque les résidus lysines peuvent être mono- (me1), di- (me2) ou tri-méthylés (me3) et les résidus arginine symétriquement ou asymétriquement di-méthylés (me2s et me2a) (Ng, 2009). Le transfert de groupements méthyles est assuré par des histones méthyltransférases (HMTs) et des histones déméthylases (HDMs) (FIGURE 6).

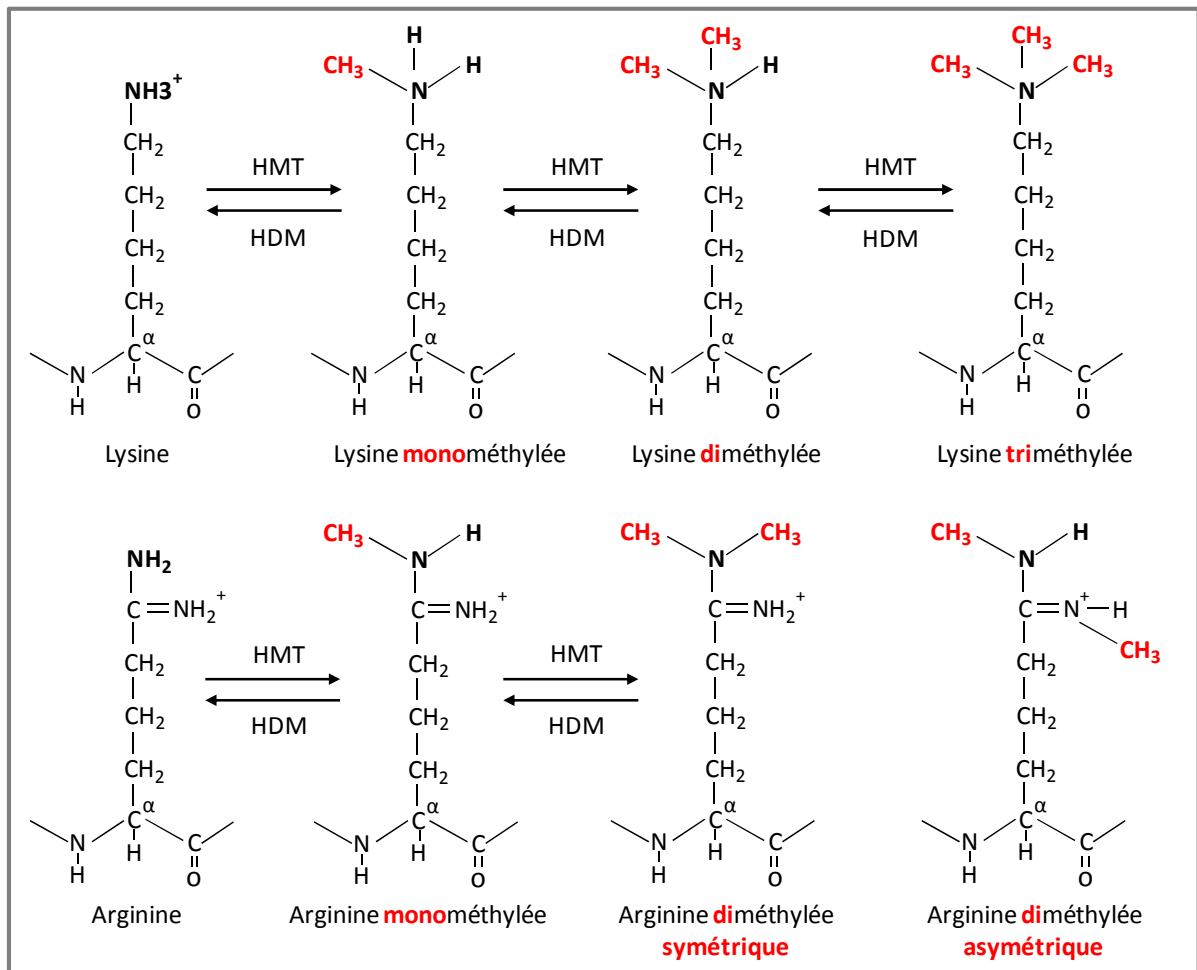


Figure 6 : Mécanismes de méthylation et de déméthylation des histones

(Shilatifard, 2006)

La famille des lysines HMTs comporte plus d'une dizaine de protéines dont la plupart d'entre elles possèdent un domaine conservé SET (*Su(var)39*, *Enhancer of zest*, *Trithorax*) dans leur structure, responsables de l'activité histone méthylase. La méthylation des résidus arginine est assurée par deux classes d'enzymes : PRMT (*protein arginine methyltransferase*) et CARM1 (*co-activator-associated arginine methyltransferase 1*). Les déméthylases ont été découvertes plus récemment et sont divisées en deux classes : la famille des monoamines oxydases dépendantes de la flavine (incluant les déméthylases spécifiques des résidus lysines LSD1 et LSD2 ou *lysine-specific histone demethylase 1 and 2*) et la famille des protéines à domaine jumonji C (JmjC) (Greer, 2012).

La méthylation des résidus lysine peut induire une activation ou une répression transcriptionnelle, en fonction du résidu ciblé et du degré de méthylation. Par exemple H3K9me_{2/3}, H3K27me_{2/3} et H4K20me₃ sont associées à une extinction génique alors que H3K4me_{2/3}, H3K36me₂, H3K79me₃ sont souvent corrélées à une activité transcriptionnelle accrue, phénomène également observé lors de la méthylation du résidu arginine (Greer, 2012). De la même manière que les HDACs/HATs, les HMTs et les HDMs régulent l'expression de gènes importants lors de processus cellulaires cruciaux et une altération de leur fonction peut conduire à des pathologies telles que le cancer (Florea, 2011).

4. Le code des histones

Strahl et Allis ont émis l'hypothèse que différentes modifications d'histones se produisant sur une ou plusieurs régions terminales pouvaient agir de façon séquentielle ou combinatoire pour dicter une fonction chromatinienne particulière (Strahl, 2000). Actuellement, de nombreuses études ont permis de confirmer la validité de cette hypothèse et la complexité du code des histones. En effet, l'immunoprécipitation de la chromatine a révélé que la phosphorylation de H3S10 favorise l'acétylation de H3K14 par Gcn5, et que ces deux modifications agissent en synergie pour activer la transcription (Cheung, 2000; Lo, 2000). Des expériences supplémentaires ont montré que la phosphorylation de H3S10 inhibent la méthylation de H3K9, et inversement, expliquant ainsi leurs effets transcriptionnels opposés. De la même manière, les modifications du domaine amino-terminal de l'histone H4 peuvent avoir des effets régulateurs les unes sur les autres. L'acétylation de H4K8 et H4K12 par le coactivateur transcriptionnel p300/CBP est augmentée lorsque H4R3 est méthylée (Wang, 2001). Ainsi, il apparaît que ces modifications ont une influence les unes sur les autres, non seulement lorsqu'elles sont présentes sur une même queue d'histones (action en *cis*), ou sur une autre queue d'histones (action en *trans*). Par exemple, chez la levure, l'ubiquitinylation de K123 de l'histone H2B (homologue de K120 chez l'homme) par Rad6 est requise pour la méthylation subséquente de H3K4 et H3K79 (Briggs, 2002) (FIGURE 7).

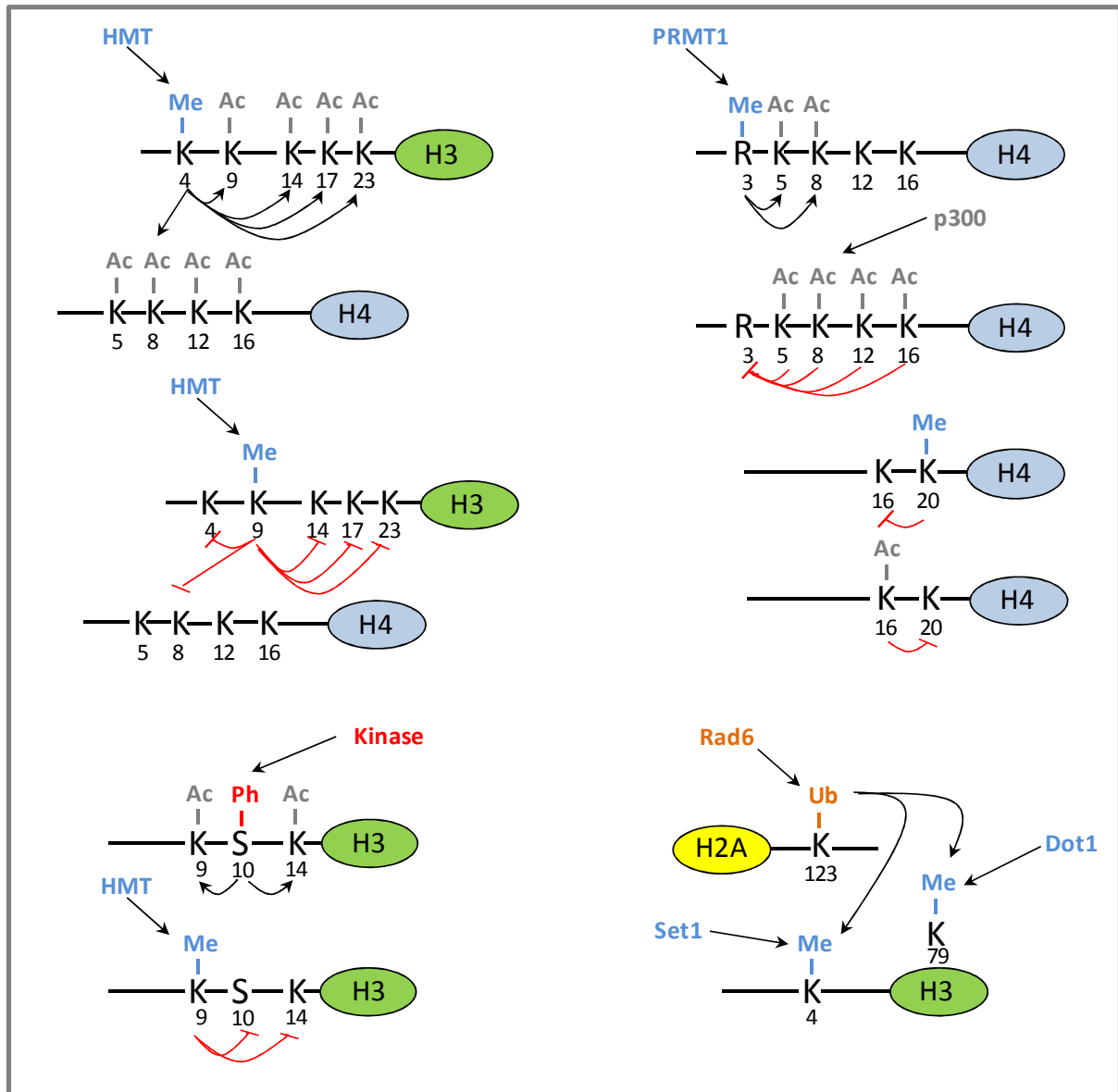


Figure 7 : Interactions entre les modifications chimiques des histones

Ac : acétylation, Ph : phosphorylation, Me : méthylation, Ub : ubiquitylation

(Margueron, 2005)

Le signal épigénétique porté par la chromatine doit être traduit pour provoquer une réponse cellulaire. Certaines protéines, responsables de cette traduction, s'agglomèrent entre elles sous forme de complexes multiprotéiques et permettent de réguler l'activité transcriptionnelle des gènes (Taverna, 2007). Elles se caractérisent par la présence de domaines structuraux particuliers servant à la reconnaissance des différentes modifications (TABLEAU 3). Les méthylation des histones peuvent être reconnues par des *chromo-like* domaines de la famille Royal (par exemple : chromo, tudor et MBT repeat ou (*malignant*

brain tumor repeat) et des domaines PHD (*plant homeo domain*). Par exemple H3K4me3 est reconnue par le domaine PHD d'un composant du complexe de remodelage de la chromatine NURF (*nucleosome remodeling factor*) (Kouzarides, 2007b; Kouzarides, 2007a). L'acétylation des lysines est reconnue par des protéines à bromodomaine telles que les HATs de la famille des GNATs ou les protéines de la famille BET (*bromodomain and extra-terminal*) (Dey, 2003) et la phosphorylation des résidus sérine par des protéines de la famille 14-3-3 (Macdonald, 2005).

Modules structuraux		Marques reconnues au niveau de H3 et H4
Bromodomaine		lysines acétylées
Royal	Chromodomaine	H3K9me2/3, H3K27me2/3
	Double chromodomaine	H3K4me1/2/3
	Chromo barrel	H3K36me2/3
	Tudor	(Rme2s)
	Double/tandem tudor	H3K4me3, H4K20me1/2/3
	MBT	H4K20me1/2, H3K4me1, H3K9me1/2
PHD		H3K4me0/3, H3K9me3, H3K36me3
WD40 répétition		H3R2/K4me2
14-3-3		H3S10ph, H3S28ph

Tableau 3 : Modules structuraux reconnaissant les modifications chimiques des histones
(Taverna, 2007)

C. Méthylation de l'ADN

Dans les cellules de mammifères, la méthylation de l'ADN a lieu au niveau du carbone 5 de cytosines (C) localisées au niveau de dinucléotides CpG et conduit à la formation de résidus 5-méthylcytosines (5mC) (FIGURE 8A). Les dinucléotides CpG sont dispersés dans le génome ou regroupés au sein d'îlots CpG (CpGi). Les CpGi, régions d'environ 200 bp contenant au moins 55 % de dinucléotides CpG, sont retrouvés dans la séquence de plus de la moitié des gènes et sont majoritairement localisés au niveau de régions promotrices ou

dans des régions intra- ou intergéniques où ils sont communément appelés « CpGi orphelins » (Deaton, 2011).

Le transfert de groupements méthyles est catalysé par une famille de méthylases spécifiques, les ADN méthyltransférases (DNMTs ou *DNA methyltransferases*), qui utilisent la S-adenosyl-méthionine (SAM) comme donneur de groupement méthyle. Les isoformes actives de DNMTs sont classées en DNMT de maintenance, représentée par DNMT1 qui assure le maintien de méthylation préexistantes au cours de la réplication de l'ADN, et en DNMTs *de novo*, DNMT3a et DNMT3b qui permettent une méthylation *de novo* au niveau de séquences CpG spécifiques. Les DNMTs *de novo* présentent une affinité pour des brins d'ADN non méthylés ou hémiméthylés, tandis que DNMT1 méthyle préférentiellement les brins d'ADN hémiméthylés (FIGURE 8B).

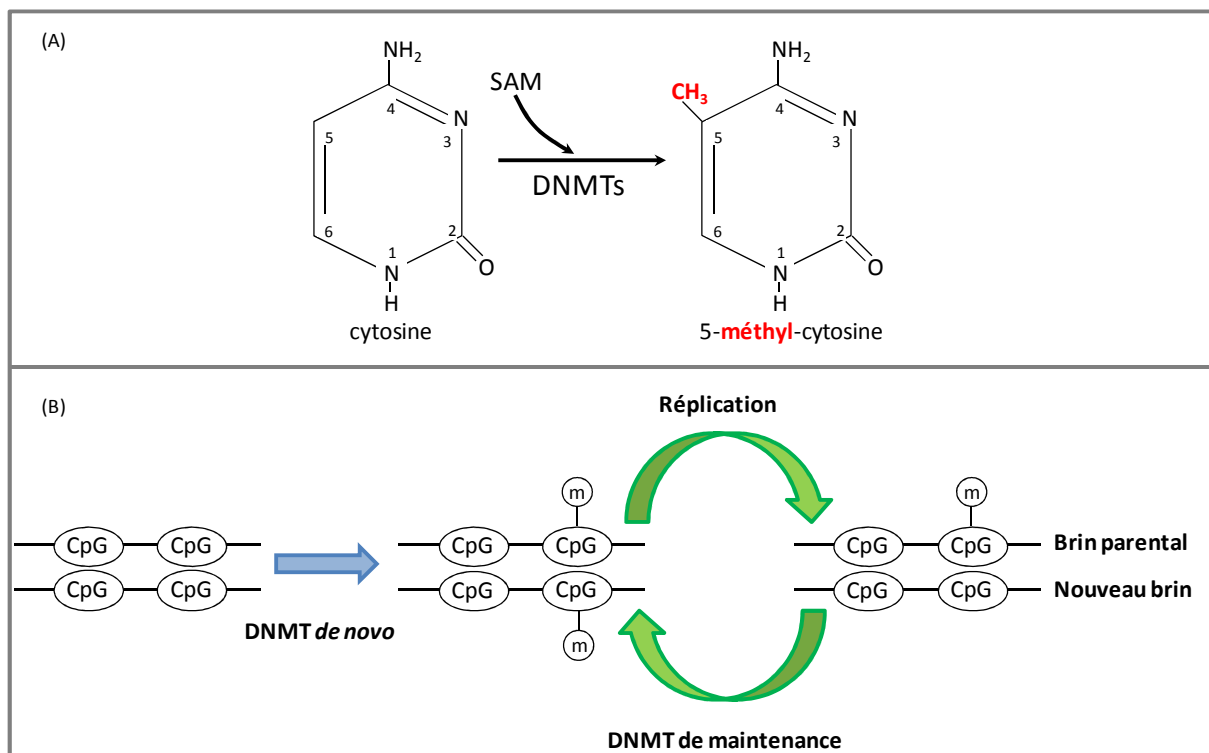


Figure 8 : Processus de méthylation de l'ADN et action spécifique des DNMTs

La méthylation de l'ADN est souvent associée à une extinction génique (Illingworth, 2009) puisqu'elle réprime la fixation de la machinerie transcriptionnelle (Esteller, 2007) ou sert d'échafaudage pour le recrutement de répresseurs transcriptionnels tels que les protéines à domaine MBD (*methyl-CpG-binding domain*) incluant MBD1-4, MeCP2 (*methyl*

CpG binding protein 2) et Kaiso (Deaton, 2011). Les protéines à domaine MBD permettent par la suite le recrutement de complexes multiprotéiques contenant des protéines de remodelage de la chromatine telles que les HDACs ou les HMTs (favorisant dans ce cas, la méthylation de H3K9 ou H3K27). L'ensemble de ces facteurs est donc impliqué dans la répression transcriptionnelle induite par la méthylation de l'ADN en augmentant le degré de compactage de la chromatine et en empêchant la fixation de facteurs de transcription. A l'inverse, une absence de méthylation des CpG est habituellement associée à une structure chromatinienne moins condensée facilitant ainsi la fixation de facteurs de transcription et activant l'expression des gènes. Cet état permissif de la chromatine est activement maintenu par des HATs et des HMTs (permettant la méthylation de H3K4), et par divers complexes coactivateurs (Berger, 2007) (FIGURE 9).

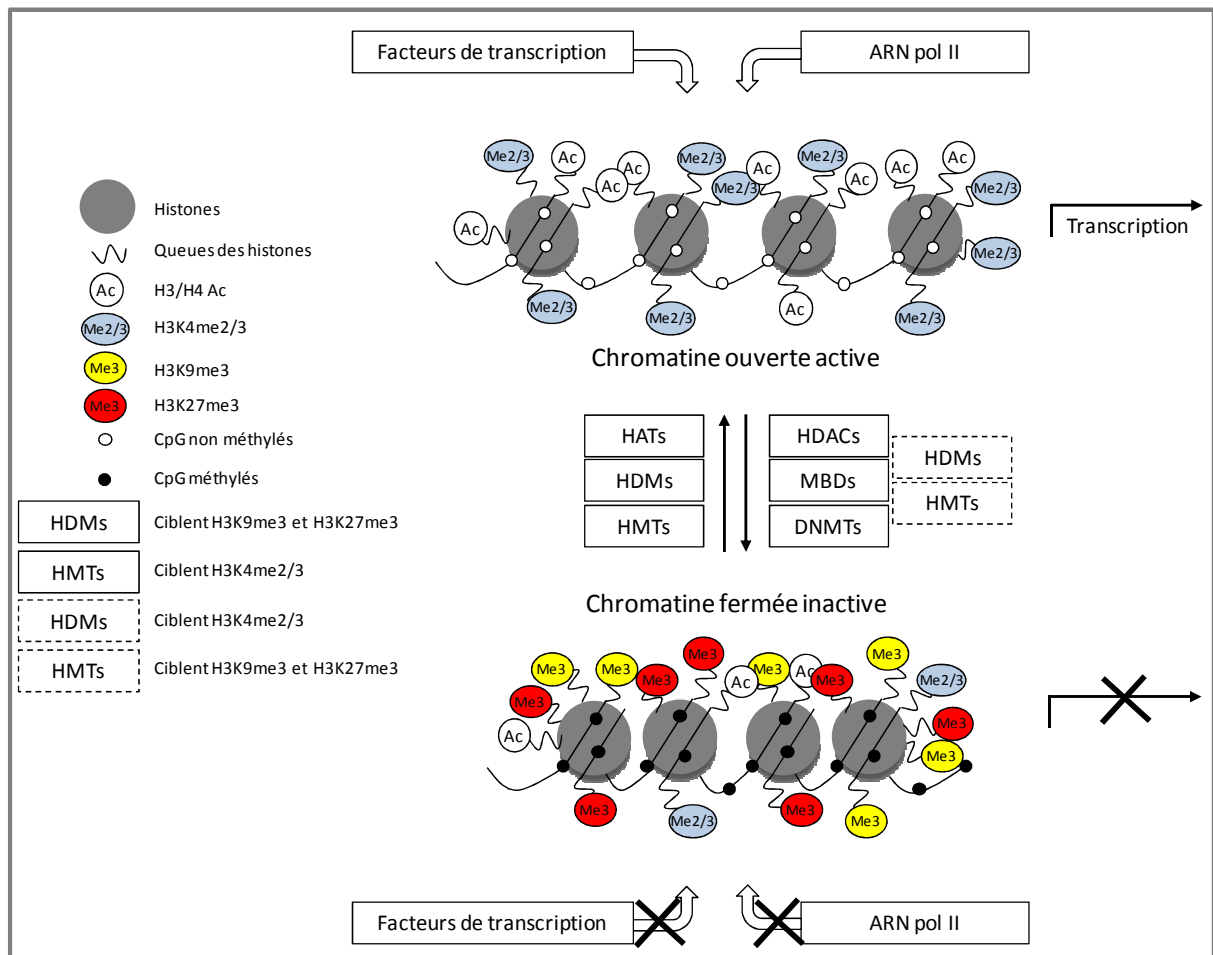


Figure 9 : Modifications épigénétiques et contrôle de l'expression des gènes

(Floean, 2011; Rodriguez-Cortez, 2011)

Les CpGi sont généralement déméthylés dans des cellules normales différenciées mais peuvent subir une vague de méthylation établie de façon tissu-spécifique (Straussman, 2009). L'extinction génique induite par la méthylation de l'ADN est impliquée dans des processus physiologiques tels que l'inactivation du chromosome X (Sharp, 2011) ou l'expression monoallélique associée à l'empreinte génomique parentale (Wilkins, 2005). De plus, une hyperméthylation de l'ADN est retrouvée au niveau de rétrotransposons, qui sont des éléments génomiques répétitifs représentant 40 % du génome de mammifères (Bire, 2012). Cette méthylation de l'ADN permet de veiller à l'intégrité du génome en empêchant des remaniements géniques associés à une instabilité chromosomique. Outre son rôle dans des processus physiologiques, la méthylation de l'ADN joue un rôle important au cours d'évènements pathologiques tels que les maladies auto-immunes (Rodriguez-Cortez, 2011) ou le cancer (Gronbaek, 2007; Jones, 2007).

IV. Mécanismes épigénétiques, MMPs et cancer

A. Mécanismes épigénétiques et cancer

L'étude des causes génétiques du cancer (mutations, amplifications ou perte de matériel chromosomique, translocations récurrentes) a longtemps occupé le devant de la scène. L'explosion des connaissances sur les acteurs moléculaires et les mécanismes sous-jacents qui, en modulant la structure de la chromatine, contrôlent l'expression des gènes a révélé le rôle prépondérant joué par les modifications épigénétiques dans le déclenchement et la progression de nombreuses maladies, en particulier le cancer. En effet, l'étude des phénomènes épigénétiques tels que la méthylation de l'ADN, les modifications post-traductionnelles des histones, le positionnement des nucléosomes et l'expression de micro-ARNs, a fourni des informations clés au cours de ces dernières années sur les mécanismes contribuant à la cancérogénèse.

1. Méthylation de l'ADN et cancer

Dans les années 80, il a été mis en évidence que le génome des cellules cancéreuses était globalement moins méthylé que celui des tissus sains correspondants (Feinberg, 1983). Cette hypométhylation globale a été observée dans différents types de néoplasies humaines

suggérant que cette différence est spécifique du phénomène de cancérogenèse et non du tissu (Ehrlich, 2002).

Dans les cellules tumorales, la perte de la méthylation génomique est souvent retrouvée au niveau de séquences d'ADN modérément ou hautement répétées telles que les séquences d'ADN répétées péri-centromériques, les rétrotransposons et les éléments rétroviraux endogènes. D'un point de vue fonctionnel, cette hypométhylation peut conduire à une instabilité chromosomique, à une activation d'éléments transposables et à une perte de l'empreinte génomique.

L'activation d'éléments transposables peut altérer la fonction d'un gène par l'insertion directe de cet élément dans un exon codant, mais aussi dans un intron, et provoquer sa transcription, ou par la génération d'un transcrit anti-sens.

Par ailleurs, une diminution de la méthylation des séquences répétées des ADN satellites augmente l'instabilité chromosomique. Cela peut conduire à des réarrangements chromosomiques résultant en des états aneuploïdes et à l'inactivation ou l'activation inappropriée de l'expression de certains gènes (Eden, 2003; Karpf, 2005).

Une hypométhylation anormale de l'ADN peut également induire une activation de proto-oncogènes et conduire à une perte de l'empreinte génomique (par exemple : activation du gène IGF2 (*insulin-like growth factor 2*) dans la tumeur de Wilms) (Ogawa, 1993; Wilson, 2007).

Cependant, une hyperméthylation aberrante des promoteurs de gènes suppresseurs de tumeurs reste une caractéristique commune des cancers humains. La perte d'expression génique par hyperméthylation spécifique a été décrite au niveau de nombreux gènes suppresseurs de tumeurs tels que le gène du rétinoblastome Rb (Greger, 1989; Sakai, 1991), p16INK4a ou BRCA1 (*breast-cancer susceptibility gene 1*) (Herman, 2003). De plus, un profil d'hyperméthylation de CpGi au niveau de tumeurs primaires humaines a été établi et permet de révéler que les profils d'hyperméthylation de ces gènes sont spécifiques du type de cancer (Costello, 2000; Esteller, 2001). Chaque type de tumeur est caractérisé par son degré de méthylation de l'ADN ou « hyperméthylome ». Ces marques épigénétiques prennent de l'ampleur par rapport aux biomarqueurs physiologiques ou cytogénétiques couramment utilisés pour la caractérisation des tumeurs et leur état de développement.

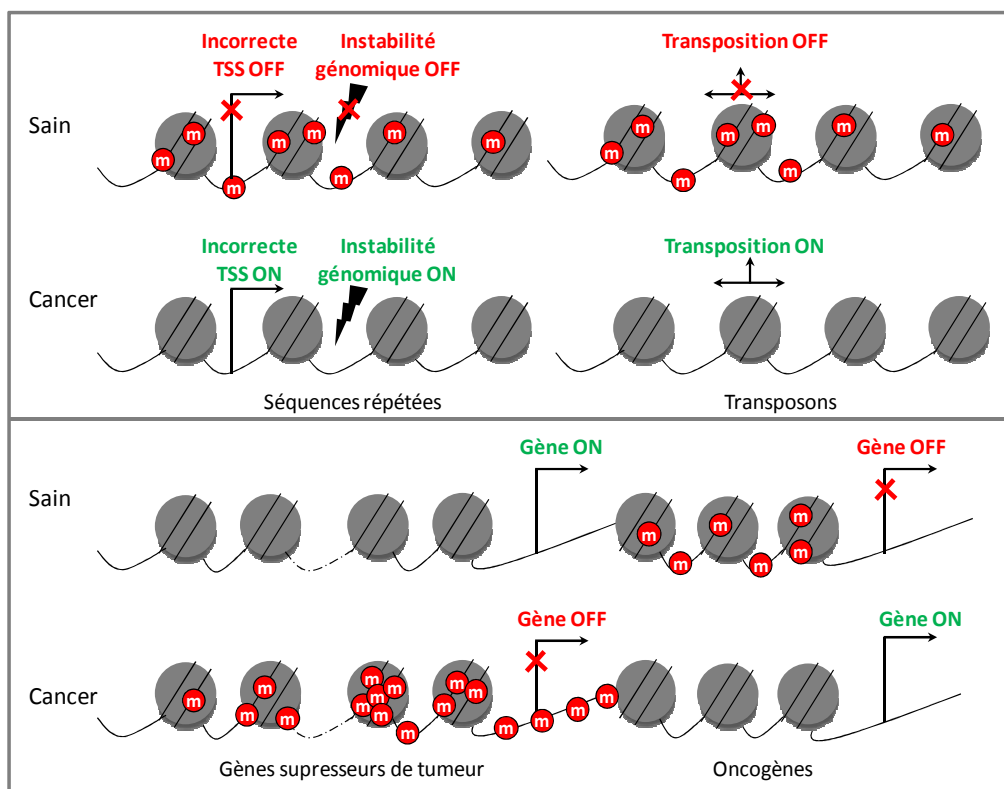


Figure 10 : Méthylation de l'ADN et cancer

(Rodriguez-Paredes, 2011)

2. Modifications post-traductionnelles des histones et cancer

Le profil des modifications chimiques des histones peut subir de profonds remaniements lors de la cancérogénèse. L'exemple le mieux caractérisé est la réduction globale de la triméthylation de H4K20 et de l'acétylation de H4K16 dans de nombreuses tumeurs primaires au niveau de régions hypométhylées (par exemple au niveau de séquences nucléotidiques répétées) (Fraga, 2005). De plus, de nombreuses altérations au niveau d'enzymes assurant le transfert ou reconnaissant des modifications chimiques des histones, ont été observées dans différents types de cancers (Berdasco, 2010). Par exemple, les lymphomes non-Hodgkinien présentent dans plus de 90 % des cas, des mutations récurrentes au niveau de MLL2 (*histone-lysine N-methyltransferase*), un gène codant pour une HMT (Morin, 2011). De manière similaire, UTX (*ubiquitously transcribed tetratricopeptide repeat, X chromosome*), un gène codant pour une H3K27 déméthylase, est muté dans plus de 12 cancers histologiquement distincts (van Haaften, 2009). De plus, des modifications au niveau d'expressions d'HDACs et d'HATs résultant de réarrangements

chromosomiques (*e-g* : établissement de protéines de fusion) ou de mutations ont été observées dans différents types de leucémies (Floean, 2011).

3. Modulateurs épigénétiques et thérapie anticancéreuse

Au vu des résultats scientifiques montrant une corrélation de plus en plus pertinente entre anomalies épigénétiques et cancers, l'élaboration de modulateurs épigénétiques comme nouvelle thérapie anticancéreuse a pris un essor important au cours de ces deux dernières décennies (Rothhammer, 2007). De nombreux agents modulant la méthylation de l'ADN ou inhibant la désacétylation des histones, ont été développés et testés cliniquement.

L'utilisation d'agents hypométhylants vise à réactiver des gènes éteints (par exemple : des gènes suppresseurs de tumeurs) dont le promoteur est hyperméthylé. La 5-azacytidine (5-azaC ou Vidaza®) et la 5-aza-2'-désoxycytidine (5-azadC ou Decitabine®), deux agents hypométhylant de l'ADN, possèdent des intérêts cliniques certains et sont déjà utilisés en pratique comme agents anticancéreux (Egger, 2004; Fenaux, 2005). Ces deux homologues de la cytosine agissent en s'incorporant dans l'ADN et en formant des complexes irréversibles avec les DNMTs, entraînant ainsi leur destruction. Ce type de molécule ne provoque donc pas directement la déméthylation de l'ADN, mais empêche sa reméthylation après la synthèse de l'ADN. En clinique, l'efficacité de ces agents hypométhylant est dépendante des paramètres de traitement (dose et espacement des soins). Un traitement par la 5-azaC (75mg/m² par jour, pendant sept jours, toutes les 4 semaines, pendant 4 mois) a été testé avec succès chez des patients atteints de syndromes myelodysplasiques (Silverman, 1993). Dans des essais cliniques de phase III, des taux de réponses élevés ainsi qu'une meilleure qualité de vie ont été observés dans un groupe de patients recevant la 5-azaC en comparaison avec un groupe de patients recevant un autre type de traitement (= « *supportive care* » ou soin de soutien) (Silverman, 2002). De ce fait, la 5-azaC et la 5-azadC présentant des résultats tout aussi promettant, ont été approuvées par la FDA (*food and drug administration*) pour le traitement des syndromes myelodysplasiques (SMD) et de certaines leucémies (Silverman, 2005). En France, le Vidaza® a reçu une autorisation de mise sur le marché (AMM) en 2008 et est indiqué dans le traitement des patients adultes non éligibles pour une transplantation de cellules souches hématopoïétiques et présentant : un SMD de risque intermédiaire-2 ou élevé selon l'index

pronostique international (*international prognostic scoring system*, IPSS), une leucémie myélomonocytaire chronique (LMC) avec 10-29 % de blastes médullaires sans syndrome myéloprolifératif, ou une leucémie aiguë myéloblastique (LAM) avec 20-30 % de blastes et dysplasie de lignées multiples, selon la classification de l'organisation mondiale de la santé (OMS). La décitabine (Dacogen®) est, quant à elle, un médicament en autorisation temporaire d'utilisation (ATU) nominative depuis juillet 2007 dans le traitement des SMD et des LAM en cas d'échec au Vidaza®. Plusieurs études de phase I-II sur des tumeurs solides ont été développées et permettent de stabiliser la progression de la maladie de patients atteints d'un cancer de la prostate (Thibault, 1998). A l'heure actuelle, le mécanisme de réactivation des gènes par l'action d'analogues nucléosidiques hypométhylants n'est pas entièrement élucidé, et d'autres classes d'inhibiteurs ne s'intégrant pas à l'ADN mais agissant directement sur les ADN méthyltransférases sont en cours de développement.

Les HDIs sont couramment testés chez des patients atteints de pathologies malignes puisqu'ils présentent différents effets anti-tumoraux. En effet, ils sont capables d'induire l'arrêt du cycle cellulaire, la différenciation et/ou l'apoptose dans des cultures de cellules transformées ou issues de tumeurs. Le phenylbutyrate (acide gras à chaîne courte) a été le premier HDI testé en étude de phase I-II sur des patients atteints de SMD, de leucémies ou présentant des tumeurs solides malignes (Gilbert, 2001; Gore, 2002). L'acide valproïque (acide gras à chaîne courte), utilisé pendant longtemps comme traitement anti-épileptique (Depakine®), a été évalué en essai clinique de phase I-II-III comme agent anticancéreux (Chavez-Blanco, 2005; Coronel, 2011; Pilatrinio, 2005). Parmi les HDIs du groupe des acides hydroxamiques, le SAHA (Vorinostat) a été approuvé par la FDA pour le traitement de lymphomes (Zolinza®) et le LBH549 (Panobinostat) et le PDX101 (Belinostat) sont en investigation clinique pour le traitement de leucémies (Mercurio, 2010). Le benzamide MS-275 (Entinostat), (actuellement en essai clinique de phase I) est utilisé en combinaison avec la 5-azaC pour augmenter la cytotoxicité dans certains types de leucémies (Gao, 2008).

B. Rôles des MMPs dans le cancer

La protéolyse extracellulaire joue un rôle fondamental dans l'homéostasie tissulaire. Au cours du cancer, une protéolyse dysfonctionnelle entraîne des dérégulations dans le remodelage tissulaire et induit des processus pathologiques tels que l'inflammation, la croissance et l'invasion tumorales ainsi que la formation de métastases (FIGURE 11).

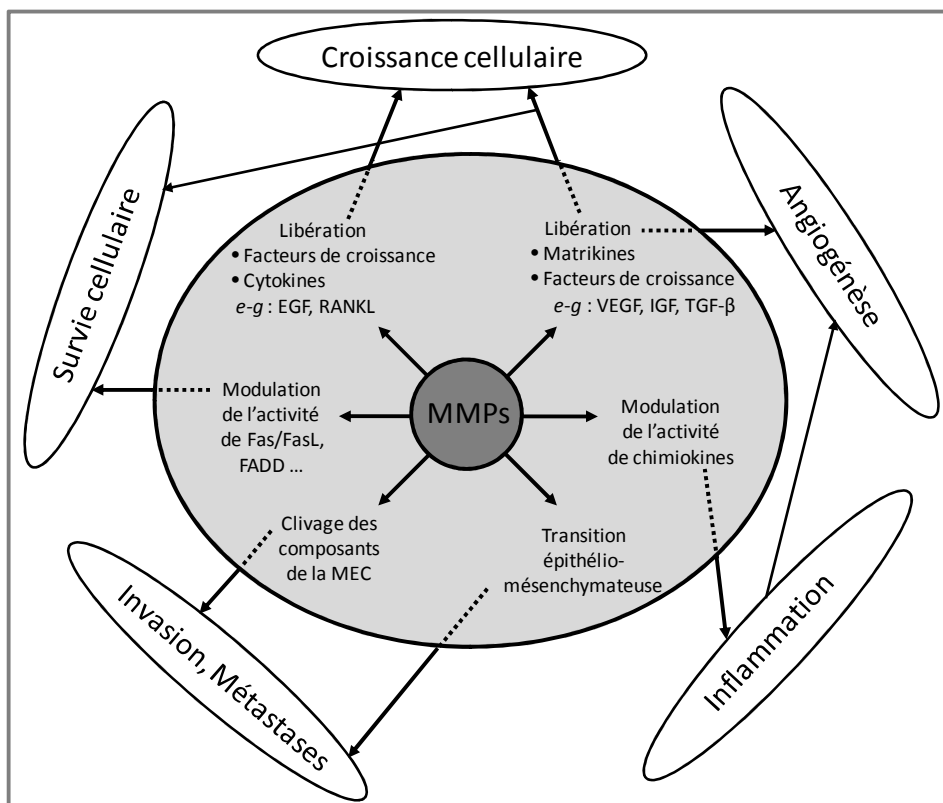


Figure 11 : Rôles des MMPs dans le cancer

(Hua, 2011)

La progression tumorale et la formation de métastases impliquent une succession d'évènements complexes au cours desquels les interactions entre les cellules tumorales, la matrice extracellulaire environnante et les cellules hôtes vont se trouver modifiées. Les MMPs vont jouer un rôle extrêmement important dans ces différents processus en remodelant le microenvironnement tumoral (FIGURE 12).

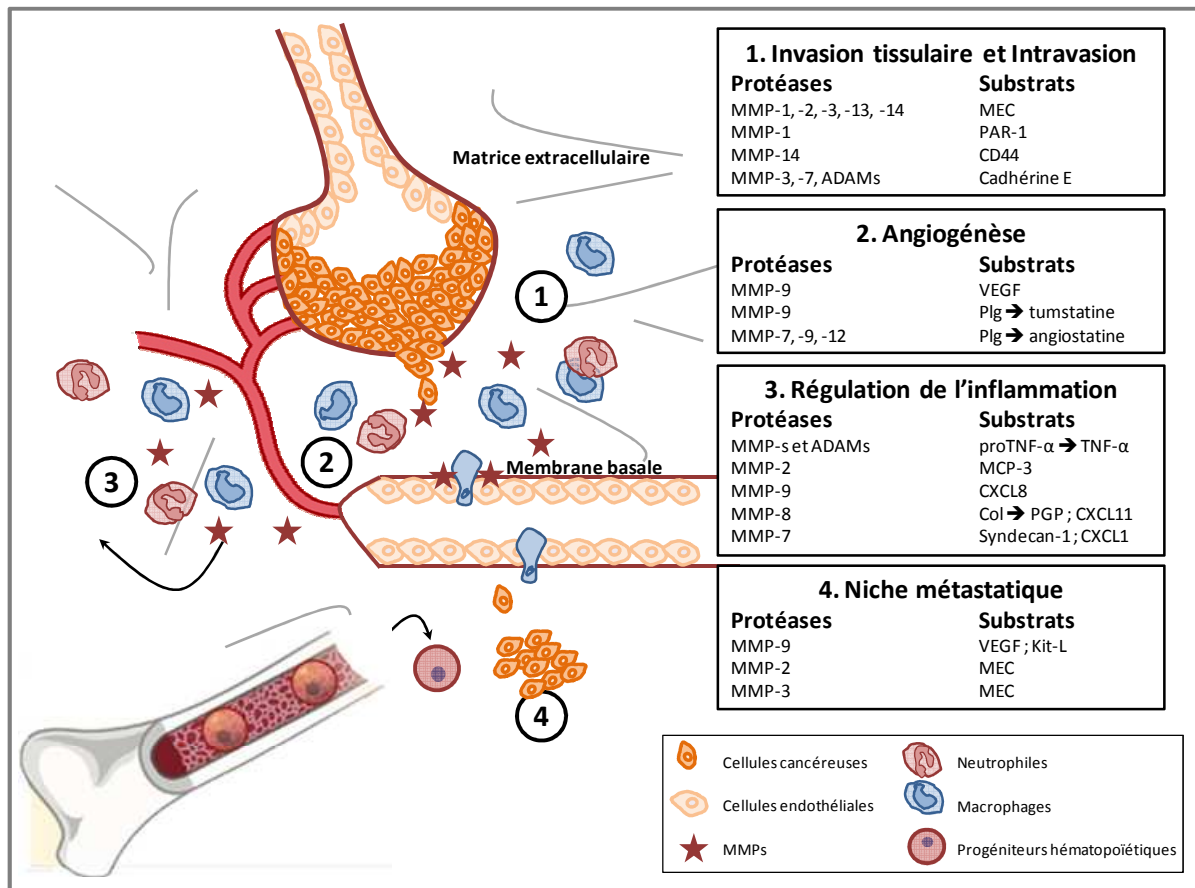


Figure 12 : Rôles des MMPs dans le remodelage du microenvironnement tumoral

(Kessenbrock, 2010)

Les MMPs sont majoritairement sécrétées par les cellules non tumorales telles que les polynucléaires neutrophiles, les macrophages, les fibroblastes ou les cellules endothéliales. L'invasion tissulaire de la tumeur (point 1, FIGURE 12) et l'intravasation des cellules cancéreuses dans la circulation sanguine nécessite un remodelage de la MEC et une perte d'adhérence des cellules. Les MMPs et d'autres protéinases (*par exemple les ADAMs ou a disintegrin and metalloproteinase domain-containing protein* et cathepsines) permettent de réguler le turnover des composants de la MEC mais aussi la migration des cellules cancéreuses, en clivant par exemple PAR-1 (*Proteinase-Activated Receptor-1*) ou en dégradant des molécules d'adhérence telles que le CD44 ou la cadhérine-E présents à la surface des cellules (Kessenbrock, 2010). Le passage des cellules tumorales métastatiques dans la circulation sanguine nécessite l'activité protéolytique de macrophages et la migration des leucocytes vers la membrane basale endothéliale (point 1, FIGURE 12). Les tumeurs sont richement vascularisées et la formation de nouveaux vaisseaux sanguins

(angiogénèse) (point 2, FIGURE 12) est initiée par la libération de VEGF (*Vascular Endothelial Growth Factor*) induite en partie par l'activité enzymatique des gélatinases. La formation de peptides angiostatiques, issus du clivage du plasminogène par les MMPs, contribue également à l'angiogénèse tumorale. Les MMPs sont de puissants régulateurs de l'inflammation et jouent un rôle critique dans le recrutement des cellules inflammatoires en libérant des cytokines telles que le TNF- α (*tumor necrosis factor- α*) ou le CXCL8 (*chemokine (C-X-C motif) ligand*) (point 3, FIGURE 12). Par ailleurs, les MMPs permettent l'établissement d'un gradient chimiotactique en libérant des peptides tels que le PGP (proline-glycine-proline) issu de la dégradation du collagène ou des chimiokines telles que le CXCL1 libérée après dégradation du syndécan. Néanmoins, certaines MMPs exercent des propriétés anti-inflammatoires en permettant, par exemple, la dégradation de chimiokines telles que MCP-3 (*Monocyte Chemotactic Protein-3*). Les métastases résultent donc d'une dissémination des cellules cancéreuses à partir d'un premier foyer (tumeur primaire) vers des foyers secondaires plus distants. Des données récentes ont révélé que ces foyers secondaires peuvent être le lieu de niches pré-métastatiques caractérisées par le regroupement de cellules inflammatoires et de progéniteurs hématopoïétiques et favorisant par la suite la colonisation de ces sites par les cellules métastatiques (Rucci, 2011) (point 4, FIGURE 12). Les gélatinases MMP-2 et -9 sont notamment impliquées dans ce processus d'initiation en permettant la libération de facteurs tels que le VEGF et le Kit-Ligand (Kit-L) responsables du recrutement des progéniteurs hématopoïétiques (Kessenbrock, 2010).

C. Mécanismes épigénétiques, MMPs et cancer

Au cours des processus pathologiques, les cellules peuvent être amenées à exprimer des niveaux très élevés de MMPs suggérant ainsi que des mécanismes additionnels vont être requis pour permettre cette modulation d'expression. Parmi ces mécanismes, les phénomènes épigénétiques vont jouer un rôle extrêmement important dans la régulation de l'expression des gènes en modulant l'activité transcriptionnelle des promoteurs ou la stabilité des ARNm.

1. Méthylation de l'ADN, MMPs et cancer

Des études récentes ont permis d'établir une relation entre le profil de méthylation des promoteurs des MMPs, l'expression de leur gène et le comportement invasif de cellules tumorales (Chernov, 2009; Sato, 2003). Par exemple, dans des lignées cellulaires hautement invasives (lignées de fibrosarcomes HT1080 et de glioblastomes U251, U87, TP98G and U373) exprimant des niveaux élevés de MMP-2 et MT1-MMP, le promoteur de ces gènes est faiblement méthylé. A l'inverse, dans des lignées faiblement invasives (carcinomes mammaires MCF-7), ces mêmes gènes sont peu exprimés en raison d'une hyperméthylation de leur promoteur (Chernov, 2009) (FIGURE 13 A). De la même manière, il a été montré que dans des cellules tumorales pancréatiques (AsPC1 ou BxPC3) exprimant peu MMP-2 et -9, le promoteur de ces gènes est complètement ou partiellement méthylé, alors que le gène de la MMP-7 est fortement exprimé en raison d'une déméthylation totale de son promoteur (Sato, 2003).

L'ensemble de ces observations permet de supposer que l'administration d'agents hypométhylants ou hyperméthylants de l'ADN serait susceptible de moduler les propriétés invasives des cellules tumorales (FIGURE 13 B et C). En effet, un traitement de cellules cancéreuses de prostate hautement invasives (lignée cellulaire PC3) par un agent donneur de groupements méthyle (SAM) ou par des oligonucléotides anti-MBD2 (MBD2 AS ou *MBD2 antisens*), diminue l'expression du gène de la MMP-2 en conséquence de la méthylation de sa région 5' régulatrice et diminue *in vitro* les capacités invasives de ces cellules (Shukeir, 2006) (FIGURE 13 B). Inversement, le traitement de cellules cancéreuses pancréatiques (lignée cellulaire AsPC1) par la 5-azadC permet de restaurer et d'amplifier l'expression des gènes des MMP-1, -2 et -14 et d'augmenter les propriétés invasives de ces cellules, augmentation abolie en présence d'inhibiteurs des MMPs (GM6001) (Sato, 2003) (FIGURE 13 C).

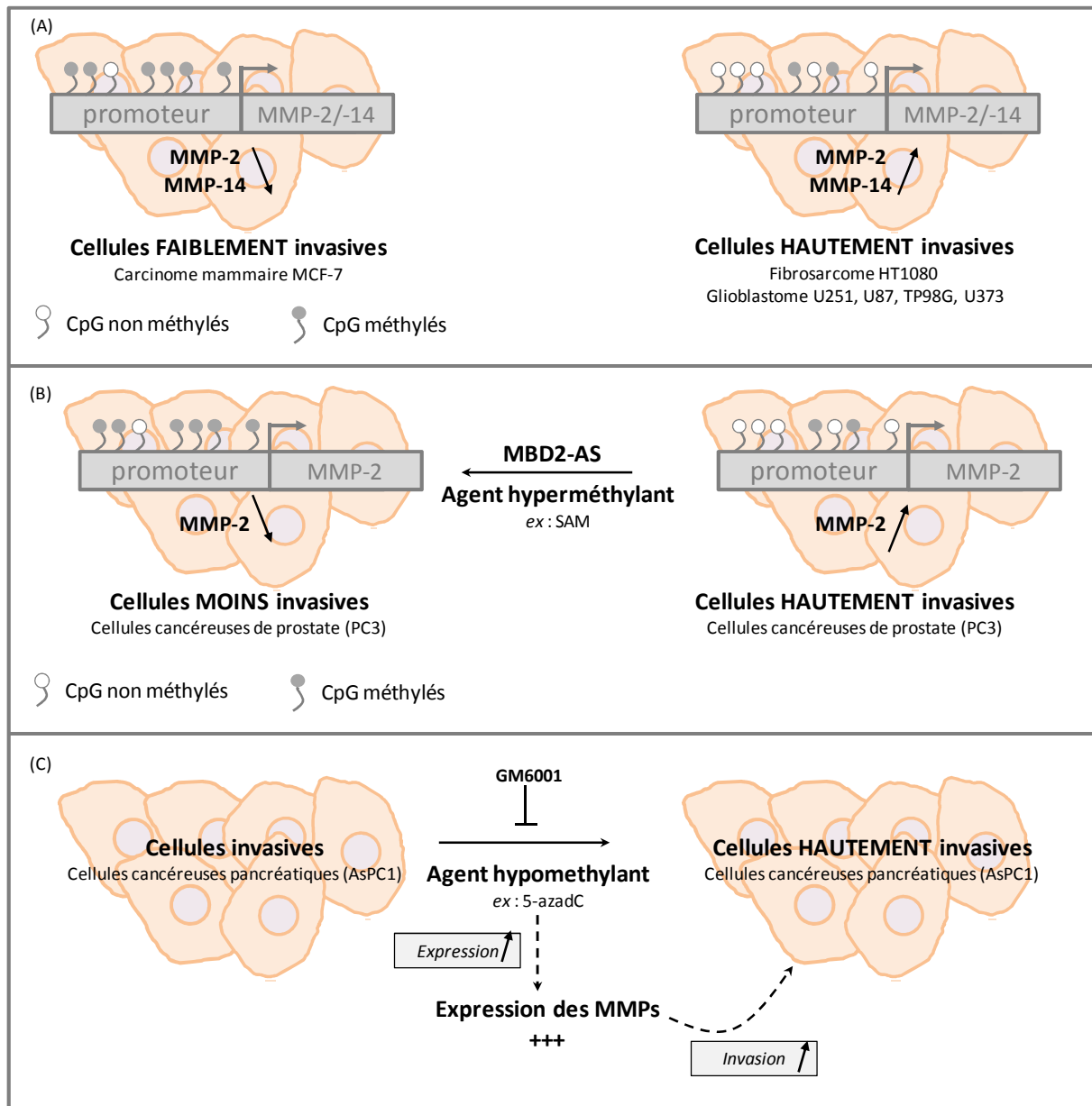


Figure 13 : Méthylation de l'ADN, expression des MMPs et invasion tumorale

(Chernov, 2009; Sato, 2003)

La 5-azadC est également capable d'augmenter l'expression de MMP-9 dans des cellules de lymphomes murins chez lesquelles le niveau d'expression de ce gène est directement en relation avec le profil de méthylation de son promoteur (Chicoine, 2002). De plus, l'équipe de Couillard *et al*, a montré qu'une double déplétion génétique des enzymes DNMT1 et DNMT3b induit une expression *de novo* de MMP-3, mais n'est pas capable d'induire l'expression de MMP-1 et-2 dans des cellules de carcinome de colon (Couillard, 2006). Un traitement par la 5-azadC ou la Zebularine® (un dérivé de l'azaC) augmente

également l'expression de MMP-3 dans des cellules de carcinome de colon mais pas dans des cellules de lymphomes, tandis-que l'expression de MMP-10 est augmentée dans ces deux types cellulaires (Couillard, 2006). Ces résultats suggèrent ainsi que l'expression des gènes des MMPs induite par hypométhylation de leur promoteur semble être cellule-spécifique, et que des phénomènes épigénétiques peuvent prédisposer les cellules cancéreuses à exprimer ou non les gènes des MMPs.

2. Modifications post-traductionnelles des histones, MMPs et cancer

Une dérégulation de l'expression des gènes des MMPs au cours du cancer peut être la conséquence de remaniements dynamiques dans la structure chromatinienne, faisant intervenir des modifications post-traductionnelles des histones et le recrutement de complexes de remodelage.

Relation entre H3K27me3, l'expression de MMP-2 et -14, et l'invasion tumorale

Des analyses *genome-wide* (« génome entier ») ont révélé que certaines modifications post-traductionnelles des histones (par exemple AcH3, AcH4, H3K4me2...) peuvent être associées à une chromatine ouverte et transcriptionnellement active : dans ce cas, ces modifications chimiques sont qualifiées de « marques actives ». A l'inverse d'autres modifications post-traductionnelles (par exemple H3K9me3, H3K27me3...) peuvent être associées à une chromatine plus condensée et à une extinction génique : dans ce cas, ces modifications chimiques sont qualifiées de « marques répressives ». L'enrichissement en ces marques « actives » et « répressives » au niveau des promoteurs des MMPs permet de refléter le phénotype invasif de cellules cancéreuses. En effet, une étude a montré que dans des lignées cellulaires faiblement invasives (MCF7), le promoteur des gènes MT1-MMP et MMP-2 dont l'expression est réduite, est fortement enrichi en marque répressive H3K27me3 (Chernov, 2009). A l'inverse, dans des cellules hautement invasives (U251), le promoteur de ces gènes est peu enrichi en cette même marque répressive (Chernov, 2009). Cependant, l'enrichissement en marques actives AcH3 et H3K4me2 au niveau du promoteur des gènes de ces MMPs, ne semble pas impliqué dans la régulation de leur expression puisque,

quelque soit le potentiel invasif des lignées, aucune différence n'est observée au niveau de cet enrichissement (FIGURE 14).

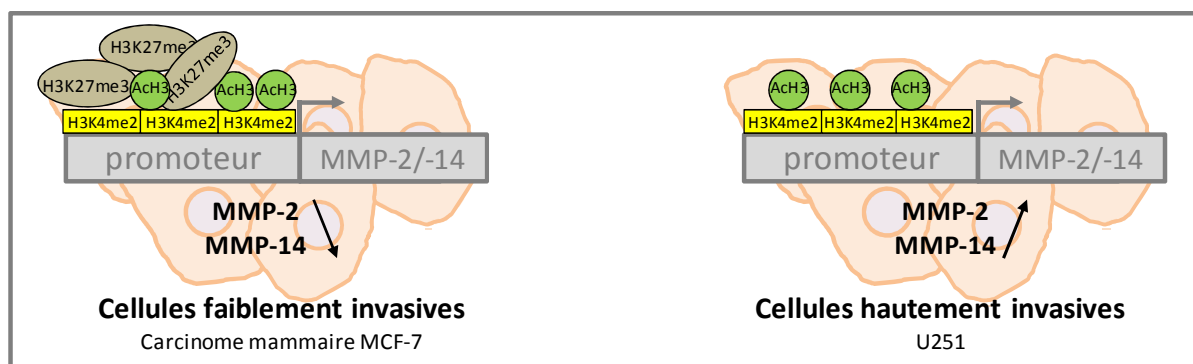


Figure 14 : H3K27me3, expression de MMP-2 et -14 et invasion tumorale

(Chernov, 2009)

Rôles des HAT dans la régulation de l'expression des MMPs et dans l'invasion tumorale

Une exposition prononcée aux ultra-violets (UV) peut être à l'origine de l'établissement de processus cancéreux en modulant l'expression des MMPs *via* des mécanismes épigénétiques (Brenneisen, 2002). L'induction de l'expression du gène de la MMP-1 par les UV requiert une hyperacétylation de l'histone H3 au niveau de son promoteur, conséquence d'une diminution de l'activité d'HDACs et d'une augmentation de l'activité d'HATs telles que p300 (Kim, 2009). Par ailleurs l'acétylation d'histones, par l'intermédiaire du recrutement du complexe p300/CBP ou par l'inhibition d'HDACs, joue un rôle critique dans l'invasion de cellules tumorales mammaires et de colon en favorisant la surexpression de MMP-13 ou de MMP-9 (Bai, 2007; Farina, 2011). Dans une autre étude, il a été montré que l'invasion de cellules tumorales de prostate était réduite après déplétion de p300 et diminution de l'expression de MMP-2 et -9 (Santer, 2011). Néanmoins, la modulation de l'activité du promoteur de MMP-9 par des mécanismes épigénétiques, semble être dépendante du type cellulaire puisque, dans un modèle de cancer cervical, le promoteur de ce gène requiert l'activité HAT de PCAF (*p300/CBP associated-factor*), et non pas celle p300/CBP, et l'activité de l'histone arginine méthyltransférase CARM1 (Zhao, 2008). L'activité de CARM1 est également impliquée dans la régulation épigénétique d'autres MMPs puisque l'expression de MMP-1, -3, -13 est considérablement diminuée dans des cellules déficientes en CARM1 (Fauquier, 2008).

Rôles des HDACs dans la régulation de l'expression des MMPs et dans l'invasion tumorale

Park *et al.* ont montré que l'expression d'isoformes d'HDACs peut moduler le comportement migratoire et invasif de carcinomes mammaires (Park, 2011). En effet, la lignée MDA-MB-231 (hautement invasive et métastatique) exprime fortement MMP-9 et présente des niveaux d'expression d'HDAC4, 6, 8 plus importants que celles exprimées dans la lignée MCF-7 (faiblement invasive et non métastatique). Un traitement des cellules MDA-MB-231 par de l'apicidine (HDI) ou par des siRNAs dirigés contre les différentes isoformes d'HDACs, révèle que les HDACs 1, 6, et 8, mais pas l'HDAC4, sont responsables de l'expression de MMP-9 et du comportement invasif de ces cellules. De plus, une surexpression des HDACs 1, 6, ou 8 dans la lignée MCF-7 augmente l'expression de MMP-9 et les capacités invasives de cette lignée (FIGURE 15).

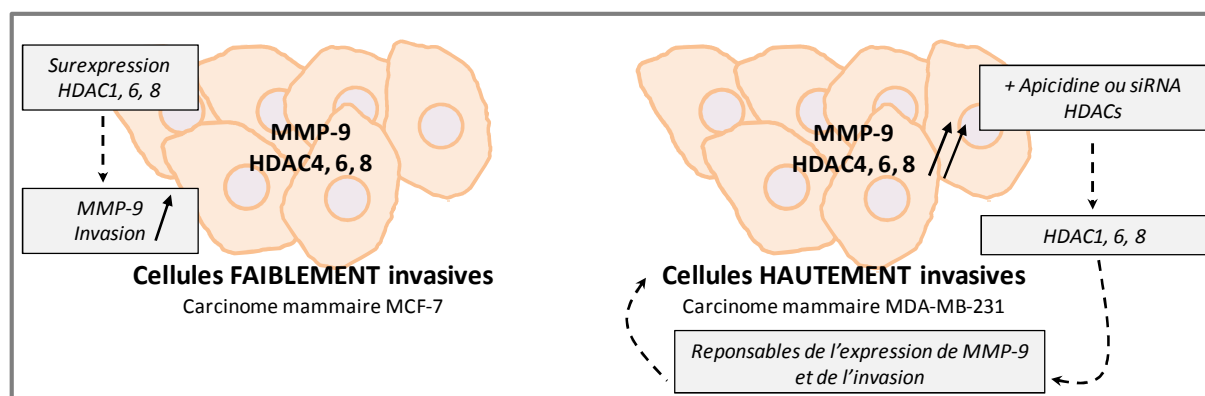


Figure 15 : HDACs, expression de MMP-9 et invasion tumorale

(Park, 2011)

Par ailleurs, dans un modèle de souris nude transplantée par des cellules de carcinomes mammaires invasifs résistant au TRAIL (*TNF-related apoptosis inducing ligand*) (lignée MDA-MB-468), il a été montré qu'un traitement par l'HDI MS-275 est capable de potentialiser les effets de TRAIL en diminuant l'expression de MMP-2 et -9 et le volume de la tumeur (Srivastava, 2010). De la même manière, les HDACs jouent un rôle primordial dans l'invasion de cellules tumorales de la thyroïde puisqu'un traitement de ces cellules par le phénylbutyrate induit une hyperphosphoacétylation d'histones suivie d'une diminution de l'expression de MMP-7 et du potentiel métastatique de ces cellules (Chen, 2012). Par ailleurs, Kim *et al.* ont montré que les effets de l'apicidine sur l'expression des MMPs, et plus particulièrement sur l'expression des gélatinases, sont dépendants du type cellulaire utilisé.

Aspects bibliographiques

En effet, l'apicidine augmente la sécrétion de MMP-2 et -9 dans des cellules murines de mélanomes, mais diminue leur sécrétion dans des fibroblastes transformés par v-Ras. Néanmoins, l'apicidine diminue les capacités invasives des deux lignées cellulaires suggérant ainsi que l'activité protéolytique des gélatinases n'est pas l'unique mécanisme ciblé au cours de ce phénomène (Kim, 2004).

Résultats

Les résultats obtenus au cours de cette thèse s'organisent en quatre parties et sont présentés sous forme de publications scientifiques.

Dans une première partie, nous analysons les effets de deux modulateurs épigénétiques, un HDI (trichostatine A ou TSA) et un agent hypométhylant de l'ADN (5-aza-2'-désoxycytidine ou 5-azadC), sur l'expression de la MMP-1 et sur l'architecture nucléaire de la lignée cellulaire de fibrosarcome humain HT1080. Ces travaux ont fait l'objet de la **PUBLICATION N°1**.

Dans une seconde partie, l'impact de ces modulateurs épigénétiques sur l'expression de MMP-2 et -9 est évalué. Les modifications observées sont ensuite comparées : (i) à celles de la texture chromatinienne, et (ii) au profil de méthylation de l'ADN dans les cellules HT1080. Ces travaux ont fait l'objet de la **PUBLICATION N°2**.

Dans une troisième partie, nous analysons l'effet de la 5-azadC sur les propriétés invasives des cellules HT1080 et nous étudions le mécanisme de régulation de l'expression génique de la MMP-1 induite par cet agent. Ces travaux ont fait l'objet de la **PUBLICATION N°3**.

Dans une dernière partie, l'effet de différents HDIs sur l'expression de la MMP-1 est analysé. De plus, nous tentons de disséquer, de manière préliminaire, le mécanisme de régulation de ce gène induit par le MS-275 (HDI). Ces travaux ont fait l'objet de la **PUBLICATION N°4**.

PUBLICATION N°1

Epigenetic regulation of proMMP-1 expression in the HT1080 human fibrosarcoma cell line.

M. Poplineau, J. Dufer, F. Antonicelli, A. Trussardi-Regnier.

Int J Oncol. 2011; 38(6): 1713-1718

Présentation de la PUBLICATION N°1

Régulation épigénétique du gène de la MMP-1 dans la lignée cellulaire de fibrosarcome humain HT1080

Contexte

La collagénase de type I ou MMP-1 est la collagénase interstitielle la plus répandue. Une dérégulation de son activité enzymatique conduit à une protéolyse matricielle anarchique observée dans des états pathologiques tels que le cancer. L'activation transcriptionnelle du gène de la MMP-1 a été largement étudiée mais les mécanismes impliqués dans cette régulation nécessitent d'être davantage élucidés (Yan, 2007). Des études ont révélé que l'expression du gène de la MMP-1 impliquait un remodelage de la chromatine, en particulier *via* des modifications post-traductionnelles des histones (Maciejewska-Rodrigues, 2010; Martens, 2003). Néanmoins, le rôle potentiel de la méthylation de l'ADN dans la régulation de l'expression de ce gène nécessite d'être élucidé. Ainsi, dans un premier temps, nous avons étudié l'effet de modulateurs épigénétiques sur l'expression génique et protéique de la MMP-1 dans la lignée de fibrosarcome humain HT1080. Ces travaux ont fait l'objet de la **PUBLICATION N°1**.

Matériel et Méthodes

Dans cette étude, des cellules de la lignée cellulaire de fibrosarcome humain HT1080 ont été traitées par un agent hypométhylant de l'ADN, la 5-aza-2' desoxycytidine (5-azadC) et/ou par un pan-inhibiteur d'HDACs (inhibiteur à large spectre), la trichostatine A (TSA). L'expression génique et protéique de la MMP-1 ont été analysées respectivement par RT-PCR en temps réel et western blot. De plus, une analyse de la texture nucléaire par cytométrie en analyse d'images a été réalisée, en vue d'établir une corrélation entre les profils d'expression de la MMP-1 et de possibles modifications de l'architecture nucléaire. Des informations supplémentaires concernant l'analyse de la texture nucléaire par cytométrie en analyse d'images sont développées en ANNEXE 1.

Résultats

Dans cette étude, il apparaît que la TSA induit une hyperacétylation de l'histone H4, mais n'est capable, à elle seule, ni d'affecter la texture nucléaire, ni de moduler l'expression du gène de la MMP-1. A l'inverse, un traitement par la 5-azadC augmente les niveaux d'ARNm et de protéine de la MMP-1. Cette augmentation de l'expression est corrélée à des changements dans la supra-organisation nucléaire, puisque la 5-azadC est capable d'induire une décondensation de la chromatine (évaluée par une augmentation de l'index d'anormalité nucléaire). De plus, la TSA et la 5-azadC semblent avoir des effets synergiques puisqu'un traitement simultané par ces deux modulateurs épigénétiques augmente fortement le degré d'anormalité nucléaire ainsi que le niveau d'expression du gène de la MMP-1. L'absence d'îlots CpG au niveau du promoteur du gène de la MMP-1, nous a amené à envisager le fait que la régulation de l'expression de la MMP-1 pourrait s'effectuer de manière indirecte et donc à étudier l'effet du blocage de la synthèse protéique *de novo* par le cycloheximide (CHX) sur l'expression de ce gène. Les résultats révèlent que le CHX abolit partiellement les effets de la 5-azadC sur l'expression de MMP-1. Ces résultats suggèrent que la 5-azadC augmente l'expression de MMP-1 après, en partie, avoir stimulé une synthèse protéique *de novo*.

Conclusion

L'augmentation de l'expression de la MMP-1 induite par la 5-azadC est corrélée à des modifications significatives de l'architecture nucléaire et semble faire intervenir une synthèse protéique *de novo*. Néanmoins une action directe de ce modulateur sur le promoteur de ce gène n'est pas à exclure. L'identification de médiateurs potentiels, ainsi que l'étude du profil de méthylation du promoteur du gène de la MMP-1, pourrait servir de base pour approfondir le mécanisme de régulation épigénétique de ce gène et l'impact potentiel de son expression induite sur les propriétés invasives des cellules HT1080.

Epigenetic regulation of proMMP-1 expression in the HT1080 human fibrosarcoma cell line

MATHILDE POPLINEAU, JEAN DUFER, FRANK ANTONICELLI and AURELIE TRUSSARDI-REGNIER

Unité MEDyC, UMR URCA-CNRS 6237, IFR53, Facultés de Pharmacie et de Médecine,
Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

Received December 2, 2010; Accepted January 28, 2011

DOI: 10.3892/ijo.2011.975

Abstract. The matrix metalloproteinase (MMP) family members play an important role in various physiological and pathological processes. Although MMP-1 (collagenase-1) has been shown to be involved in tumor invasiveness, the regulation of its expression is still not fully elucidated and could implicate epigenetic mechanisms. The aim of this study was to analyze the effects of the Histone Deacetylase Inhibitor (HDI) trichostatin A (TSA) and the inhibitor of DNA methylation 5-aza-2'-deoxycytidine (5-azadC) on the proMMP-1 expression in the human HT1080 fibrosarcoma cell line. Real-time RT-PCR revealed that 5-azadC or 5-azadC + TSA but not TSA alone, despite global histone H4 hyperacetylation, increased *proMMP-1* mRNA levels. This transcription activation was correlated with chromatin decondensation determined by nuclear texture image analysis technique. Western blot analysis of cell culture conditioned media revealed a significant increase in proMMP-1 secretion after 5-azadC or 5-azadC + TSA treatment compared to untreated cells. These results suggested that epigenetic mechanisms could be involved in *proMMP-1* gene expression including chromatin supra-organization changes. Indeed, although the *proMMP-1* gene promoter does not appear to contain CpG islands, its expression can be induced by the demethylating agent 5-azadC. Further experiments revealed that inhibition of protein neosynthesis by cycloheximide decreased 5-azadC-induced *proMMP-1* mRNA, suggesting that epigenetically regulated intermediate molecules could be involved in proMMP-1 expression regulation in these cells.

Introduction

Matrix metalloproteinases (MMPs) are zinc depending endopeptidases involved in the modeling and remodeling of tissue.

Correspondence to: Dr Aurélie Trussardi-Régnier, Unité MEDyC CNRS UMR 6237, UFR Pharmacie, 1 avenue du Maréchal Juin 51100 Reims, France
E-mail: aurelie.trussardi@univ-reims.fr

Key words: proMMP-1, epigenetics, trichostatin A, 5-azadC

The degradation of Extra Cellular Matrix (ECM) macromolecules such as collagens, fibronectin, laminin, and proteoglycans by MMPs plays a crucial role in physiological and pathological processes (1). MMP-1 (collagenase-1) is the most ubiquitous interstitial collagenase and is able to initiate the breakdown of types I, II and III collagens (2,3). Matrix modeling by MMP-1 is considerably implicated in embryonic development, tissue morphogenesis and wound repair (4). Furthermore, MMP-1 overexpression is associated with several pathological conditions such as irreversible degradation of cartilage, tendon and bone in arthritis, and tumor invasion and metastasis (5-7). Transcriptional activation of *proMMP-1* has been extensively studied, whereas the mechanisms involved in its expression remain largely unclear (7,8).

Accumulating evidence highlights the important role of epigenetic mechanisms in the regulation of various genes that determine the biologic behavior of cells (9-11). DNA methylation and post-translational modifications of histones appear as two of the main mechanisms of chromatin remodeling regulating the accessibility of DNA to transcriptional machinery (12). DNA methylation/demethylation, which involves DNA methyltransferases (DNMTs) and demethylases, occurs on the C5 of cytosine in CpG islands often located in or near the promoter region of approximately 50% of genes. In malignancies, DNA methylation is frequently dysregulated. By interfering with the transcription initiation, the methylation of CpG islands inhibits gene expression and then represses suppressor tumor genes (13). The covalent modifications of the nucleosome core histones H3, H4, H2A and H2B consist in the addition on their N terminal tails of various groups (methyl, acetyl, phosphoryl, ubiquitin, etc). The most characterized modification is the acetylation/deacetylation catalyzed by the Histone Acetyl Transferases (HATs) and the Histone Deacetylases (HDACs). The transfer of acetyl group modifies the affinity between DNA and histones and then modulates chromatin condensation. Thereby, acetylation of histones may induce the activation of gene transcription (14). Furthermore, there is substantial evidence that a key link exists between cytosine methylation and post-translational modification of histones (15-17).

It has been established that the *proMMP-1* gene expression requires chromatin remodeling in part via histone post-translational modifications (18-22). A previous study showed that *proMMP-1* gene expression induced by TPA in T98G cells involved a dynamic and ordered recruitment of enzymes

allowing H3/H4 acetylation, H3K4 di- and trimethylation, H3S10 phosphorylation and chromatin opening (22). Furthermore, it was shown that the production of MMP-1, in rheumatoid arthritis, is mediated by small ubiquitin-like modifier (SUMO) which triggers HDAC4 allowing histone hyperacetylation and gene expression (20,21). However, the potential role of DNA methylation in the regulation of *proMMP-1* expression remained unclear.

The aim of this study was therefore to analyze the effects of the inhibitor of DNA methylation 5-aza-2'-deoxycytidine (5-azadC) and the Histone Deacetylase Inhibitor (HDI) trichostatin A (TSA) on the proMMP-1 expression, both at the mRNA and protein levels. By using nuclear texture image, the relationships between higher-order chromatin supra-organization and *proMMP-1* gene expression were also analyzed.

Materials and methods

Cell culture and treatment. Human fibrosarcoma HT1080 cells (ATCC, CCL-121) were grown at 37°C in RMPI medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified 95% air, 5% CO₂ atmosphere. Before stimulation, cells were washed twice with PBS, and then incubated in serum-free medium for 24 h. Cells were treated or not 2 or 5 µM of 5-aza-2'-deoxycytidine (5-azadC) and/or 100 ng/ml of trichostatin A (TSA) and/or 10 µg/ml of cycloheximide (CHX) for the indicated times.

ProMMP-1 gene expression analysis. After stimulation, cells were washed twice in ice-cold PBS and total RNAs were isolated using TRIzol reagent (Invitrogen). For quantitative RT-PCR, 1 µg of RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen), and each cDNA sample was analyzed by real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) on the LightCycler system (Roche Diagnostics). *ProMMP-1* transcripts were amplified using the following primers: *proMMP-1* forward 5'-GAGCAAACACATCTGACCTACAGGA-3'; *proMMP-1* reverse 5'-TTGTCCCGATGATCTCCCCTGACA-3'. Relative quantification was performed by using the housekeeping gene β_2 macroglobulin as a reference: β_2m forward 5'-ATCTTCAAACCTCCATGATG-3'; β_2m reverse 5'-ACCCCCACTGAAAAAGATGA-3'.

ProMMP-1 Western blot analysis. Cells culture media were harvested and concentrated using Amicon Ultra centrifugal filters (Millipore). Total proteins (35 µg) were heated 5 min at 100°C in Laemmli sample buffer, separated on 10% SDS PAGE under reducing conditions and transferred to PVDF membrane by using I-Blot system (Invitrogen). The membranes were placed in blocking solution [5% (w/v) Blotto in Tris-Buffered-Saline/Tween 20] for 2 h at room temperature and incubated overnight at 4°C with anti-MMP-1 (Mouse, Ab-1, Calbiochem/Merck, 1:1000) or anti- β -actin (Mouse, clone AC-15, Sigma Aldrich, 1:10000) primary antibodies. Proteins were revealed using a peroxidase-conjugated secondary antibody (Goat anti-mouse IgG, Millipore, 1:10000) and the ECL Plus detection kit (Amersham).

Histone extraction and immunoblotting. Histones were acid extracted with HCl and precipitated with acetone. Total histones (15 µg) were separated on 15% SDS PAGE and transferred to PVDF membrane. The membranes were blocked during 2 h and incubated overnight with a primary antibody against acetylated total histone H4 (Rabbit, 06-866, Upstate, 1:2,000) and a peroxidase-conjugated anti rabbit secondary antibody (GE Healthcare, 1:100,000).

Image cytometry. Cells were grown and treated with TSA in LabTek chambered slides (Nunc). Cells were air-dried and fixed in alcohol-formalin mixture (95% ethanol, 3% formaldehyde in saline 3:1). After 5N HCl hydrolysis, slides were stained by the Feulgen method. Image cytometry was performed as described with an image analysis system (SAMBA 2005, Samba Technologies) coupled to a color 3CCD camera (XC-007P, Sony Corp. Japan) and a microscope (Axioscop, Karl Zeiss) (23). G₀/G₁ nuclei were extracted as previously reported (23). Nine texture parameters were computed from G₀/G₁ nuclei after reduction to 16 gray levels by linear rescaling. Four features were calculated on the gray levels co-occurrence matrix: local mean of gray levels (LM), energy (E), entropy (ENT), and inertia (I). Five parameters were calculated on the run-length matrix: short run-length emphasis (SRE), long run-length emphasis (LRE), gray level distribution (GLD), run-length distribution (RLD) and run-length percentage (RPC). The distribution, mean, and SD of the nuclear parameters were calculated for each cell population.

In order to perform multiple cell groups comparisons, a deviation index was calculated, by computing, for each parameter measured in a given nucleus, the difference in value to the feature value observed in control untreated nuclei (24). These differences are then standardized by dividing them by the corresponding standard deviations in the control cells data sets. Significance of the differences between indexes values was estimated by t-test after Bonferroni correction for multiple variables. Another data reduction method is computing an average nuclear abnormality index (NAI) which was calculated for each cell line as the arithmetic mean of the absolute values of the deviation indexes (z-scores) observed overall included features for all nuclei in a data set (24,25).

Statistical analysis. All experiments were performed in triplicate. Results are expressed as mean \pm SEM. Statistical analysis was realized using Kruskal-Wallis test except otherwise specified. The results were considered significantly different when $p < 0.05$.

Results

Epigenetic modulation of proMMP-1 gene expression. In order to know whether DNA methylation mechanisms could be involved in *proMMP-1* gene expression, fibrosarcoma HT1080 cells were treated with the DNA methylation inhibitor 5-azadC (2 or 5 µM) and/or the Histone Deacetylase Inhibitor (HDI) TSA (100 ng/ml) for various times. Levels of endogenous *proMMP-1* mRNA were monitored by real-time RT-PCR. Compared to control cells, TSA induced histone H4 acetylation after 0.5 h of treatment but did not modulate *proMMP-1* mRNA levels (Fig. 1A and B). After 48 h of treatment with 2 or

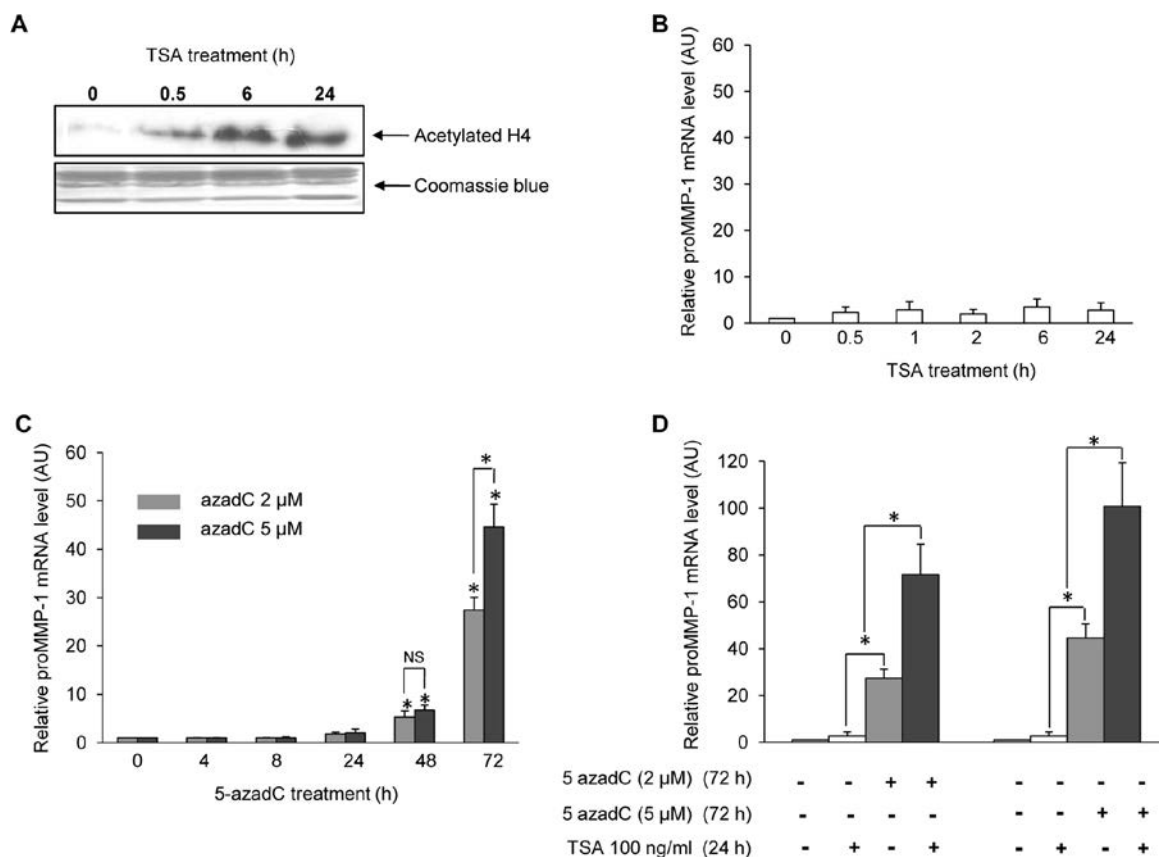


Figure 1. Effects of 5-azadC and TSA on *proMMP-1* gene expression. HT1080 cells were treated or not with TSA (100 ng/ml), 5-azadC (2 or 5 μ M), or both drugs for various times as indicated on the figures. (A) Histones were acid extracted and Western blotting using total acetylated H4 histone antibody was performed. (B-D) Levels of endogenous mRNA were monitored by real-time RT-PCR. β_2 microglobulin (β_2m) was used as internal control. Results are expressed as the mean \pm SEM of normalized ratio *proMMP-1*/ β_2m . * p <0.05 (Kruskal Wallis statistical test).

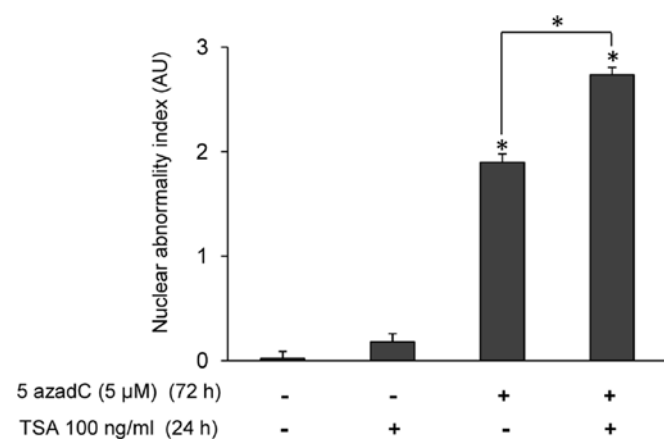


Figure 2. Effects of 5-azadC and TSA on chromatin supraorganization. HT1080 cells were treated or not with TSA (100 ng/ml 24 h) and/or 5-azadC (5 μ M 72 h). After Feulgen staining, 9 nuclear texture features were analyzed on G_0/G_1 nuclei using image cytometry. Results are expressed as nuclear abnormality index. * p <0.05 (t-test after Bonferonni correction).

5 μ M of 5-azadC, *proMMP-1* gene expression was increased (6-fold increased, p <0.05). *ProMMP-1* expression was further increased after 72 h of 5-azadC treatment (30-fold increased with 2 μ M p <0.05, 45-fold increased with 5 μ M p <0.05) (Fig. 1C). When cells were simultaneously treated with TSA

(100 ng/ml 24 h) and 5-azadC (2 or 5 μ M 72 h), *proMMP-1* was strongly expressed (70-fold increased with 2 μ M, 100-fold increased with 5 μ M) compared to 5-azadC alone (Fig. 1D).

Nuclear texture. To examine the effects of these drugs on nuclear phenotype, image cytometry was performed on G_0/G_1 nuclei of HT1080 cells treated by TSA (100 ng/ml 24 h) and/or 5-azadC (5 μ M 72 h). As shown in Fig. 2, these treatments induced significant chromatin higher-order organization changes, as evaluated by NAI computing. Moreover, simultaneous treatments with 5-azadC and TSA resulted in a synergistic effect on nuclear phenotypic alterations. In 5-azadC treated cells, analysis of individual deviation indexes (Table I) revealed that these changes corresponded to a global chromatin decondensation, as evidenced by a decrease in LM, with a less compact (increase in E, decreases in ENT and I) and more homogeneously and finely distributed chromatin (decreases in SRE and RPC, increases in LRE and GLD). The intensity of these changes was further increased by TSA addition. On the other hand, TSA alone induced a very slight but significant decompaction of chromatin (increase in E) which appeared containing reduced heterochromatin areas (increase in LRE, decrease in RPC).

Analysis of *proMMP-1* secretion. To correlate gene expression with protein secretion after treatment by epigenetic modulators, Western blot analysis was performed on cell conditioned media.

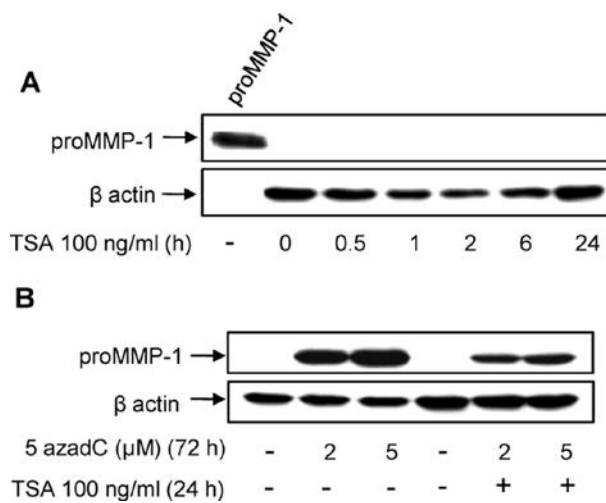


Figure 3. Effects of 5-azadC and TSA on proMMP-1 secretion. HT1080 cells were treated or not with TSA and/or 5-azadC as indicated on the figures. (A and B) Western blot analyses of cell concentrated conditioned media: 35 μ g of total proteins were analyzed. Panel A (lane 1), recombinant proMMP-1 was used as positive control.

Table I. Values of deviation indexes in 5-azadC and TSA treated HT1080 cell nuclei.

Parameters	Treatments		
	TSA	5-azadC	5-azadC + TSA
LM	-0.09±0.06	-1.94±0.03 ^a	-2.17±0.02 ^{a,b}
E	0.21±0.05	2.81±0.08 ^a	4.36±0.13 ^{a,b}
ENT	-0.13±0.05	-2.19±0.04 ^a	-2.84±0.04 ^{a,b}
I	-0.14±0.06	-1.26±0.04 ^a	-1.68±0.02 ^{a,b}
SRE	-0.14±0.06	-1.42±0.02 ^a	-1.96±0.02 ^{a,b}
LRE	0.46±0.06 ^a	1.84±0.05 ^a	3.74±0.10 ^{a,b}
GLD	0.02±0.05	2.64±0.06 ^a	3.64±0.08 ^{a,b}
RLD	-0.13±0.06	-1.40±0.02 ^a	-1.90±0.02 ^{a,b}
RPC	-0.29±0.05 ^a	-1.58±0.03 ^a	-2.34±0.03 ^{a,b}

Values are expressed as mean \pm SEM. ^a $p < 0.05$ as compared to untreated cells (t-test after Bonferroni correction). ^b $p < 0.05$ as compared to 5-azadC alone (t-test after Bonferroni correction).

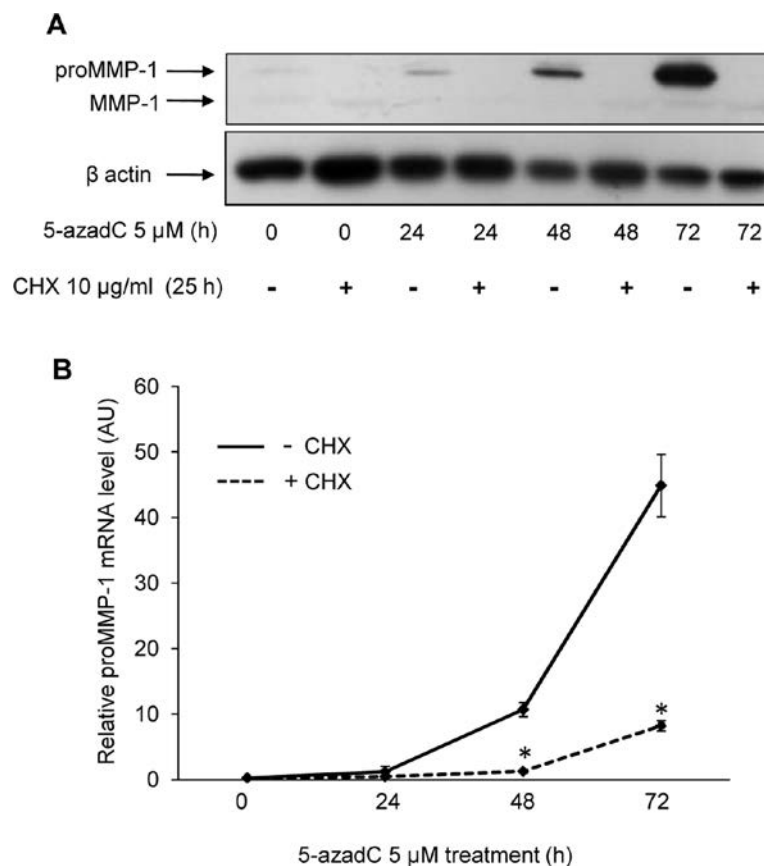


Figure 4. Effect of protein neosynthesis inhibition on proMMP-1 mRNA and protein levels. HT1080 cells were treated or not with 5-azadC and/or CHX as indicated in the figures. (A) Western blot analysis of cell-concentrated conditioned media: 35 μ g of total proteins were analyzed. (B) Real-time RT-PCR of *proMMP-1* mRNA. β_2 microglobulin (β_2m) was used as internal control. Results are expressed as the mean \pm SEM of normalized ratio *proMMP-1*/ β_2m . ^a $p < 0.05$ as compared to CHX untreated cells values (Kruskal Wallis statistical test).

The results show that TSA alone did not induce proMMP-1 secretion (Fig. 3A). 5-azadC or 5-azadC and TSA induced proMMP-1 secretion in supernatants (Fig. 3B). However, the association of both drugs induced a weaker secretion than 5-azadC alone.

Indirect epigenetic regulation of proMMP-1 expression. Despite its induction by 5-azadC, *proMMP-1* gene does not contain CpG islands. In order to investigate if other intermediate molecules could be involved in the regulation of proMMP-1

expression, the neosynthesis of proteins was blocked with cycloheximide (CHX). HT1080 were treated or not with 5-azadC (5 μ M 72 h) and CHX (10 μ g/ml 25 h). CHX totally abolished proMMP-1 secretion induced by 5-azadC, thus confirming the efficiency of the CHX treatment (Fig. 4A), and reduced strongly *proMMP-1* mRNA levels up to 72 h (Fig. 4B).

Discussion

In most tumor cells, *proMMP-1* mRNA levels are very low but can be induced by a wide variety of stimuli (7,8,26-28). For instance, interleukin-1 β , UV radiations or phorbol esters, increase *proMMP-1* gene expression through histone post-translational modifications (22,29). In this study we observed that accumulation of hyperacetylated histones is not a sufficient trigger to enhance either *proMMP-1* gene expression or chromatin global decondensation in HT1080 fibrosarcoma cells. Similarly, it was previously reported that an increase in histone acetylation level, induced by HDI treatment, did not seem to have any effect on unstimulated levels of *MMPs* whereas it could enhance already stimulated *MMP* production (30). This suggests that HDI effects could target the signaling pathways induced by exogenous stimuli (e.g., inflammatory cytokines, growth factors and phorbol esters) rather than the *proMMP-1* gene itself.

These histone post-translational modifications are only one possible mechanism and accumulating evidence supports epigenetic cross-talk between DNA methylation and histone modifications in the regulation of gene expression (31). During this cross-talk, DNA methylation appears to be the initial signal that triggers events leading to non-permissive chromatin. In HT1080 cells, we showed that a treatment with the DNA demethylating agent 5-azadC induced a global chromatin decondensation and an increase in *proMMP-1* expression. Interestingly, this gene promoter, unlike other *MMPs*, does not appear to contain CpG islands (32), suggesting that 5-azadC effects occur via either demethylation of other genes, or different mechanisms (33). Moreover, histone hyperacetylation by TSA activates *proMMP-1* expression only after chromatin demethylation and both epigenetic mechanisms display synergistic effects on gene expression and nuclear architecture alteration. These ordered re-expression and synergy, involving DNMTs, MBDs and HDACs (16,34), have been reported with other genes (35-38), but not concerning the *proMMP-1* gene.

The simultaneous treatment with TSA increased *proMMP-1* mRNA levels, but surprisingly, decreased MMP-1 protein secretion. Such a TSA-induced decrease in MMP-1 secretion was also observed in mesenchymal cells treated with IL-1 β (30). Post-transcriptional regulatory processes including mRNA stability, protein translational efficiency, and microRNA-based mechanisms have been recently described as modulators of *MMPs* expression (39). Several non-histone proteins have been identified as acetylation targets and hyperacetylation of these proteins could play important roles in the mRNA stability regulation, protein localization and degradation, and protein-protein and protein-DNA interactions. HDI treatment may thus destabilize mRNA and decrease the resulting protein level, as reported here for *proMMP-1* (40,41). Indeed the *MMP* mRNA transcripts harbor specific sequences in their 5' or 3' untranslated regions

(UTRs) which are potential targets of regulatory proteins involved in mRNA stability (39). On the other hand, HDI treatment may activate miRNA expression and modulate tumor cell invasiveness (42-45). For instance, miR22 was shown to be upregulated by TSA treatment but not by 5-azadC (42). Target sequence analyses revealed that this TSA-inducible miRNA could target and potentially repress *proMMP-1* expression. Therefore it could play, as many other miRNAs, a significant role in the regulation of *proMMP-1* expression (43).

Finally, inhibition of protein neosynthesis abolishes the 5-azadC-induced *proMMP-1* mRNA increase, a phenomenon not observed for *MMP-2* or *MMP-9* genes (data not shown). These data suggest that some intermediary molecules could be involved in the regulation of *proMMP-1* gene expression and that their production could be specifically triggered by DNA demethylation. For instance, *proMMP-1* expression could be mediated by autocrine mechanisms (46) involving cytokines and growth factors (7) whose expression in HT1080 cells could be upregulated by 5-azadC.

Identification of such *proMMP-1* inducers could therefore shed new light on the impact of epigenetic regulation on invasive properties of cancer cells.

Acknowledgements

This study was supported by grants from the Comité Départemental de l'Aube de la Ligue Nationale Contre le Cancer.

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PUBLICATION N°2

Epigenetically-induced changes in nuclear textural patterns and gelatinase expression in human fibrosarcoma cells.

M. Poplineau, C. Doliwa, M. Schnekenburger, F. Antonicelli, M. Diederich, A. Trussardi-Régnier and J. Dufer.

Article en révision pour publication dans Cell Proliferation

Présentation de la PUBLICATION N°2

Effets de modulateurs épigénétiques sur l'architecture nucléaire et l'expression de gélatinases dans la lignée cellulaire de fibrosarcome humain HT1080

Contexte

Dans la mesure où les modifications épigénétiques entraînent des remaniements de la structure chromatinienne, leur impact sur la texture nucléaire pourrait refléter des changements dans le fonctionnement cellulaire. Les mesures des différents paramètres de texture nucléaire sont des éléments importants de diagnostic qui, entre autres, ont déjà permis la discrimination entre des cellules saines et des cellules cancéreuses, et de classer ces cellules cancéreuses en fonction du stade de la progression tumorale. De plus, l'analyse de la texture nucléaire par cytométrie en analyse d'images a révélé son potentiel pour la caractérisation de changements cellulaires induits par des agents toxiques (Mello, 2003) ou thérapeutiques (Poplineau, 2011b), phénomènes difficilement observables par microscopie conventionnelle. L'établissement d'une « signature nucléaire » associée à l'expression de MMP-2 et -9, gélatinases fortement impliqués dans l'invasion tumorale, pourrait permettre d'obtenir des informations pertinentes sur le pronostic et le diagnostic de cancers. De ce fait, nous avons analysé l'impact de modulateurs épigénétiques sur la texture chromatinienne que nous avons tenté de corrélérer à l'expression de MMP-2 et -9, et au profil de méthylation de l'ADN dans la lignée de fibrosarcome humain HT1080. Ces travaux ont fait l'objet de la **PUBLICATION N°2**.

Matériel et Méthodes

Dans cette étude, des cellules de la lignée cellulaire HT1080 ont été traitées par la 5-azadC et/ou par la TSA. L'expression génique de MMP-2 et -9 a été analysée par RT-PCR en temps réel. L'effet de la 5-azadC sur le degré de méthylation global de l'ADN a été analysé par MSRA (*methylation-sensitive restriction assay*). Le profil de méthylation des promoteurs

de MMP-2 et -9 a été étudié par MSP, après conversion au bisulfite de l'ADN génomique (*methylation-specific PCR*). De plus, une analyse de la texture nucléaire par cytométrie en analyse d'images a été réalisée, en vue d'établir une corrélation entre (1) les profils d'expression des gélatinases, (2) les profils de méthylation global de l'ADN et spécifiques des promoteurs de MMP-2 et -9, et (3) de possibles modifications de l'architecture nucléaire. Des informations supplémentaires concernant la technique de MSRA, le principe de la conversion au bisulfite et la technique de MSP sont développées respectivement en ANNEXES 2, 3 et 4.

Résultats

Dans cette publication, nous montrons qu'un traitement par la 5-azadC entraîne des variations significatives de la texture chromatinienne associées à une déméthylation globale de l'ADN (ANNEXE 2). De plus la 5-azadC induit une augmentation des niveaux d'ARNm de MMP-2 et -9. L'analyse du profil de méthylation par MSP des promoteurs de ces gènes révèle que (1) les promoteurs de ces gènes sont partiellement méthylés dans les cellules HT1080 et que (2) la 5-azadC induit une déméthylation d'environ 50 % du promoteur du gène de la MMP-9 tandis que le profil de méthylation du promoteur du gène de la MMP-2 est relativement peu affecté par un tel traitement. Par ailleurs la TSA, à elle seule, n'est pas capable de moduler les paramètres de texture nucléaire et l'expression des gélatinases. Cependant, la TSA présente des effets différentiels sur l'expression des gélatinases lorsqu'elle est ajoutée sur des cellules HT1080 préalablement traitées par la 5-azadC : (1) elle n'affecte pas les niveaux d'ARNm de MMP-2 induits par la 5-azadC et (2) elle diminue les niveaux d'ARNm de MMP-9 induits par la 5-azadC.

Conclusion

L'analyse des paramètres de texture nucléaire, par cytométrie en analyse d'images, permet de définir une « signature nucléaire » au sein des cellules HT1080. Cette signature varie en fonction du profil de méthylation de l'ADN et pourrait permettre l'établissement d'une relation entre l'organisation structurale de la chromatine et le niveau d'expression des gélatinase



Epigenetically-induced changes in nuclear textural patterns and gelatinase expression in human fibrosarcoma cells

Journal:	<i>Cell Proliferation</i>
Manuscript ID:	Cellprol-1139-12.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Poplineau, Mathilde; University of Reims, UFR Pharmacy Doliwa, Christelle; University of Reims, UFR Pharmacy Schnekenburger, Michael; Hôpital Kirchberg, Laboratoire de Biologie Moléculaire du Cancer Antonicelli, Frank; University of Reims, UFR Medecine Diederich, Marc; Hôpital Kirchberg, Laboratoire de Biologie Moléculaire du Cancer Trussardi-Regnier, Aurelie; University of Reims, UFR Pharmacy Dufer, Jean; University of Reims, UFR Pharmacy
Keywords:	chromatin texture, DNA methylation, 5-aza-2'-deoxycytidine, trichostatin A, MMP-2, MMP-9

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2 **Epigenetically-induced changes in nuclear textural patterns and gelatinase expression in**
3 **human fibrosarcoma cells.**

4 M. Poplineau*, C. Doliwa*, M. Schnekenburger†, F. Antonicelli‡, M. Diederich†, A.
5 Trussardi-Régnier* and J. Dufer*

6 *Unité MEDyC, URCA-CNRS FRE 3481, SFR Cap-Santé, Facultés de Pharmacie et de
7 Médecine, Université de Reims, Reims, France,

8 †Laboratoire de Biologie Moléculaire du Cancer, Hôpital Kirchberg, Luxembourg,
9 Luxembourg,

10 ‡Laboratoire de Dermatologie, UFR Médecine, Reims, France

11

12 Correspondance: Prof. J. Dufer, Unité MEDyC, URCA-CNRS FRE 3481, Faculté de
13 Pharmacie, 1 avenue du Maréchal Juin, 51096 Reims, France.

14 Tel. : +33 326 918 015 ; Fax : +33 326 913 550 ;

15 E-mail : jean.dufer@univ-reims.fr

16

17 Short title: Nuclear texture and gelatinase expression

18

19

1 **Abstract**

2 *Objective:* Chromatin texture patterns of tumour cell nuclei can serve as cancer biomarkers,
3 either to define diagnostic classifications or to obtain relevant prognostic information in a large
4 number of human tumours. Epigenetic mechanisms, mainly DNA methylation and histone
5 post- translational modifications, have been shown to influence chromatin packing states, and
6 therefore nuclear texture. The aim of this study was to analyse the effects of these two
7 mechanisms on chromatin texture and gelatinase expression in human fibrosarcoma tumour
8 cells.

9 *Materials and methods:* We investigated the effects of the DNA demethylating agent 5-aza-2'-
10 deoxycytidine (5-azadC) and the histone deacetylase inhibitor trichostatin A (TSA) on the
11 nuclear textural characteristics of human HT1080 fibrosarcoma cells, evaluated by image
12 cytometry, and the expression of the gelatinases MMP-2 and MMP-9, two metalloproteinases
13 implicated in cancer progression and metastasis.

14 *Results:* 5-azadC induced significant variations in chromatin higher-order organization,
15 particularly a chromatin decondensation, associated with a decrease in global DNA
16 methylation, concomitantly to an increase in MMP-9, and to a lesser extent, MMP-2
17 expressions. TSA alone did not display any effect on HT1080 cells but exhibited differential
18 activity when added to cells treated with 5-azadC. When treated by both drugs, nuclei showed
19 increased texture abnormalities. In this setting, a decrease of MMP-9 expression was observed,
20 whereas MMP-2 expression remained unaffected.

21 *Conclusions:* These data show that the hypomethylating drug 5-azadC and the histone
22 deacetylase inhibitor TSA were able to induce modulations in higher-order chromatin
23 organisation and in gelatinase expressions in the human HT1080 fibrosarcoma cells.

24

1 **Introduction**

2 During the past decade, computerized microscopic image analysis has been widely used in
3 pathology and cytology, either for the identification of benign and malignant tumour cells,
4 the evaluation of subtle morphologic cellular changes, which cannot be recognized by
5 conventional microscopy, *i.e.* malignancy associated changes (MACs), or the detection of
6 relevant tumour prognostic markers (1,2). Moreover, this technique proved efficient to
7 examine chromatin remodelling induced by various drugs and therapeutic agents (3–5). This
8 chromatin remodelling appear controlled, at least partly, by two key epigenetic mechanisms,
9 DNA methylation and post-translational modifications of histones, which both regulate the
10 accessibility of the transcriptional machinery to DNA (6). They could result in
11 modifications of chromatin texture as evaluated by image cytometry. For instance, DNA
12 methylation level has been associated with changes in the distribution of heterochromatin
13 regions (7) and histone acetylation with interphase chromatin decondensation (8,9).
14 Numerous data now strengthen the major role of these epigenetic mechanisms in the
15 regulation of expression of various genes, particularly in neoplastic diseases (10). The DNA
16 methylation/demethylation process of cytosines of gene promoters is frequently deregulated
17 in several malignancies, where the methylation of CpG islands inhibits gene expression and
18 can repress tumour suppressor genes (11,12). Post-translational modifications of the
19 nucleosome core histones H3, H4, H2A, and H2B consist in the addition on their N-terminal
20 tails of different chemical groups. One of the most characterized modifications is the
21 acetylation/deacetylation process mediated by the enzymes Histone Acetyl Transferases
22 (HATs) and Histone Deacetylases (HDACs). These histone N-terminal tail changes modify
23 the affinity between DNA and histones, and then modulate chromatin condensation.
24 Thereby, acetylation of histones may induce the activation of gene transcription (13).

1 In cancer, matrix metalloproteinases (MMPs) are involved in the formation of a
2 microenvironment that contributes to tumour cell progression (14). Among these MMPs,
3 gelatinases (Gelatinase A or MMP-2 and Gelatinase B or MMP-9) play a major role mainly by
4 controlling the activity of molecules that target specific receptors regulating cell growth,
5 migration, inflammation and angiogenesis (15). MMP-2 and -9 expressions are dysregulated in
6 a variety of malignancies, and an increase in their expression and activity are generally
7 associated with a poor prognosis (16,17). This overexpression correlates with an increased
8 invasive potential of the tumours, indicating the crucial role of these MMPs in the metastatic
9 processes (14). MMP-2 and -9 activities are regulated by cytokines, including interleukins,
10 growth factors, and tumour necrosis factor- α at different levels, including gene transcription
11 (14). Besides classical transcription factors such as AP-1, recent studies demonstrated the
12 involvement of other transcriptional regulators. For instance, *MMP-2* has been described as a
13 target for FoxO3 (18) and Runx2 (19), whereas *MMP-9* could be regulated by Reck (20) and
14 SMYD3 (21). Epigenetic mechanisms such as DNA methylation, histone acetylation or
15 methylation at the *MMP* promoter could also be implicated in this regulation (22). Furthermore,
16 there is evidence of a crosstalk between DNA methylation and histone post-translational
17 modifications, where histone modifications provide a labile transcriptional repression and DNA
18 methylation results in a highly stable gene silencing (23,24). Therefore, in this study, we
19 analysed the effects of the hypomethylating agent 5-aza-2'-deoxycytidine (5-azadC) and the
20 Histone Deacetylase Inhibitor (HDI) trichostatin A (TSA) on the higher-order chromatin
21 organisation and the expression of the gelatinase genes in the invasive HT180 fibrosarcoma
22 tumour cell line.

1 **Material and methods**

2 *Cell culture and treatments*

3 Highly invasive human fibrosarcoma HT1080 cells (ATCC, CCL-121) were grown at 37°C in
4 RPMI medium supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and 100
5 µg/ml streptomycin. All cultures were maintained under a fully humidified atmosphere of 95%
6 air/5% CO₂ at 37°C. Cultures were passaged twice weekly. Exponentially growing cells were
7 used in all experiments. Cell viability was assessed by the ability to exclude trypan blue.
8 Before experiments, cells were washed twice with PBS, and then incubated in serum-free
9 medium for 24 h. Cells were treated or not with up to 5 µM of 5-aza-2'-deoxycytidine (5-
10 azadC, Sigma-Aldrich, France) and/or 100 ng/ml of trichostatin A (TSA, Sigma-Aldrich,
11 France) for indicated times.

12 *Image cytometry*

13 Cells were grown and treated with 5-azadC and/or TSA in LabTek chambered slides (Nunc,
14 Denmark). Nuclear texture analysis was performed as reported (8). In brief, cells were air-
15 dried, fixed in alcohol-formalin mixture (95% ethanol, 3% formaldehyde in saline; 3:1) for 10
16 min. After hydrolysis with 5N HCl for 30 min, cells were stained by the Feulgen method.
17 Image cytometry was performed with an image analysis system (SAMBA 2005, Samba
18 Technologies, France) coupled to a colour 3CCD camera (XC-007P, Sony Corp. Japan) and a
19 microscope (Axioscop, Karl Zeiss, Germany). As nuclear textural features observed by image
20 cytometry are closely related to the location of a given cell in the cell cycle, comparisons of
21 textures should therefore be performed on cells selected within a specific cycle phase. For this
22 purpose, G₀/G₁ nuclei were isolated on the basis of their Integrated Optical Density value,
23 which corresponds to their DNA content, and a minimum of 200 G₀/G₁ nuclei were extracted
24 for each cell population as previously reported (8). Nine texture parameters were computed

1 from these nuclei. Four features were calculated on the gray levels co-occurrence matrix: local
2 mean of gray levels (LM), energy (E), entropy (ENT), and inertia (I). Five parameters were
3 calculated on the run-length matrix: short run-length emphasis (SRE), long run-length
4 emphasis (LRE), gray level distribution (GLD), run-length distribution (RLD) and run-length
5 percentage (RPC) (25). It has been previously reported that the variations of these parameters
6 could be related to chromatin condensation, distribution or organisation (25,26).

7 *MMP1, -2 and -9 gene expression analyses*

8 After drug treatments, cells were washed twice in ice-cold PBS and total RNAs were isolated
9 using NucleoSpin RNA II kit (Macherey Nagel, France). For quantitative RT-PCR, 1 µg of
10 RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, France), and
11 each cDNA sample was analyzed by real-time PCR using Platinum SYBR Green qPCR
12 SuperMix-UDG kit (Invitrogen, France) on the LightCycler system (Roche Diagnostics,
13 France). *MMP-1*, *MMP-2* and *MMP-9* transcripts were amplified using the primers described
14 in Table 1. *MMP-1* was used as a control gene as we showed previously that this gene
15 expression was induced by 5-azadC (27). Relative quantification was performed by using the
16 housekeeping gene *β2microglobulin* as internal control.

17 *Global DNA methylation analysis*

18 Global DNA methylation level after 5-azadC treatments was analyzed by Methylation-
19 Sensitive Restriction Assay (MSRA) as previously described (28). Briefly, 0.5 µg of genomic
20 DNA were incubated overnight at 37°C with 20 units of *HpaII* (methyl-sensitive) or *MspI*
21 (methyl-insensitive) restriction enzymes (New England BioLabs, UK). The next day, 10 units
22 of each enzyme were added for 2 more hours. The DNA digestion level was checked by
23 electrophoresis on 0.8% agarose gel. Electrophoretic images were analyzed with ImageJ
24 software (29).

1 *Methylation-specific PCR*

2 Methylation status of *MMP-2* and *MMP-9* promoters was determined by Methylation-Specific
3 PCR (MSP). Genomic DNA was isolated using the QIAamp DNA Mini kit (Qiagen, France).
4 0.5 µg of genomic DNA were bisulfite-converted using the EpiTect Bisulfite kit (Qiagen,
5 France) according to manufacturer's instructions. The bisulfite-modified DNA samples were
6 amplified by primers specific for both methylated and unmethylated sequences of the -224 to -
7 51 region of the *MMP-2* promoter, *or* the 3382 to 3488 region of the *MMP-9* gene (Table 1).
8 Primers were designed using the Methyl Primer Express software (Applied Biosystems). After
9 optimization using EpiTect PCR Control DNA kit (Qiagen, France), PCR was performed on 2
10 µl of converted DNA using the Hot-Start Taq polymerase (Qiagen, France). After
11 amplification, PCR products were analyzed on 12% polyacrylamide gel and revealed by
12 ethidium bromide and UV light.

13 *Statistical analysis*

14 For image cytometry data, the mean, standard deviation and range values of the nuclear
15 parameters were calculated for each cell population. Variability of parameter measurements in
16 each cell population was analysed by one-way analysis of variance. Significance of the
17 differences between parameter values was estimated by Kolmogorov-Smirnov test on
18 cumulated frequencies (30).

19 In order to perform comparisons between multiple cell groups, a deviation index (z-
20 score) was calculated, as previously reported (8). For each parameter measured in a given
21 nucleus, the differences in value to the feature value observed in control untreated nuclei were
22 computed and standardized by dividing them by the corresponding standard deviations in the
23 control cells data sets (31). An average nuclear abnormality index (NAI) was then calculated
24 for each cell line as the arithmetic mean of the absolute values of the deviation indexes
25 observed overall included features for all nuclei in a data set. This NAI represents a data

1 reduction technique that permits to take into account all z-scores that constitute the
2 nuclear textural signature (31). Significance of the differences between indexes values was
3 estimated by Student's t test.

4 Multivariate analysis was performed with the Samba software. Two main kinds of
5 multidimensional analyses were used: supervised classification by discriminant analysis and
6 non-supervised classification (32). The linear stepwise discriminant analysis was based on use
7 of the F-test to select variables. Its purpose was to select the textural features whose variations
8 characterise the different cell populations. This discriminant analysis could be completed by a
9 canonical analysis, which derives canonical variables (linear combinations of independent
10 variables). Significance of the differences between canonical variables values computed in the
11 various cell populations was estimated by Kolmogorov-Smirnov test on cumulated
12 frequencies. The unsupervised multidimensional partitioning (cluster analysis) was applied to
13 separate cell groups that differed in the distribution of the texture parameters. This analysis
14 successively looked for the most heterogeneously distributed feature, split the population into
15 subgroups, and fused the non significant subgroups. This process is iterated for all the
16 parameters and does not require any a priori knowledge about the different cell types
17 eventually to be found in the population. It preserves total objectivity about the status of a
18 given cell (33).

19 RT-PCR results were expressed as mean \pm SEM of at least 3 independent experiments.
20 Significance of the differences observed was analysed by Kruskal-Wallis test.

21 **Results**

22 *Nuclear texture and DNA methylation changes induced by 5-AZAdC*

23 Nine textural features were computed on each nuclear image. Values of these textural
24 parameters in control cells and cells treated with 5 μ M of 5-azadC for 72h appear on Table 2.

1 5-azadC-treated cells displayed significant variations as compared to control HT1080 cells: a
2 less compact chromatin (increase of E and decrease in LM, ENT, and I), with an increase of the
3 distance between isodensity points (decrease of SRE and increase of LRE), confirming a more
4 homogeneous distribution of the chromatin within the nucleus (increase of GLD measuring the
5 uniformity of distribution of optical densities, decrease of RLD and RPC). For all the analysed
6 parameters, the distribution in 5-azadC-treated cells and in control cells was significantly
7 different ($p < 0.05$). However, due to the great sensitivity of texture features, we looked at the
8 variability of their measurements in three separate control and treated cells groups. None of the
9 nine features displayed any significant variation between these groups (Table 3).

10 All textural parameters might be involved in the definition of a nuclear phenotype. The
11 features enabling optimal separation between control and 5-azadC-treated cells were then
12 selected by performing a linear discriminant analysis. The most discriminant features, ranked
13 according to their discriminatory power, appear in Table 4. Three features (GLD, LM, and
14 LRE) were predominant for the identification of 5-azadC-treated cells and adding new features
15 in the discriminant analysis did not increase classification accuracy. This accuracy at the first
16 step (87.7%) was highly significant. This analysis was followed by a canonical transformation
17 of data, resulting in the definition of canonical variables. Usually the first canonical variable
18 (CV1) is the most important as it always shows the most discriminant power between the
19 groups. The values of this canonical variable observed in cells treated with various
20 concentrations of 5-azadC appear on Figure 1. A significant increase of this CV1 could be
21 observed in cells treated with 1 μM of 5-azadC and this CV1 value is still enhanced with higher
22 concentrations of 5-azadC.

23 To overcome the apriori knowledge about the analysed cell populations (control or
24 treated), the changes observed in 5-azadC-treated cells could be objectively evaluated by
25 unsupervised classification of cells. All data from control and 5-azadC-treated cells were

1 merged into a single group of cells, which was then submitted to unsupervised
2 multidimensional partitioning. This method partitioned the cells in 2 significant subgroups, A
3 and B. The distribution of the 9 textural parameters in the 2 subgroups (Figure 2) showed the
4 same variations as those observed between control cells and cells treated with 5 μ M of 5-azadC
5 (Table 2). A discriminant analysis computed using A and B subsets as learning sets and the
6 constituting primary cell populations (control cells and cells treated with different
7 concentrations of 5-azadC) as test sets lead to the results displayed in Table 5. These data
8 showed that treatment of cells with increasing concentrations of 5-azadC corresponded to a
9 progressive increase in the number of “B-subset cells” in the respective cell populations. This
10 was a non-subjective evidence for a dose-related appearance of nuclear texture changes in
11 HT1080 cells treated with 5- azadC.

12 In order to compare these nuclear textural changes to modifications of DNA methylation
13 induced by 5-azadC, the level of global DNA methylation in the various cell populations was
14 estimated by MSRA. As shown on Figure 1, a strong decrease in DNA methylation (more than
15 60%) could be observed even with a low concentration (0.5 μ M) of 5-azadC in HT1080 cells.

16 *MMP-2 and -9 expressions induced by 5-AZAdC in HT1080 cells*

17 In order to assess whether these nuclear textural and DNA methylation changes could be
18 associated with modifications in gene expression, the expression of gelatinases (MMP-2 and
19 MMP-9) was evaluated in 5-azadC-treated HT1080 cells. Cells were incubated with 5-azadC (5
20 μ M) for various times, and levels of endogenous *MMP-2* and *MMP-9* mRNAs were monitored
21 by real-time RT-PCR. As shown in Figure 3a, compared to control cells, *MMP-2* gene
22 expression was significantly increased only after 72h of treatment with 5 μ M of 5-azadC (3-
23 fold increase). *MMP-9* expression was significantly increased after 24 h of 5-azadC treatment
24 and continued to increase up to 72h of treatment (8-fold increase).

25 MSP was then performed to determine whether these expression patterns were correlated

1 to changes in methylation of *MMP-2*, or *MMP-9* genes. As shown in Figure 3b, treatment
2 of HT1080 cells with 5 μ M of 5-azadC for 72h was associated with changes in *MMP-9* gene
3 methylation status. ImageJ analysis of these results showed that 5-azadC treatment induced an
4 increase in unmethylated (U) and a decrease in methylated (M) areas on MSP blots. The ratio
5 M/U+M was about 70% in control cells but 48% in 5-azadC-treated cells. Besides, *MMP-2*
6 MSP profile seemed to remain unchanged in 5-azadC-treated cells, with a ratio of 54% in
7 control and 51% in 5-azadC-treated cells.

8 *Differential modulation of 5-AZAdC-induced nuclear textural changes and MMP expression by* 9 *TSA*

10 In order to determine whether histone acetylation could influence the modifications induced by
11 5- azadC in HT1080 cells, a subsequent treatment by the histone deacetylase inhibitor TSA
12 (100 ng/mL for 24h) was applied to cells previously treated or not with 5-azadC. Nuclear
13 textural characteristics were evaluated on these cells as well as the expressions of *MMP-2* and -
14 *9*, and *MMP-1* as control. In order to compare the various treatments, textural changes were
15 expressed as Nuclear Abnormality Index (NAI). As shown in Figure 4a, TSA alone did not
16 induce any significant increase in NAI. When TSA was applied on cells treated previously with
17 5 μ M of 5-azadC, a significant increase in the NAI value could be observed (TSA-induced NAI
18 = 0.87 ± 0.08 , $p < 0.05$ as compared to cells treated with 5-azadC alone, Student's t test).
19 However, this enhancement occurred in cells treated previously with 5 μ M but not with 2 μ M
20 of 5-azadC. In this latter case, TSA induced only a slight NAI increase (TSA-induced NAI =
21 0.29 ± 0.06 , not significant as compared to cells treated with 5-azadC alone), similar to the one
22 observed in cells incubated with TSA alone (NAI = 0.27 ± 0.11). At the *MMP* gene level, TSA
23 alone did not display any effect on *MMP-2*, or *MMP-9* expressions (Figure 4b). When applied
24 on cells previously incubated with 5 μ M of 5-azadC, TSA induced a decrease in *MMP-9* gene
25 expression as compared to 5-azadC alone. On the other hand, this subsequent treatment with

1 TSA did not modulate *MMP-2* expression, but strongly increased *MMP-1* gene expression.

2 **Discussion**

3 Numerous studies have demonstrated the potential of chromatin texture analyses to the
4 identification, or follow-up of neoplastic processes. In particular, 2D analyses of Feulgen-
5 stained cells and tissues have been widely applied to the diagnosis or prognosis of tumours
6 (1,34–37) and have been used either to characterize tumour cells displaying specific properties,
7 such as drug resistance (32), or to evaluate drug effects on cancer cells (3–5). Other image
8 cytometry methods have been successfully applied to nuclear texture analysis, including 3D
9 analysis on either fluorescence confocal images or optical absorption tomographic images, or
10 low coherence interferometry on living cell specimens (38–40). However, the effect of the cell
11 cycle on nuclear texture is an important criterion which was not always considered in these
12 studies and, in this context, texture analysis of Feulgen-stained nuclei could offer an easy-to-
13 perform and efficient method. Recently, we compared data obtained by conventional 2D
14 texture analysis and Raman microspectroscopy on nuclei from Jurkat leukemic cells and both
15 techniques appeared able to discriminate between cells treated or not with different HDIs (41).
16 Regardless of the technique used, the functional significance of the variations in textural
17 features is sometimes unclear. For instance, changes in chromatin higher-order organisation
18 have been attributed to chromosomal abnormalities, transcription, or proliferation (42).
19 Moreover, the functional relevance of nuclear structure needed extensive studies on the
20 interplay between nuclear architecture and gene expression. Epigenetic abnormalities
21 frequently observed in cancer might also be implicated in these textural irregularities. In this
22 work, we showed that the DNA hypomethylating agent 5-azadC was able to induce significant
23 changes in chromatin higher-order organisation evaluated through image cytometry in HT1080
24 fibrosarcoma cells. Three features (GLD, LM, and LRE) appear discriminant in 5-azadC-
25 treated cells identification. Interestingly, these features concern both chromatin condensation

1 and distribution within the nucleus (26). Their variations suggest that cells treated with 5-azadC
2 display a decondensed, loosely packed and homogeneously distributed chromatin, and it has
3 been reported that 5-azadC drastically inhibit condensation in mammalian constitutive
4 heterochromatin (43). These variations appear to be linked to the 5-azadC treatment itself as the
5 variability within control or experimental groups appear quite low (Table 3). Indeed, the
6 presence of textural artefacts induced by the analytical method used cannot be entirely ruled
7 out. For instance, it has been shown that processing techniques (dewaxing, fixation) could
8 influence measurements of nuclear structure (44). However, fixation combining ethanol and
9 formaldehyde was successfully applied to the analysis of nuclear textural patterns linked to
10 cellular phenotypes (32,45,46) or to drug treatments (4,8). It could represent a good
11 compromise for optimal DNA staining after hydrolysis (47), therefore enabling G₀/G₁ nuclei
12 selection and convenient textural analysis of these nuclei (48), and gave rise to reproducible
13 results (Table 3). The first textural changes were observed after treatment with 1 μM of 5-
14 azadC whereas global DNA demethylation could be detected with a lower dose of the drug.
15 These data fit well with other reports indicating that 5-azadC concentration able to induce DNA
16 hypomethylation could be very low (49,50). Although chromatin higher-order changes induced
17 by 5-azadC were mainly related to its hypomethylating properties (51), the relative low
18 sensitivity of nuclear texture analysis could indicate that a significant concentration of this
19 drug, or some additional mechanisms would be needed for the induction of textural chromatin
20 alterations. In the same way, in promyelocytic leukaemia, texture analysis was not able to
21 detect chromatin alterations in leukemic cells from patients displaying methylation of specific
22 genes (52). In HT1080 cells, a treatment with TSA increased the intensity of the nuclear
23 changes (evaluated by NAI) induced by 5-azadC. It has been shown that panabinstat or TSA
24 acted in synergy with 5-azadC and that the combination of both drugs induced a marked
25 increase in histone acetylation (49,53). However, adding TSA on 5-azadC-treated HT1080 cells

1 induced a significant effect on nuclear texture, but only when cells were previously incubated
2 with the higher concentration of 5-azadC. This could suggest that texture analysis may only
3 detect TSA-induced changes in chromatin distribution patterns if cells have been sufficiently
4 sensitised through hypomethylation by 5-azadC. However, it must be stressed that these data
5 apply to G₀/G₁ HT1080 cells only, as it has been reported that the extent of TSA-induced
6 chromatin changes was both cell cycle and cell line dependent (54).

7 Significant changes in chromatin higher-order organization, evaluated through nuclear
8 texture, have already been reported as markers of tumour cell aggressiveness or metastatic
9 potential (34,55). Since gelatinases are MMPs implicated in tumour metastasis processes, we
10 looked at their expression in HT1080 fibrosarcoma cells treated with 5-azadC and/or TSA.
11 Treatment with 5-azadC induced an increase in *MMP-9* expression, and to a lesser extent
12 *MMP-2* expression. Similarly, we previously observed that 5-azadC enhanced strongly *MMP1*
13 expression in these cells (27). However, comparisons of data issued from nuclear texture
14 analysis on one hand, and gene expression analysis by RT-PCR on the other hand could be
15 difficult. Indeed the first technique constitutes a cell-by-cell type method, whereas RT-PCR in
16 this study was applied to RNA pooled from large numbers of cells, without selecting G₀/G₁
17 cells. Therefore, further analyses might compare texture from isolated living cells with single-
18 cell gene expression profiling (56). Induction of *MMP-2* and *MMP-9* genes by 5-azadC has
19 already been reported in breast cancer (57), pancreatic cells (58), or lymphomas (59). In our
20 results, this expression was associated with a significant modulation of DNA methylation of
21 *MMP-9* gene but not of *MMP-2* gene. Large CpG islands (4.65 kb) are present in the *MMP-9*
22 gene-coding region, which correlates with a key role of DNA methylation in the regulation of
23 its expression (22). CpG islands size is more limited (1.5 kb) in *MMP-2* gene and knockout of
24 DNMT1 and DNMT3b in mice was unable to induce *MMP-2* expression whereas it could
25 induce *MMP-3* (60). This could indicate some level of resistance of *MMP-2* to

1 hypomethylating drugs, as resistance to drug-induced hypomethylation is mostly
2 overcome in DNMT1; DNMT3B double knockout cells (61). On the other hand, TSA alone did
3 not display any effect on these genes. Similar results have already been observed for *MMP-1* or
4 *MMP-2* in HT1080 cells (27,62). Interestingly, further treatment with TSA associated to 5-
5 azadC induced different effects on *MMP* genes. TSA was inactive on *MMP-2*, while it
6 decreased the 5-azadC induction of *MMP-9*. This was different from the already described
7 enhancement of *MMP-1* expression induced by this combination of drugs (27). The variations
8 observed for *MMP-9* fit well with previous data showing that TSA was able to decrease *MMP-9*
9 expression in gastric cancer (63), in thyroid cancer cells (64) or in human endometrial stromal
10 cells (65). Moreover, it has been shown that MCT1, a new HDI, was able to inhibit *MMP-9*
11 expression induced by 5-azadC in HL60 hematopoietic cells (66).

12 Our data indicate that the hypomethylating drug 5-azadC and the histone deacetylase
13 inhibitor TSA were able to induce modulations in higher-order chromatin organisation and in
14 gelatinase expressions in the human HT1080 fibrosarcoma cells. These results concern only
15 *MMP-2* and *-9* genes, but epigenetic events induce multiple gene regulations. These data must
16 therefore be completed by analyses on a larger spectrum of cancer-related genes and
17 complimentary approaches must be used to address the complex interplay between nuclear
18 architecture and functions like gene expression.

19 **Acknowledgements**

20 MP is the recipient of a fellowship from the french “Ministère de l’Enseignement Supérieur et
21 de la Recherche”. MS is supported by a Waxweiler grant for cancer prevention research from
22 the Action Lions “Vaincre le Cancer”. This work was supported by grants from the Comité
23 Départemental de l’Aube de la Ligue Nationale contre le Cancer. The work performed at
24 Laboratoire de Biologie Moléculaire et Cellulaire du Cancer was supported by Télévie

1 Luxembourg, the Recherche Cancer et Sang fondations and the Recherches Scientifiques
2 Luxembourg association.

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- 13
14

1 **Figure legends**

2 **Figure 1. Variations of nuclear texture and DNA methylation in 5-azadC-treated cells.**

3 Textural variations are expressed as Canonical Variable 1 (mean \pm SEM), as described in
 4 Material and Methods. CV1 was calculated for each selected cell using the discriminant
 5 features and the coefficients described in Table 4. DNA methylation level was assessed by
 6 MSRA. The amounts of *HpaII*-undigested DNA were evaluated by electrophoresis and
 7 quantified using ImageJ software. Results are expressed as % of methylation decrease (mean of
 8 2 separate experiments). Closed dots, CV1 values; open dots, MSRA data. * $p < 0.05$,
 9 Kolmogorov-Smirnov test on cumulated frequencies.

10

11 **Figure 2. Variations of textural parameters in the subsets defined after unsupervised**
 12 **multidimensional partitioning.**

13 Each bar represents mean + SEM of parameter value in subsets. LM: local mean; E: energy;
 14 ENT: entropy; I: inertia; SRE: short run length emphasis; LRE: long run length emphasis;
 15 GLD: grey-level distribution; RLD: run-length distribution; RPC: run-length percentage. White
 16 columns, subset A; black columns, subset B. For all these parameters, distributions in subset A
 17 and subset B are significantly different ($p < 0.05$, Kolmogorov-Smirnov test on cumulated
 18 frequencies)

19

20 **Figure 3. Effects of 5-azadC on MMP-2 and MMP-9 gene expression.**

21 (a) HT1080 cells were treated or not with 5-azadC (5 μ M) for various times as indicated on the
 22 figure. Levels of endogenous mRNAs were monitored by real-time RT-PCR. *β 2 microglobulin*
 23 (*β 2m*) was used as internal control. After normalization to *β 2m*, results are expressed as the

1 mean \pm SEM of fold induction relative to control. * $p < 0.05$ (Kruskal Wallis test, $n=5$). (b)
2 Methylation status of the *MMP-2* and *-9* genes evaluated by MSP. HT1080 cells were treated or
3 not with 5-azadC (5 μ M) for 72h. U: unmethylated; M: methylated. Pictures are representative
4 of 2 independent experiments.

5

6 **Figure 4. Effects of 5-azadC and TSA on nuclear texture and *MMP-2/-9/-1* gene**
7 **expressions.**

8 HT1080 cells were treated or not with TSA (100 ng/mL for 24h), 5-azadC (5 μ M for 72h), or
9 both drugs. (a) Results of nuclear texture changes in G_0/G_1 nuclei are expressed as the mean \pm
10 SEM of Nuclear Abnormality Index (NAI). (b) Levels of endogenous mRNAs were monitored
11 by real-time RT-PCR. *$\beta 2$ microglobulin ($\beta 2m$)* was used as internal control. After
12 normalization to *$\beta 2m$* , results are expressed as the mean \pm SEM of fold induction relative to
13 control. Open bars: control cells; dotted bars: cells treated with TSA alone; hatched bars, cells
14 treated with 5-azadC alone; black bars, cells treated with 5-azadC and TSA. * $p < 0.05$ (Kruskal
15 Wallis test, $n=3$).

16

Tables

Table 1. PCR Primer sequences

Target	Forward and reverse primer sequences	
<i>MMP-1</i>	Forward	5'-GAGCAAACACATCTGACCTACAGGA-3'
	Reverse	5'-TTGTCCCGATGATCTCCCCTGACA-3'
<i>MMP-2</i>	Forward	5'-TCTTCCCCTTCACTTTCCTG-3'
	Reverse	5'-ACTTGCGGTCGTCATCGT-3'
<i>MMP-9</i>	Forward	5'-TGACAGCGACAAGAAGTGG-3'
	Reverse	5'-GGCGAGGACCATAGAGGTG-3'
<i>β2-microglobulin</i>	Forward	5'-ATCTTCAAACCTCCATGATG-3'
	Reverse	5'-ACCCCCACTGAAAA AGATGA-3'
MSP <i>MMP-2</i> methylated	Forward	5'-ATTGTTAGGATTTGCGGC-3'
	Reverse	5'-CTCGATACGTATAACCGCCT-3'
MSP <i>MMP-2</i> unmethylated	Forward	5'-GGGATTGTTAGGATTTGTGGT-3'
	Reverse	5'-AACTCAATACATATAACCACCTC-3'
MSP <i>MMP-9</i> methylated	Forward	5'-ATTAAGGTAGGCGTGGTTTC-3'
	Reverse	5'-AAACCTAAAACGTAAACGCC-3'
MSP <i>MMP-9</i> unmethylated	Forward	5'-GGATTAAGGTAGGTGTGGTTTT-3'
	Reverse	5'-AAACCTAAAACATAAACACCAA-3'

Table 2. Values of the textural features in HT1080 cells treated with 5 μ M of 5-azadC for 72h.

Parameters	Cell treatment			
	None (n=320)		5-azadC 5 μ M (n=276)	
LM	33.4 \pm 7.4	<i>(12.3-61.0)</i> ^b	16.5 \pm 4.6	<i>(7.7-33.2)</i>
E	5.0 \pm 0.7	<i>(2.0-19.8)</i>	12.2 \pm 4.2	<i>(4.3-31.5)</i>
ENT	33.7 \pm 4.5	<i>(18.7-42.5)</i>	24.5 \pm 3.2	<i>(14.2-34.1)</i>
I	31.7 \pm 9.0	<i>(15.7-75.4)</i>	20.6 \pm 2.8	<i>(13.8-36.4)</i>
SRE	60.8 \pm 3.5	<i>(47.7-76.3)</i>	52.8 \pm 2.2	<i>(47.3-60.5)</i>
LRE	6.0 \pm 1.9	<i>(3.3-13.7)</i>	9.3 \pm 1.7	<i>(5.3-16.7)</i>
GLD	12.2 \pm 4.6	<i>(6.9-34.5)</i>	23.4 \pm 5.1	<i>(11.5-46.3)</i>
RLD	36.0 \pm 5.9	<i>(23.7-53.9)</i>	28.1 \pm 2.1	<i>(22.8-35.2)</i>
RPC	10.3 \pm 1.2	<i>(7.1-13.3)</i>	8.5 \pm 0.6	<i>(6.7-10.4)</i>

Textural features were evaluated in G₀/G₁ cell nuclei. LM: local mean; E: energy; ENT: entropy; I: inertia; SRE: short run length emphasis; LRE: long run length emphasis; GLD: grey-level distribution; RLD: run-length distribution; RPC: run-length percentage.

For each parameter, value is expressed as mean + SD, and numbers in italic represent the distribution range of each parameter in the population. For all these parameters, distributions in control and treated cells are significantly different (p<0.05, Kolmogorov-Smirnov test on cumulated frequencies).

Table 3. Variability of the measurements of nuclear texture parameters

Parameters	Variability within control groups from 3 experiments		Variability within treated groups from 3 experiments	
	F value	P	F value	P
LM	1.80	0.16	0.15	0.86
E	1.37	0.25	0.23	0.79
ENT	1.06	0.34	0.10	0.90
I	1.07	0.34	0.18	0.83
SRE	0.48	0.61	0.07	0.93
LRE	1.17	0.31	0.61	0.54
GLD	1.12	0.32	0.11	0.89
RLD	0.80	0.45	0.03	0.97
RPC	1.10	0.33	0.08	0.91

Nuclear texture parameters were computed on G₀/G₁ nuclei from control cells and cells treated for 72h with 5μM 5-azadC from 3 separate experiments. LM: local mean; E: energy; ENT: entropy; I: inertia; SRE: short run length emphasis; LRE: long run length emphasis; GLD: grey-level distribution; RLD: run-length distribution; RPC: run-length percentage.

Values within control or treated groups were compared by one-way analysis of variance.

Table 4. Ranking of the best discriminant parameters and calculation of the canonical variable

Parameter	Discriminatory Power	Good classification rate (%)	Coefficient of CV1
GLD	754	87.7	63.1
LM	87	91.1	-14.6
LRE	53	92.2	347.8

The most discriminant features were selected after linear discriminant analysis applied to files from control cells and cells treated with 5 μ M of 5-azadC for 72h. These parameters were then used in computation of the canonical variable. GLD: grey-level distribution; LM: local mean; LRE: long run length emphasis.

Table 5. Distribution of the 5-azadC-treated cells among the subsets defined after unsupervised partitioning

Cell Populations	Cells classified in subsets (%)	
	A	B
Control	254 (88)	65 (12)
5-azadC 0.5 μ M	156 (60)	102 (40)*
5-azadC 1 μ M	107 (38)	177 (62)*
5-azadC 2 μ M	43 (15)	250 (85)*
5-azadC 5 μ M	25 (9)	250 (91)*

All G₀/G₁ cell data files from control cells and cells treated with 5 μ M of 5-azadC for 72h were merged and the resulting population was submitted to unsupervised classification which identified 2 subsets, A and B. Cells from the constituting populations were classified in these subsets by linear discriminant analysis. *p<0.05 when compared with values in control cells (Chi²-test)

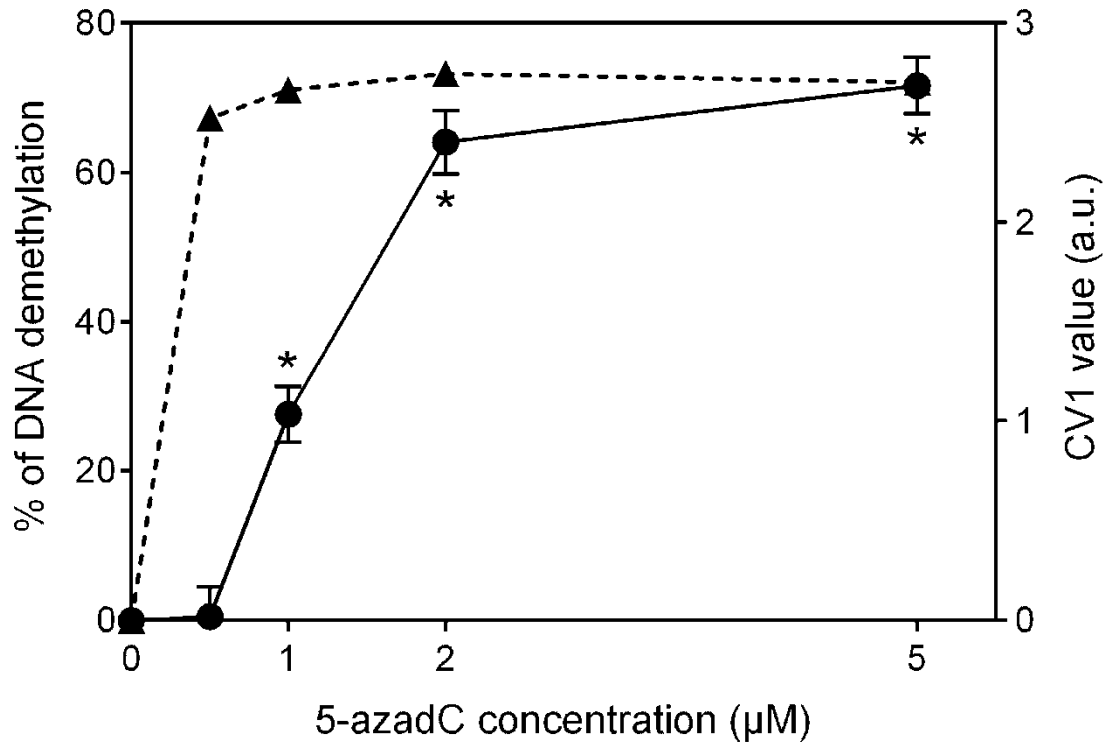


Figure 1

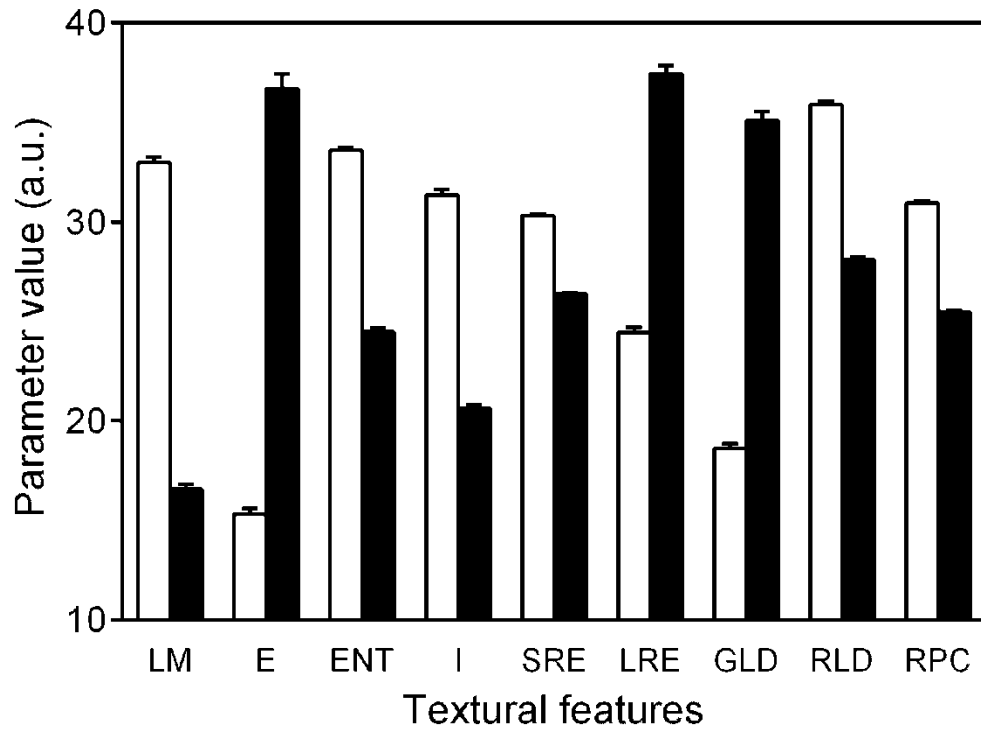


Figure 2

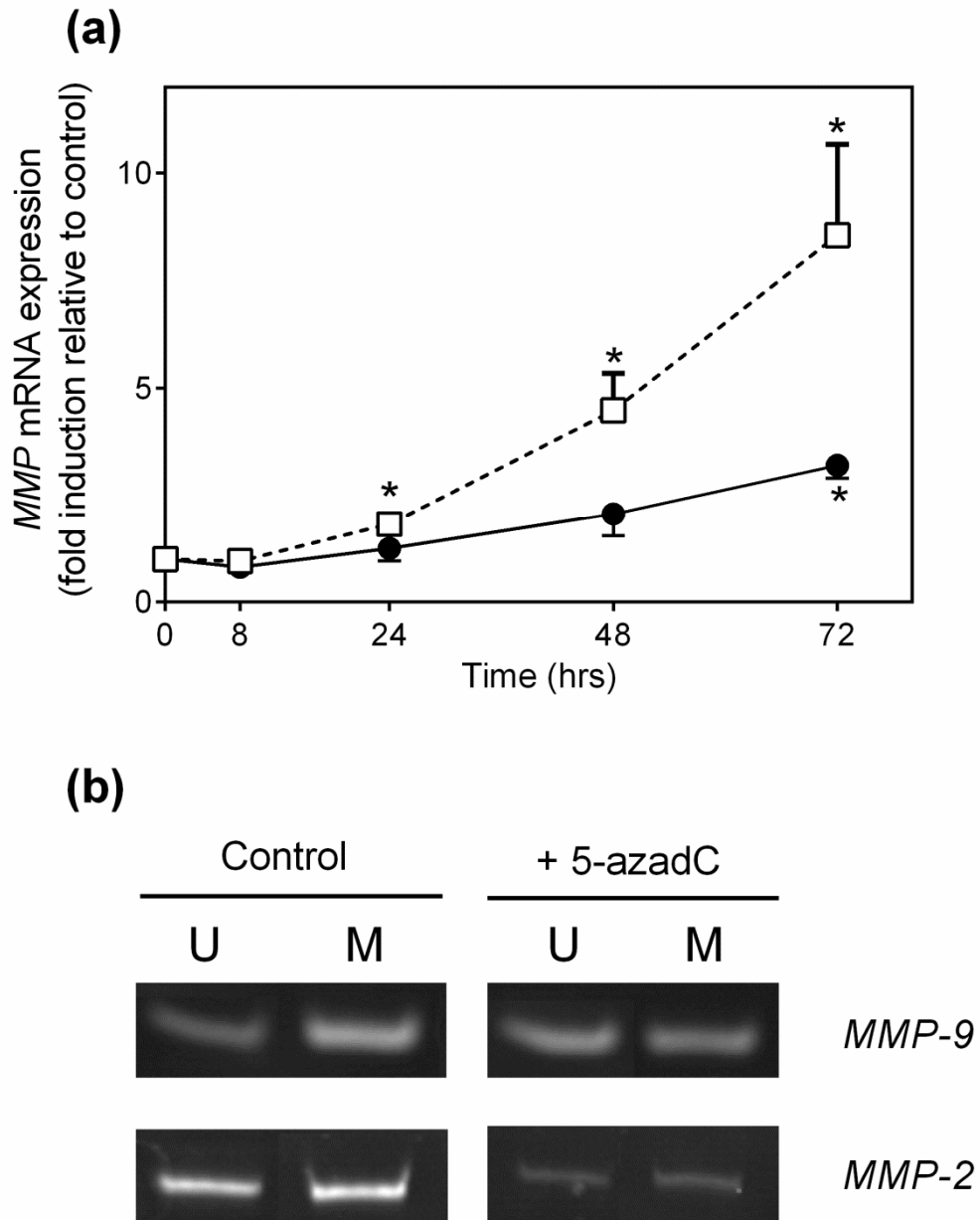


Figure 3

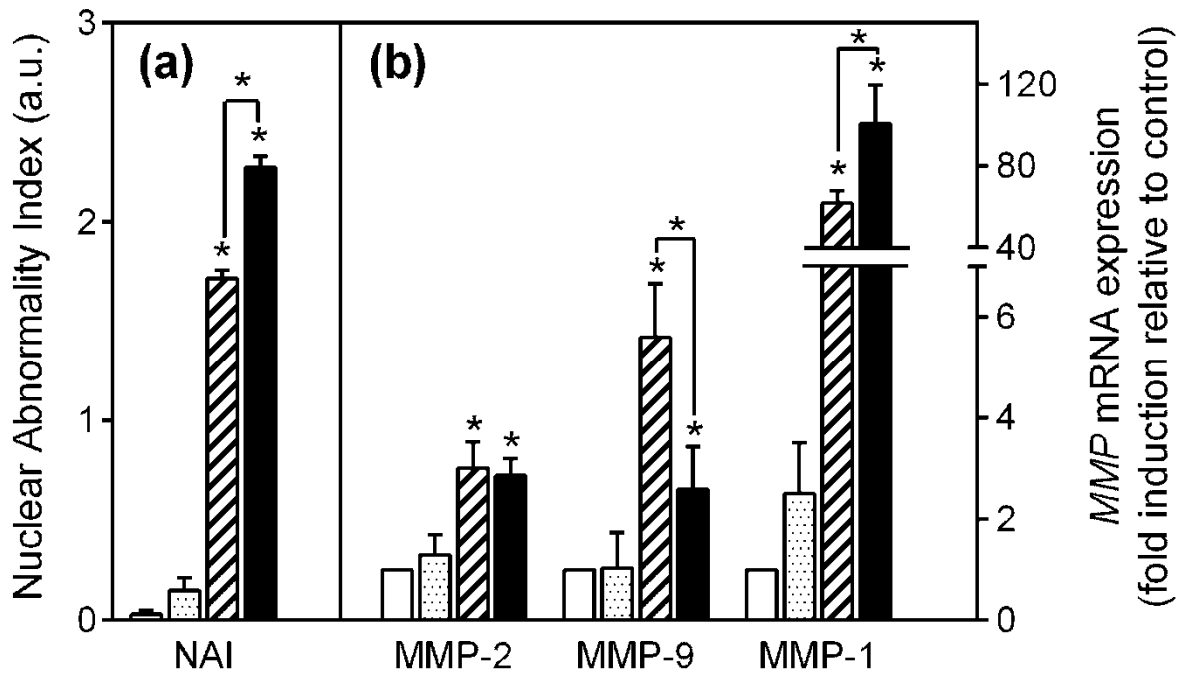


Figure 4

PUBLICATION N°3

The DNA hypomethylating agent 5-aza-2'-deoxycytidine enhances tumor cell invasion through MMP-1 transcription-dependent modulation in human fibrosarcoma cells.

M. Poplineau, M. Schnekenburger, A. Kościarz, J. Dufer, F. Antonicelli, M. Diederich, A. Trussardi-Régnier.

Manuscrit soumis pour publication dans Molecular Oncology

Présentation de la PUBLICATION N°3

La 5-aza-2'-deoxycytidine, un agent hypométhylant de l'ADN, augmente l'invasion des cellules de fibrosarcome humain HT1080 via une modulation transcriptionnelle de l'expression de la MMP-1

Contexte

Une protéolyse dysfonctionnelle de la matrice extracellulaire conduit à des dérégulations dans le remodelage tissulaire facilitant la prolifération et la perte d'adhérence des cellules tumorales et conduisant ainsi à l'invasion des tissus environnants et à la formation de métastases. L'activité protéolytique des métalloprotéinases matricielles (MMPs) et en l'occurrence, de MMP-1, -2 et -9 joue un rôle primordial au cours de ces processus pathologiques c'est pourquoi l'expression de ces protéases tend à être augmentée dans de nombreux cancers (Kessenbrock, 2010). Par ailleurs nous avons montré dans les publications précédentes que l'expression de ces MMPs était fortement induite par la 5-azadC. Il est à prendre en compte que la 5-azadC ou Décitabine® est actuellement utilisée en clinique pour le traitement de leucémies et de syndromes myélodysplasiques et en essai clinique pour le traitement de tumeurs solides (Silverman, 2005). De ce fait, nous avons voulu tester l'effet de ce modulateur épigénétique, utilisé à l'heure actuelle pour le traitement de certains cancers, sur les propriétés invasives des cellules HT1080. Ces travaux ont fait l'objet de la PUBLICATION N°3.

Matériel et Méthodes

Dans cette étude, des cellules de la lignée cellulaire HT1080 ont été traitées par la 5-azadC. L'invasion des cellules HT1080 a été étudiée par la technique de la chambre de Boyden modifiée par un « coating » avec du Matrigel®. L'expression génique et protéique de MMP-1, -2 et -9 ont été analysées respectivement par RT-PCR en temps réel et western blot ou zymographie. L'effet de la 5-azadC sur le profil de méthylation de l'ADN total et de celui du promoteur du gène de la MMP-1 ont été analysés respectivement analysés par MSRA et

séquençage au bisulfite ou par analyse CoBRA (*combined bisulfite restriction analysis*). Les niveaux nucléaires des facteurs de transcription connus pour être impliqués dans la régulation de l'expression du gène de la MMP-1 ont été analysés par western blot. Des expériences d'immunoprécipitation de la chromatine (*chromatin immunoprecipitation* ou CHIP) ont été réalisées en vue d'analyser la dynamique transcriptionnelle et le remodelage chromatinien au niveau du promoteur du gène de la MMP-1. Des informations supplémentaires concernant la technique de séquençage au bisulfite et l'analyse CoBRA sont développées respectivement en ANNEXES 5 et 6.

Résultats

Dans cet article, il apparaît que différents inhibiteurs de l'activité protéolytique des MMPs, ne sont pas capables de moduler l'invasion spontanée de cellules HT1080 au travers du Matrigel®. Un traitement de ces cellules par la 5-azadC augmente leur invasion d'environ 25 %. L'ajout d'inhibiteurs à large spectre des MMPs et d'un inhibiteur spécifique de MMP-1 abolit totalement l'invasion induite par la 5-azadC. Un inhibiteur spécifique de MMP-2 et -9 n'a aucun effet sur cette invasion induite. L'utilisation d'actinomycine D, un inhibiteur de l'assemblage des ribosomes, révèle que la 5-azadC affecte la transcription du gène de la MMP-1 mais n'a aucun effet sur la stabilité de ses ARNm. Bien que la 5-azadC soit capable de déméthylater la quasi totalité de l'ADN dans les cellules HT1080, elle n'induit qu'une très faible déméthylation du promoteur du gène de la MMP-1 (moins de 10 % au niveau de quelques dinucléotides CpG). Ces résultats semblent confirmer l'hypothèse émise dans la publication n°1 et suggérant que la régulation de l'expression de ce gène pourrait s'effectuer de manière indirecte. De plus la 5-azadC augmente les niveaux nucléaires des facteurs de transcription Sp1 et Sp3. Par la technique de CHIP, nous montrons que la 5-azadC entraîne le recrutement de Sp1 et d'ARN polymérase II au niveau du promoteur du gène de la MMP-1. Ce recrutement s'accompagne d'un enrichissement en marques actives (H4ac, H3K4me3, H3S10P) et d'une diminution en marque répressive H3K27me3 au niveau de ce promoteur.

Conclusion

Dans cet article, nous montrons que l'invasion spontanée des cellules HT1080 ne requiert pas l'activité protéolytique des MMPs. Un traitement par la 5-azadC augmente l'invasion de ces cellules, en partie par l'intermédiaire d'une augmentation de l'expression de la MMP-1. La modulation de l'expression du gène de la MMP-1 par la 5-azadC est régie par un mécanisme transcriptionnel et ne requiert pas la déméthylation totale de son promoteur. Le facteur de transcription Sp1 ainsi que l'établissement d'une chromatine plus permissive caractérisée par un enrichissement en marques actives (H4ac, H3K4me3, H3S10P) et une déperdition de la marque répressive H3K27me3, semble participer à l'induction du gène de la MMP-1 par la 5-azadC.

The DNA hypomethylating agent 5-aza-2'-deoxycytidine enhances tumor cell invasion through MMP-1 transcription-dependent modulation in human fibrosarcoma cells

Mathilde Poplineau¹, Michael Schnekenburger², Aleksandra Kościarz¹, Jean Dufer¹, Frank Antonicelli³, Marc Diederich², Aurélie Trussardi-Régnier^{1, *}

¹Unité MEDyC, URCA-CNRS FRE 3481, SFR Cap Santé, Faculté de Pharmacie, Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

²Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, L-2540 Luxembourg, Luxembourg

³Laboratoire de Dermatologie, UFR Médecine, 1 avenue du Maréchal Juin, 51096 Reims, France

* corresponding author: Tel: +33 326918016; Fax: +33 326913550; E-mail address: aurelie.regnier@univ-reims.fr; address: Unité MEDyC, URCA-CNRS FRE 3481, SFR Cap Santé, Faculté de Pharmacie, Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

Abstract

Under physiological conditions, the transcriptional activity of matrix metalloproteinase (MMP) genes results in low basal MMP protein levels. However, in pathologies such as cancer, cells need to degrade the extracellular matrix (ECM) and therefore to express high levels of proteases. Thus, aberrant tissue degradation is associated with MMP overexpression that results from different mechanisms such as epigenetic events. One of the most characterized epigenetic mechanisms is DNA methylation, which causes changes in chromatin conformation, thereby downregulating the accessibility to the transcriptional machinery and resulting in a highly stable gene silencing. The level of DNA methylation could be modulated by DNA hypomethylating agents such as 5-aza-2'-deoxycytidine (5-azadC), which is widely used for epigenetic anticancer treatments. Here, we focus on the effects of this drug on the expression level of MMP-1, -2 and -9 in the human HT1080 fibrosarcoma cell line. We demonstrated that 5-azadC increases MMP expression levels both at the mRNA and protein levels, and promotes invasion of HT1080 cells. Using broad-spectrum and specific MMP inhibitors, we established that MMP-1, but not MMP-2 and -9, play a key role in 5-azadC-enhanced cell invasion. We showed that 5-azadC enhances MMP-1 expression through a transcriptional mechanism but did not affect MMP-1 promoter methylation status. This 5-azadC treatment increased the nuclear levels of Sp1 and Sp3 transcription factors, and modulated their recruitment to MMP-1 promoter together with a chromatin remodeling evidenced by histone active mark deposition.

Highlights

- The hypomethylating agent 5-azadC induces MMP-1, -2 and -9 expression levels
- 5-azadC increases fibrosarcoma HT1080 cell invasion
- The invasion enhancement requires a transcription-dependent increase of MMP-1 expression
- 5-azadC induces Sp1 recruitment and chromatin remodeling at MMP-1 promoter

1. Introduction

Extracellular proteolysis mediates tissue homeostasis. In cancer, altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis. The matrix metalloproteinases (MMPs) represent the most prominent family of proteinases associated with tumorigenesis (Kessenbrock, et al., 2010). They belong to a family of 24 zinc-dependent endopeptidases characterized by highly conserved pro-peptide and catalytic domains, and by a carboxy terminal hemopexin domain conferring substrate specificity (Raffetto and Khalil, 2008). MMPs are classified in four classes according to their substrate specificity: collagenases (*e.g.*, MMP-1), which degrade the interstitial collagens type I, II, III; gelatinases (MMP-2 and MMP-9), which degrade gelatin, collagen type IV from the basement membrane and proteoglycans; stromelysins (*e.g.*, MMP-3), which degrade several non-collagen matrix proteins such as laminin or fibronectin; and other MMPs (matrilysins, membrane-type MMPs, elastases...).

Under normal physiological conditions, genes encoding MMPs are primarily regulated at transcriptional level and often result in low basal mRNA levels since they are considered as inducible genes and could be targeted by a wide variety of stimuli (Fanjul-Fernandez, et al., 2010). To invade the surrounding tissue, cancer cells need to express high level of proteases that can degrade the extracellular matrix (ECM), activate matrix proteins and induce the release of bioactive molecules such as growth factors, cytokines, receptors and adhesion molecules (Hua, et al., 2011). Thus, to enhance their invasive properties, cancer cells require critical changes in MMP expression. In this line, previous reports have established a correlation between the epigenetic fingerprint of some MMP promoters and the invasive properties of cancer cell lines. Three relevant points have been developed: there is a correlation between

(i) MMP promoter methylation, their gene expression and tumor cell invasion; (ii) histone chemical modifications on MMP promoters, their gene expression and tumor cell invasion; (iii) chromatin remodeling enzyme, MMP expression and tumor cell invasion. (Chernov, et al., 2009; Park, et al., 2011; Sato, et al., 2003; Shukeir, et al., 2006).

Epigenetic mechanisms such as posttranslational modifications of histones and/or DNA methylation allow the cells to tightly regulate gene expression (Florea, et al., 2011). These modifications not only regulate chromatin structure but also recruit remodeling enzymes to reposition nucleosomes, thereby modulating processes such as transcription, DNA repair, replication and recombination (Bannister and Kouzarides, 2011). Chemical modifications of N-terminal tail of histones are responsible for the epigenetic fingerprint of promoters. These DNA-packaging proteins regulate the underlying DNA sequences through complex modifications such as lysine acetylation, serine phosphorylation, arginine and lysine methylation.

Genome-wide studies revealed distinct combinations of histone modifications, called “histone code” and that lead to a more closed or open chromatin state, thereby regulating gene activity (Berger, 2002). For instance, global acetylation of lysine residues on histone H4 (H4ac) and trimethylation of lysine K4 on histone H3 (H3K4me3) are commonly considered as “active marks” since they promote gene transcription. In contrast, trimethylation of lysine K9 and K27 on histone H3 (H3K9me3 and H3K27me3) are considered as “repressive marks” since they are associated to a silenced chromatin (Berger, 2007).

In mammalian cells, DNA methylation occurs on cytosine residues within CpG dinucleotides and involves the catalytic activity of DNA methyl transferases (DNMTs). DNA methylation is usually associated with gene silencing

(Illingworth and Bird, 2009), since it represses gene expression either by interfering with the transcriptional machinery (Esteller, 2007) or by recruiting chromatin repressors such as proteins with methyl-CpG-binding domains (MBD including MBD1-4, MeCP2 and Kaiso proteins), histone deacetylases (HDACs) or histone methyl transferases (HMTs) (Deaton and Bird, 2011). In cancer, two paradoxal phenomena have been observed: (1) a specific gene silencing through aberrant hypermethylation of gene promoters, particularly tumor suppressor genes, and (2) a genome-wide hypomethylation that could reactivate proto-oncogene expression and generate genomic instability (Florea, et al., 2011). Hypomethylating agents are already used in clinical practice (Seidel, et al.) but they could aggravate the situation by further decreasing the global level of DNA methylation and activate genes potentially implicated in oncogenesis or in metastasis formation (Yoo and Jones, 2006). So, their impact on the regulation of MMP gene expression associated with tumor invasion needs to be clarified. Thus, in this study we focused our attention on the effects of the hypomethylating agent 5-aza-2'-deoxycytidine (5-azadC) on the invasive behavior and the regulation of MMP expression in the human fibrosarcoma cell line (HT1080).

2. Material and Methods

2.1. Chemicals

Actinomycin D (ActD), cycloheximide (CHX), GM6001, GM1489, MMP-2/MMP-9 inhibitor III and 5-aza-2'-deoxycytidine (5-azadC) were purchased from Sigma-Aldrich (France).

2.2. Cell culture

Human fibrosarcoma HT1080 cells (ATCC, CCL-121) were grown at 37 °C in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified 95% air, 5% CO₂ atmosphere.

2.3. Real-time RT-PCR

Cells were washed twice in ice-cold 1x PBS and total RNAs were isolated using NucleoSpin® RNA II kit (Macherey Nagel, France). For real-time RT-PCR, 1 µg of RNA was reverse transcribed using MML-V reverse transcriptase (Fisher Scientific, France), and each cDNA sample was analyzed by real time PCR using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, France) on the LightCycler system (Roche Diagnostics, France). MMP transcripts were amplified using primers represented in table 1. Relative quantification was performed by using the housekeeping gene β_2 microglobulin as internal control.

2.4. Western blot

For MMP western blot analyses, serum-free media from HT1080 cell cultures were harvested and proteins concentrated using Amicon Ultra centrifugal filters (Millipore, France). For transcription factor western blot analyses, total nuclear proteins were extracted using the Nuclear Extract kit (Active Motif, Belgium). 35 µg of total proteins were heated 5 min at 100 °C in Laemmli sample buffer, separated on 10% SDS PAGE under reducing conditions and transferred to PVDF membrane by using I-Blot

system (Invitrogen, France). The membranes were immersed in blocking solution for 2 h at room temperature, and incubated with the primary antibodies of interest (table 2). Proteins were revealed using a peroxidase-conjugated secondary antibody (table 2) and the ECL Plus detection kit (Amersham, France).

2.5. Zymography

For MMP-2 and MMP-9 zymography analyses, 20 µl of serum-free cell culture media were analyzed on 9% SDS-PAGE containing 0.1% gelatin. ProMMP-2 (72kDa) and proMMP-9 (92 kDa) were used as positive markers. After the electrophoretic migration, the gels were incubated in Triton X100 (2%) for 1h (2x30 min) and in incubation buffer (Tris 50 mM, NaCl 200 mM, CaCl₂ 5 mM, pH 7.6) at 37 °C for 18 h in order to activate both gelatinases. MMP-2 and MMP-9 were revealed using Coomassie Blue staining and destaining solutions. Zymography revealed proteolytic activity which appeared as clear zones, demonstrating lysis of the gelatin in the gels, against the blue background of stained gelatin.

2.6. *In vitro* invasion assay

Invasive properties of HT1080 cells were analyzed using modified Boyden chambers with a size pore of 8 µm (Transwell Greiner, Dutscher, France) and coated with 10 µg of Matrigel® (BD Biosciences, France). Cells were seeded onto the upper chamber and treated with 5-azadC for 48 h. Cells were then washed twice with D-PBS and incubated in 200 µl of serum-free RPMI containing 0.2% BSA and 5 µM 5-azadC. After 8 h, 800 µl of chemoattractive medium (RPMI containing 10% FCS and 2% BSA) were added in the lower chamber. After 16 h, cells were fixed 20 min in methanol and stained with crystal violet. Non-invasive cells (on the filter upper side) and invasive cells (on the filter lower side) were counted. Invasive properties of

HT1080 were expressed as percentage of cells having crossed the Matrigel® coating.

2.7. DNA methylation analyses

Genomic DNA was isolated using QIAamp DNA Mini kit (Qiagen, France). Global DNA demethylation after 5-azadC treatment was assessed by Methyl Sensitive Restriction Assay (MSRA). 0.5 µg of genomic DNA was incubated at 37 °C overnight with 20 units of the following restriction enzymes (New England BioLabs, UK): *HpaII* (methyl-sensitive) or *MspI* (methyl-insensitive). The next day, 10 units of both enzymes were further added for 2 h. The DNA digestion level was checked on 0.8% agarose gel. Methylation status of MMP-1 promoter was analyzed by bisulfite sequencing (BSP) or combined bisulfite restriction assay (CoBRA). BSP was performed using 16 couples of primers allowing amplification of MMP-1 distal and proximal promoters. Primer sets for CoBRA and BSP are represented in table 1. After optimization of the primers, 0.5 µg of HT1080 genomic DNA were bisulfite converted, amplified using Hot-Start enzyme (Qiagen, France) and purified using the QIAquick PCR Purification Kit (Qiagen, France). Then, the converted genomic DNA was sequenced at the Plateforme Régionale de Biologie Innovante (PRBI, Reims, France) on the 3130X sequencer (Life technologies, France). Sequences were analyzed using MEGA4 and Chromas softwares, and compared to MMP-1 reference sequence (NCBI reference sequence: NG_011740.1). For CoBRA analysis, PCR products (150-200 ng) from converted genomic DNA were restricted with 10 units of *TaqI* (Invitrogen, France) in a reaction volume of 20 µl, at 65 °C overnight. The restriction pattern was analyzed on 0.8% agarose gel.

2.8. Chromatin immunoprecipitation (ChIP)

ChIP experiment was performed using Magna ChIP™ A/G kit (Millipore, France) according to the manufacturer protocol. In brief, $5 \cdot 10^6$ cells were cross-linked with 1% formaldehyde (Sigma, France) for 8 min at room temperature. After cross-linking, the reaction was quenched with 0.125 M of glycine for 10 min at room temperature. Cells were washed twice with ice cold D-PBS, pelleted by centrifugation, resuspended in 500 μ L of lysis buffer containing 1X protease inhibitor cocktail II and incubated 15 min at 4 °C under agitation. After centrifugation, nuclei were resuspended in 250 μ L of nuclear lysis buffer containing 1X protease inhibitor cocktail II and incubated for 10 min on ice. A pool of two nuclei suspension (500 μ L), were sonicated on ice using the high intensity Ultrasonic processor (Bioblock scientific, France) (time: 50 s, pulse on: 2 s, pulse off: 9.9 s, amplitude: 40). After centrifugation, to remove cell debris, chromatin was diluted in the IP dilution buffer, and 10% of the diluted supernatant was used as input. The diluted chromatin was incubated overnight at 4 °C with the antibodies of interest and with 20 μ L of resuspended protein A/G magnetic beads (table 2). The precipitated immune complexes (DNA-proteins-antibodies-beads) were washed sequentially with the ice-cold buffers of the kit: once wash with low salt, high salt and LiCl buffers and twice washes with the TE buffer. Then, the cross-linking between DNA and proteins was reversed. For this step, the immune complexes were resuspended in the ChIP elution buffer containing 1% of proteinase K (v/v) and incubated 2 h at 62 °C with shaking and 10 min at 95 °C. DNA was purified using spin columns. Purified DNA samples were analyzed by real-time PCR using the Power SYBR® Green PCR Master Mix and the StepOnePlus™ real-time PCR system (Life Technologies, France). PCRs were performed using primers that covered different regions of the MMP-1 promoter (table 1).

2.9. Quantification of data and statistical analyses

All experiments were performed at least in triplicate. Results are expressed as mean \pm SEM. Statistical analysis was realized using Kruskal-Wallis test. The results were considered significantly different when $p < 0.05$. Electrophoretic images were analyzed with ImageJ software (Schneider, et al., 2012).

3. Results

3.1. 5-azadC increases HT1080 invasion through MMP-1

The cell invasion potential of HT1080 cells after 5-azadC treatment was analyzed in modified-Boyden chambers coated with Matrigel®. HT1080 cells were treated or not during the last 16 h of incubation by 10 μ M of the following MMP inhibitors: GM6001 (broad-spectrum MMP inhibitor), GM1489 (MMP-1 inhibitor) and MMP-2/MMP-9 inhibitor III. In these conditions, these inhibitors did not modulate HT1080 invasive properties (figure 1A). After 5-azadC exposure (5 μ M for 72 h), HT1080 invasion was significantly enhanced (figure 1B). This increase was abolished by addition of both GM6001 broad-spectrum MMP inhibitor and GM1489 MMP-1-specific inhibitor, but not with the MMP-2/-9 inhibitor, suggesting that 5-azadC could increase HT1080 invasion mainly *via* MMP-1 activity (figure 1B). At the DNA level, MSRA revealed that this treatment induced a global demethylation in these cells (around 80% as evaluated by Image J quantification of electrophoretic data) (figure 1C). In order to elucidate how these changes in DNA methylation could increase HT1080 invasiveness, MMP gene expression levels were monitored after 5-azadC treatments (figure 1D). 5-azadC markedly increased mRNA level of MMP-1 (44.6 fold) and to a lesser extent levels of MMP-2 (1.87 fold), and -9 (8.56 fold) (figure 1D). Analyses of extracellular secretion of these MMPs by western blot or zymography revealed that the 5-azadC-enhanced MMP gene expression was correlated with an increase of MMP protein levels (figure 1E).

3.2. 5-azadC increases MMP-1 gene expression through a transcriptional mechanism

To analyze the mechanisms by which 5-azadC modulates MMP-1 gene expression, cells were simultaneously treated with actinomycin D (ActD) and 5-azadC for 48 h (figure 2A). Results revealed that ActD completely abolished 5-azadC-enhanced MMP-1 gene expression, suggesting that 5-azadC increased MMP-1 mRNA level in a transcription-dependent manner. Then, cells were treated with 5-azadC for 72 h, followed by ActD for different times (from 0 to 40 h), in order to evaluate MMP-1 mRNA stability (figure 2B). Untreated cells and 5-azadC-treated cells exhibited similar MMP-1 mRNA half-life times (25 h and 23 h for untreated and 5-azadC-treated cells, respectively).

Since the previous experiment suggests that 5-azadC strongly increases MMP-1 expression through a transcription-dependent mechanism, its effects on MMP-1 promoter methylation was investigated. *In silico* analysis of the MMP-1 promoter sequence between -3500 and +1 bp failed to reveal the presence of a CpG island. Nevertheless, this region contains 33 isolated CpG dinucleotides (NCBI reference sequence: NG_011740.1). Therefore, we analyzed the status of methylation of those individuals CpG using bisulfite sequencing. Results demonstrate that 5-azadC induced a low demethylation (less than 10%) of 12 out of 33 isolated CpG dinucleotides only (-3273, -3262, -3187, -3096, -3076, -2951, -2821, -2791, -2768, -1304, -941, -202 bp) (figure 2C). CoBRA confirmed that 5-azadC did not deeply affect MMP-1 promoter methylation profile (figure 2D).

3.3. 5-azadC induces RNA pol II and Sp1 recruitments to MMP-1 promoter

As the low variation in DNA methylation induced by 5-azadC at MMP-1 promoter could alone hardly explain the 44-fold increased gene transcription, we sought for

other mechanisms potentially implicated in 5-azadC-induced MMP-1 gene expression.

In order to investigate whether protein neosynthesis could be implicated in MMP-1 overexpression in HT1080 cells after 5-azadC treatment, we co-incubated cells with 5-azadC and with the protein synthesis inhibitor cycloheximide CHX (figure 3A).

Results show that CHX partly reduced 5-azadC-induced MMP-1 mRNA expression after 48 h (1.5 fold decrease) and 72 h (5.5 fold decrease) of treatment indicating that protein neosynthesis could play a role in 5-azadC-enhanced MMP-1 expression.

We then evaluated the nuclear levels of transcription factors known to be implicated in MMP-1 gene regulation (Fanjul-Fernandez, et al., 2010; Wallace, et al., 2012) (figure 3B). Results reveal that treatment with 5-azadC for 24 h or 48 h failed to affect the nuclear level of c-Fos, c-Jun, p50, p65, p53 and PEA3, but increased the nuclear level of the Sp-family members Sp1 and Sp3, 39 and 31 fold change increase respectively, after 48h of treatment respectively, ImageJ analysis of electrophoretic data).

To evaluate whether the effect of 5-azadC-enhanced Sp1 and Sp3 protein expression levels play a role in 5-azadC-mediated MMP-1 overexpression, we carried out ChIP analyses with antibodies against Sp1, Sp3, RNA polymerase II (RNA pol II) as well as for several histone modifications known to be involved in gene expression regulation.

These assays were associated with a systematic mapping of the MMP-1 promoter using several primer sets between -15 kb and the transcription start site (TSS) (figure 4A). MMP-1 promoter in untreated cells was characterized by an important recovery of RNA pol II and active marks such as acetylated H4 (H4ac) and trimethylation of H3K4 (H3K4me3) in the regions situated between -1 kb and the TSS (figure 4B).

Primers targeting an intergenic region located 15 kb upstream of the TSS were used as a negative control and, as expected, this region was not enriched for proteins related to active transcription (RNA pol II, H4ac, H3K4me3). Under basal conditions, MMP-1 promoter was very weakly enriched in Sp1 and Sp3 transcription factors (figure 4C). In contrast, treatment of HT1080 cells with 5-azadC resulted in a significant increase of RNA pol II recruitment to the TSS (2.5 fold) (figure 4D). Mapping the effects of 5-azadC on the recruitment of Sp1 and Sp3 along the MMP-1 promoter using the same specific primers upstream of the TSS (figure 4E) revealed a significant increase of the binding of Sp1 to the -2.2 Kb and -0.5 kb regions, whereas Sp3 binding was significantly decreased at the -2.2 kb site. Finally, we analyzed the chromatin remodeling on the TSS to define histone marks associated with the 5-azadC-enhanced MMP-1 transcriptional activity (figure 4F). The deposition of the following marks was analyzed: H4ac, H3K4me2, H3K4me3, H3S10P as active marks, and H3K27me3 as a repressive mark. Cells treated with 5-azadC- exhibited important changes in histone modification marks, which globally correspond to a shift to a more permissive chromatin state. An increase of H4ac, H3K4me3 and H3S10P enrichments to the MMP1 promoter after 5-azadC treatments was observed, whereas H3K27me3 enrichment was decreased. H3K4me2 level was not significantly modulated by 5-azadC treatment.

4. Discussion

The lethal outcome of the vast majority of all cancers is due to the dissemination of metastatic tumor cells and the outgrowth of secondary tumors at distant sites. MMPs are implicated in this metastatic process by initiating the turnover of ECM components and by regulating cancer cell migration (Kessenbrock, et al., 2010). It is therefore important to understand how cells tightly modulate, particularly via epigenetic mechanisms, MMP expression levels in order to modulate their invasive properties. In this study, we focused on the role of DNA methylation in the regulation of MMP expressions and in the modulation of the invasive behavior of a human fibrosarcoma cell line (HT1080).

Tumor cell invasion is a multi-step mechanism, which mainly requires both cell adhesion and cell migration (Friedl and Wolf, 2009). In the present study, we showed that, in absence of any epigenetic modulation, the spontaneous crossing of HT1080 cells through the Matrigel® did not require MMP-1, -2 or -9 activities. Similarly, Fisher *et al* showed that these MMP activities were not required for lipid agonist-induced HT1080 cell motility on 2D collagen coating (Fisher, et al., 2006). The mechanisms by which cells may maintain migratory dissemination in the absence of ECM degrading capacity remain unclear. As one possibility, proteolytic compensation could be provided by enzymes not inhibited in this study (*e.g.*; cathepsin) (Puxbaum and Mach, 2009). Alternatively, cells could sustain motility via protease-independent compensation strategies (*e.g.* amoeboid migration) (Sabeh, et al., 2009; Van Goethem, et al., 2010).

5-azadC induced DNA hypomethylation and increased the invasion of HT1080 cells. This suggests that epigenetic events could play a key role in the regulation of this invasive behavior. Since the broad spectrum MMP inhibitor completely abolished 5-

azadC-increased HT1080 cell invasion, induction of MMP expression may be an important mediator of these effects. More thoroughly, we showed that the epigenetically-enhanced HT1080 cell invasion required MMP-1 activity, but did not involve gelatinase activities (MMP-2 and MMP-9). Previous reports revealed an increased invasion of several pancreatic tumor cell lines *in vitro* (Sato, et al., 2003) and described the acquisition of a metastatic phenotype in murine tumor cells *in vivo* (Frost, et al., 1987) after 5-azadC exposure. Since invasion is a complex process and considering that many genes are induced by 5-azadC in cancer cell lines (Liang, et al., 2002), it is likely that the expression of other genes in addition to MMPs contributes to the enhanced invasion by 5-azadC treatment *in vitro*. Nevertheless, it was previously reported that MMP gene expression and tumor cell invasion could be correlated with either (i) MMP promoter methylation status, (ii) histone repressive mark enrichment on MMP promoters or (iii) HDAC expression levels (Chernov, et al., 2009; Park, et al., 2011; Sato, et al., 2003; Shukeir, et al., 2006).

MMP mRNA level analysis reveals that, in HT1080 cells, 5-azadC strongly increased MMP-1 expression, to a lesser extent MMP-9 and very weakly MMP-2 expression. The low induction of MMP-2 by 5-azadC could be related to the fact that its promoter lacks AP-1 binding site and TATA box resulting in its constitutive expression (Yan and Boyd, 2007). Remarkably, MMP-1 promoter contains isolated CpG dinucleotides. In contrast, MMP-2 and -9 promoters display CpG islands which are partially demethylated in the HT1080 cells (Poplineau 2012, submitted). Treatment of these cells with 5-azadC decreases the methylation pattern of MMP-9 promoter only, and not that of MMP-2 (Poplineau, 2012, submitted). MMP-1 is mainly regulated at the transcriptional level, but also by its mRNA stability due to the presence of specific sequences in the 3'-untranslated regions (UTRs) of MMP-1 transcript

(Vincenti, et al., 1996). However, up to now, little is known about the effects of 5-azadC on the molecular mechanisms involved in this gene regulation. We show that 5-azadC increased MMP-1 mRNA level in a transcriptional-dependent manner but does not affect the stability of MMP-1 transcripts. Moreover, a full demethylation of the MMP-1 promoter is not required for MMP-1 gene expression. It was previously reported that genes with low CpG promoters did not display significant correlation between gene activity and the abundance of methylated cytosines within their promoter (Weber, et al., 2007). Since blocking protein neosynthesis by cycloheximide partially abolishes 5-azadC-induced MMP-1 gene expression, some indirect effects of 5-azadC on this gene could not be ruled out. Indeed, we show that the 5-azadC treatment increases the nuclear levels of Sp1 and Sp3 transcription factors, and modulates their recruitment to MMP-1 promoter. Sequence analysis suggests that Sp1 could potentially bind close to the -2.2 Kb region on the MMP-1 promoter, whereas Sp3 did not seem to have consensus binding site but rather used in competition Sp1 consensus binding sequence (Li, et al., 2004). BSP analysis indicates that the methylation status of this region remains unaffected after 5-azadC treatment, but a previous report showed that methylation within the consensus Sp1-binding site did not reduce Sp1/Sp3 binding (Zhu, et al., 2001). Consistent with these observations, a significant recruitment of Sp1 after 5-azadC exposure is observed at the -2.2 Kb region, whereas Sp3 binding is diminished probably due to the competitive effect of Sp1. The additional recruitment of Sp1 to -0.5 kb region was rather unexpected. Further experiments are needed to define whether Sp1 could trigger a chromatin looping between the -2.2 kb and 0.5 kb regions as previously reported for other genes (Deshane, et al., 2010; Nolis, et al., 2009). Sp1 is described as a typical activator of transcription (Li, et al., 2004) of multiple target genes and can

dynamically recruit and form complexes with many other factors, which can cause region-specific changes in histone acetylation and RNA pol II recruitment within gene promoters (Jang and Steinert, 2002). Indeed, treatment of HT1080 cells with 5-azadC results, at the level of MMP-1 promoter, in epigenetic changes which globally correspond to a shift to a more permissive chromatin state (increase of AcH4, H3K4me3, H3S10P enrichment) correlated with an increased of RNA pol II binding. These changes in gene expression have significant impact on the *in vitro* invasive properties of HT1080 cells (figure 5). This observation may initially appear surprising based on the use of 5-azadC as a chemotherapeutic agent but previous studies have demonstrated that DNA hypomethylation can promote tumor dissemination (Frost 1987). Noteworthy, 5-azadC is not currently used for the treatment of human fibrosarcoma and the treatment applied to HT1080 cells appears different from the hypomethylating drug regimens active in myelodysplastic syndromes and acute myeloid leukemia (Seidel 2012). Consequently, the results obtained with 5-azadC may be specific for the HT1080 cells and the conditions of the experiment. However, our data suggest that the DNA hypomethylating agent 5-azadC may have a significant impact on tumor invasion through MMP modulation. Thus, the potential adverse effects of this drug need to be carefully evaluated before clinical applications in new types of solid neoplasms.

5. Acknowledgements

Michael Schnekenburger is supported by a “Waxweiler grant for cancer prevention research” from the Action Lions “Vaincre le Cancer”. Research in Marc Diederich’s laboratory is supported by the “Recherche Cancer et Sang” foundation, the “Recherches Scientifiques Luxembourg” association, by the “Een Häerz fir kribbskrank Kanner” association, by the Action LIONS “Vaincre le Cancer” association and by Télévie Luxembourg. Research in Unité MEDyc’s laboratory is supported by grants from the Comité Départemental de l’Aube de la Ligue Nationale Contre le Cancer.

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Legends of Figure

Figure 1: Effect of 5-azadC on HT1080 invasion and MMP gene expression.

(A)(B) HT1080 cells were treated or not with 5-azadC (5 μ M) for 72 h. Cell invasion was analyzed using a Boyden-modified chamber coated with Matrigel®. Results are expressed as mean \pm SEM. (A) Spontaneous crossing of HT1080 cells through the Matrigel®. (B) 5-azadC effect on HT1080 cell invasion. *** $p < 0.001$ compared to untreated cell value; ** $p < 0.01$ compared to 5-azadC-treated cell values; NS: not significant. (C) Methylation Sensitive Restriction Assay (MSRA). ND: no digestion; *MspI*: insensitive to DNA methylation; *HpaII*: sensitive to DNA methylation. (D) mRNA levels monitored by RT real time PCR. β_2m : internal control. Results are expressed as mean \pm SEM of normalized MMPs/ β_2m ratio. * $p < 0.05$ compared to untreated cells. (E) MMP secretions were analyzed by western blot (MMP-1) and zymography (MMP-2/-9) using HT1080 culture media.

Figure 2: 5-azadC enhances MMP-1 expression through a transcriptional mechanism, but does not induce full demethylation on its promoter. (A)(B)

mRNA levels were monitored by RT real time PCR. β_2m : internal control. Results are expressed as mean \pm SEM of normalized MMPs/ β_2m ratio. * $p < 0.05$; NS: not significant. (A) HT1080 cells were simultaneously treated for 48 h with 5-azadC (5 μ M) and ActD (1 μ g/ml). (B) HT1080 were previously treated for 48 h with 5-azadC (5 μ M) and then incubated for various times with ActD (1 μ g/ml). (C)(D) HT1080 cells were treated for 72 h with 5-azadC (5 μ M) and DNA methylation profile of MMP-1 promoter was analyzed by BSP (C) and CoBRA (D). Genomic DNA was extracted, bisulfite-converted and amplified using several sets of primers (table 1).

Figure 3: 5-azadC-induced MMP-1 expression requires protein neosynthesis.

(A) HT1080 cells were treated for 72 h with 5-azadC (5 μ M) and for 25 h with CHX (10 μ g/ml). mRNA levels were monitored by RT real time PCR. β_2m : internal control. Results are expressed as mean \pm SEM of normalized MMPs/ β_2m ratio. * p < 0.05; NS: not significant. (B) HT1080 cells were treated for various times with 5-azadC (5 μ M). c-Fos, c-Jun, p50, p65, p53, PEA3, Sp1 and Sp3 nuclear levels were analyzed by western blot. Lamin-B was used as loading control.

Figure 4: 5-azadC induces chromatin remodeling, RNA pol II and Sp1

recruitments to MMP-1 promoter. (A) Schematic representation of the MMP-1 5'upstream region with the position of specific PCR primer sets used to map the promoter. (B)(C)(D)(E) ChIP analyses on MMP-1 promoter. ChIPs were performed as mentioned in material and methods. The enrichment was measured by real-time PCR and results are expressed as recovery (% of total input) = $((Ct_{IP} - Ct_{IgG})/Ct_{input})$. Data are the mean \pm SEM of 5 independent experiments. (B) Characterization of the basal MMP-1 promoter. (C)(D)(E) Chromatin dynamics induced by 5-azadC. HT1080 cells were treated for 36 h with 5-azadC (5 μ M).

Figure 5: Schematic representation of 5-azadC effects on MMP-1 expression and on the invasive behavior of HT1080 cells

Supplementary figure 1: Positions of the set primers used to map MMP-1 promoter in BSP and CoBRA

Figures

Figure 1

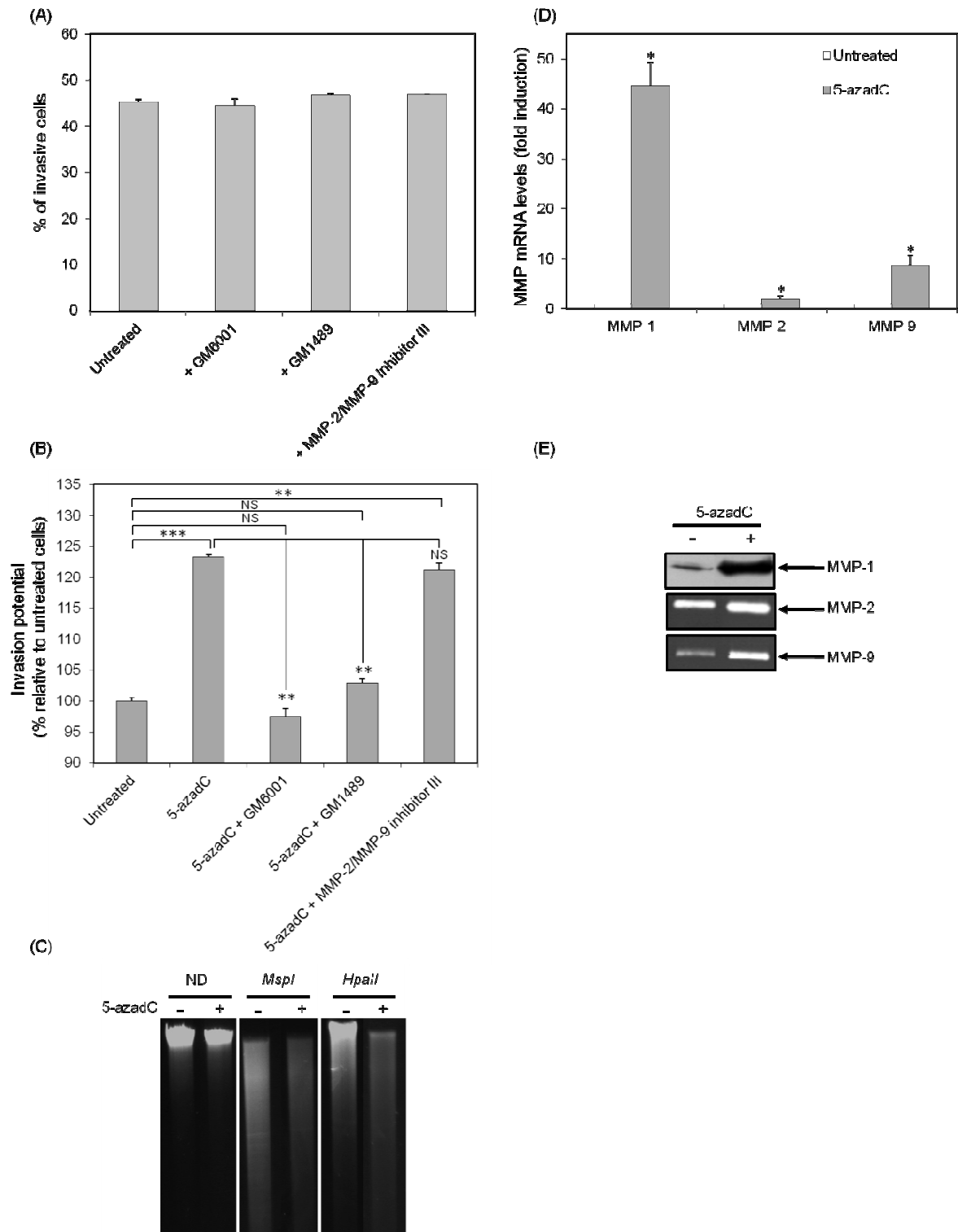


Figure 2

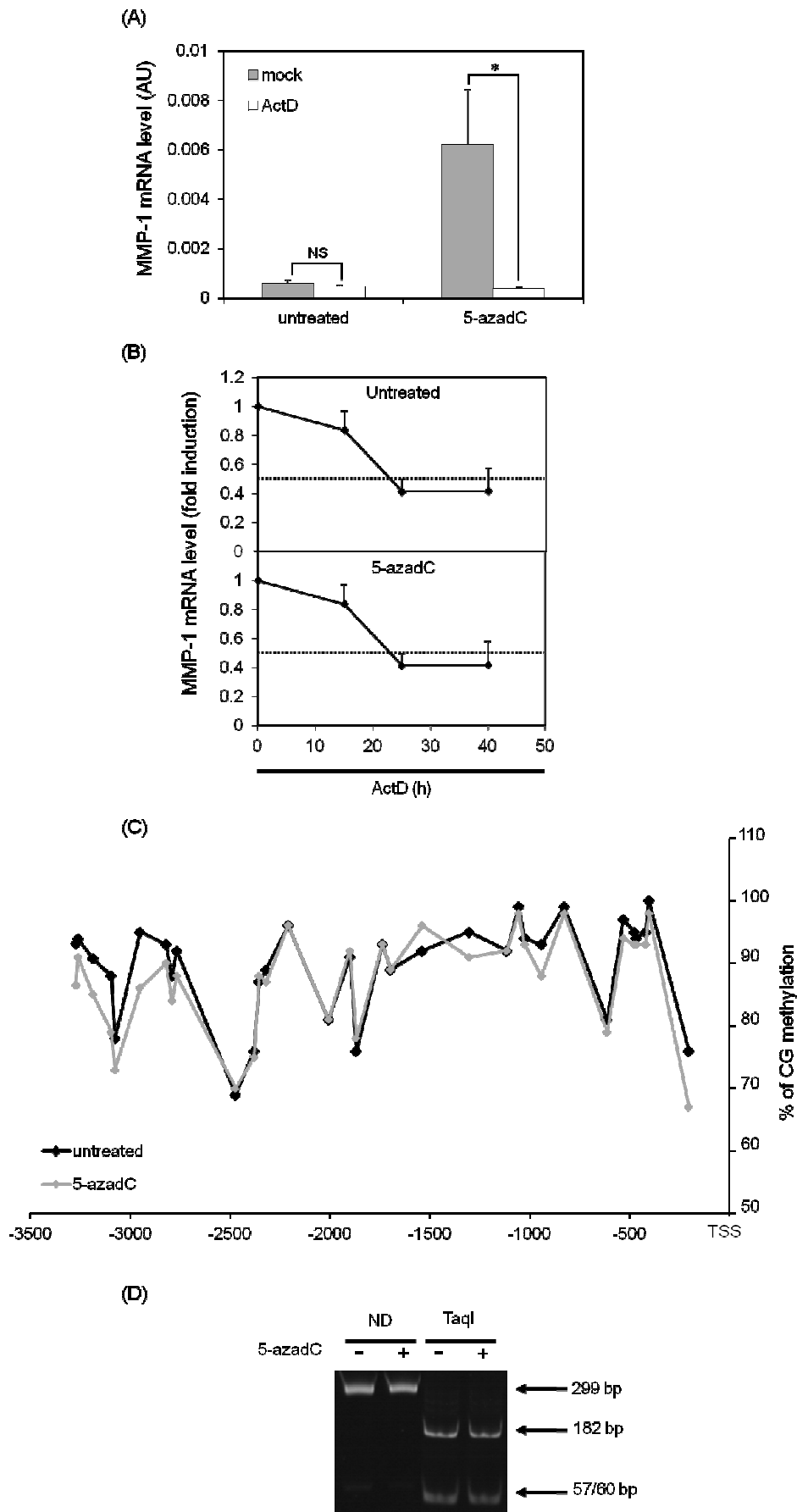


Figure 3

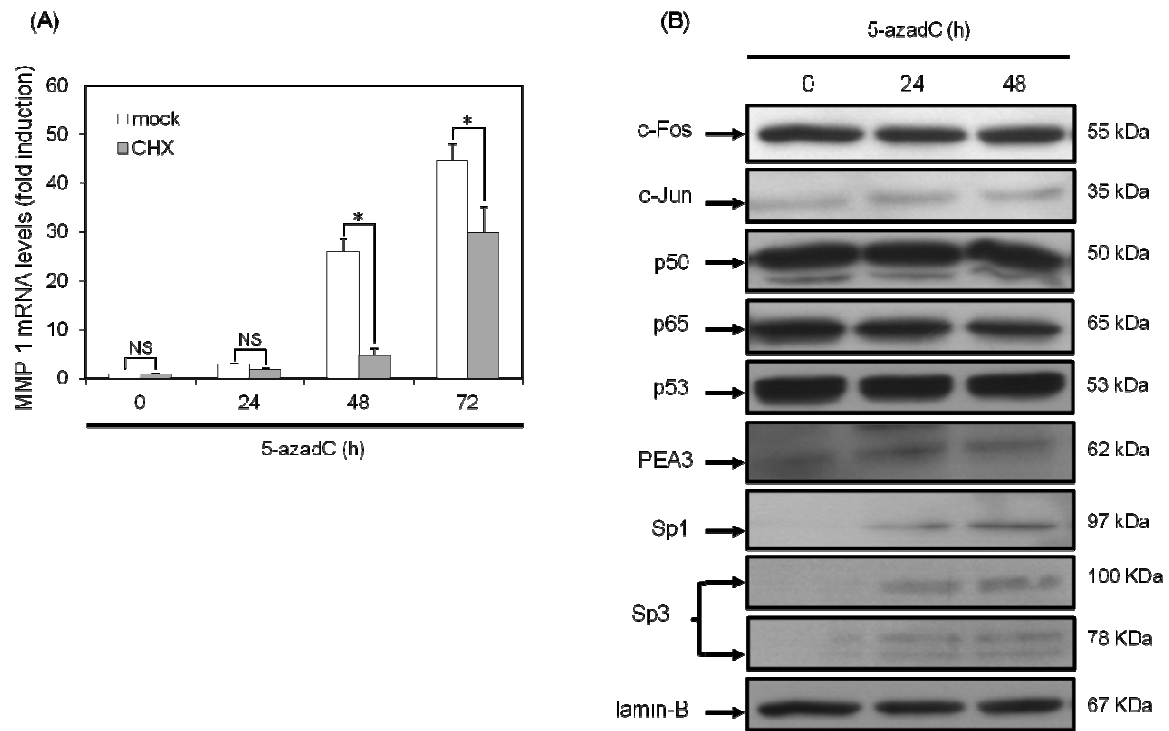


Figure 4

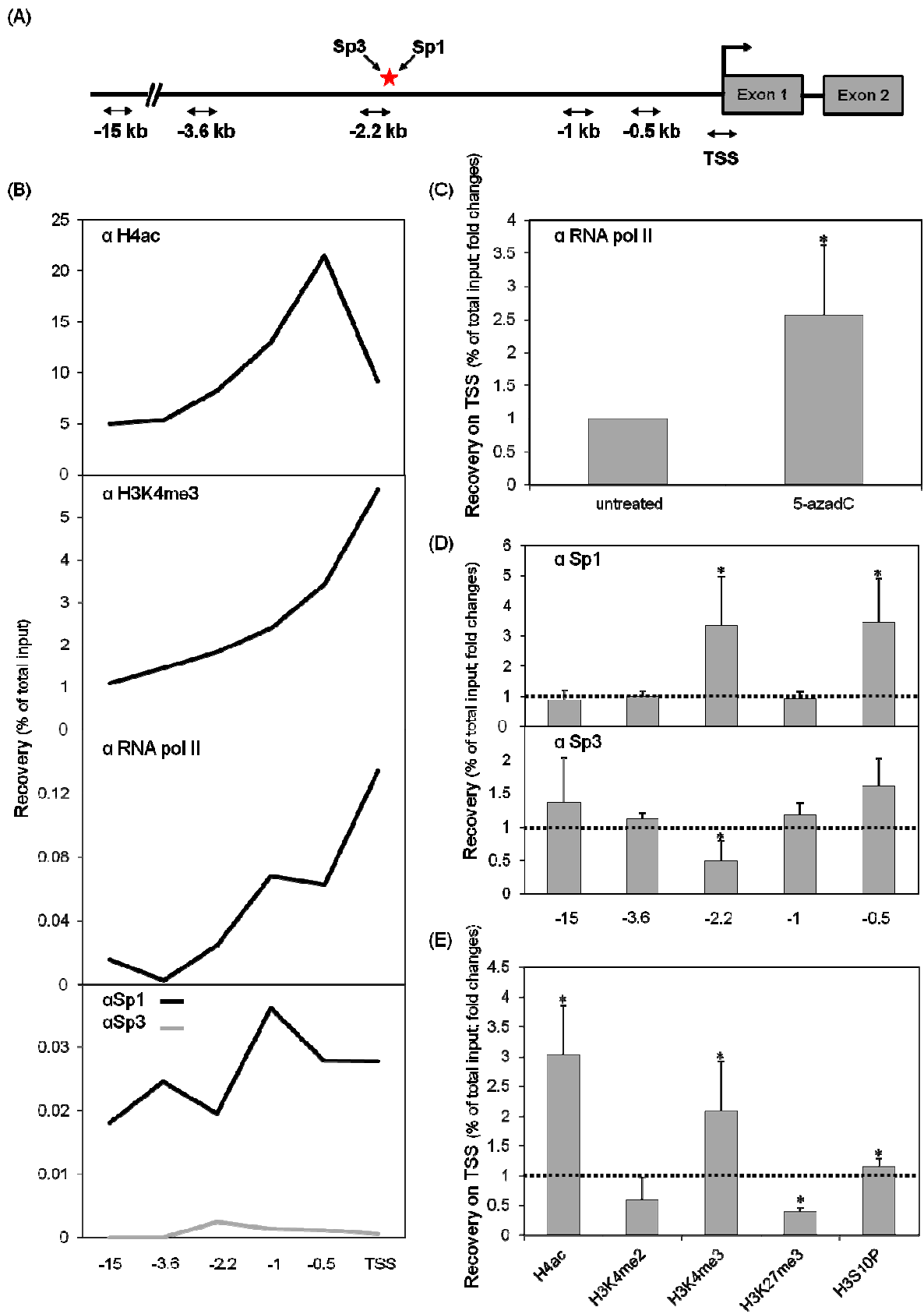
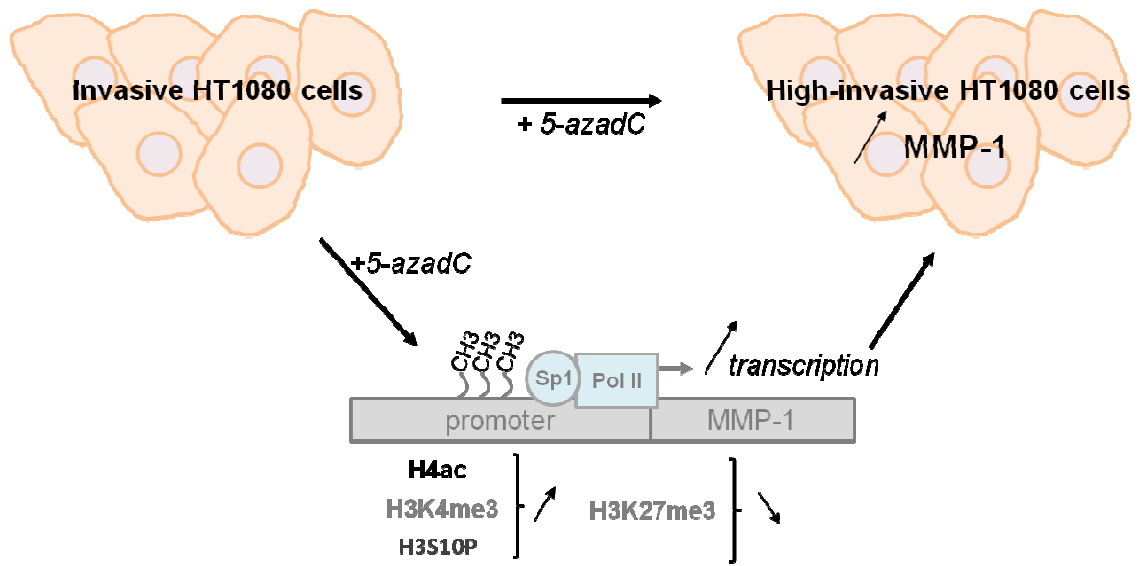
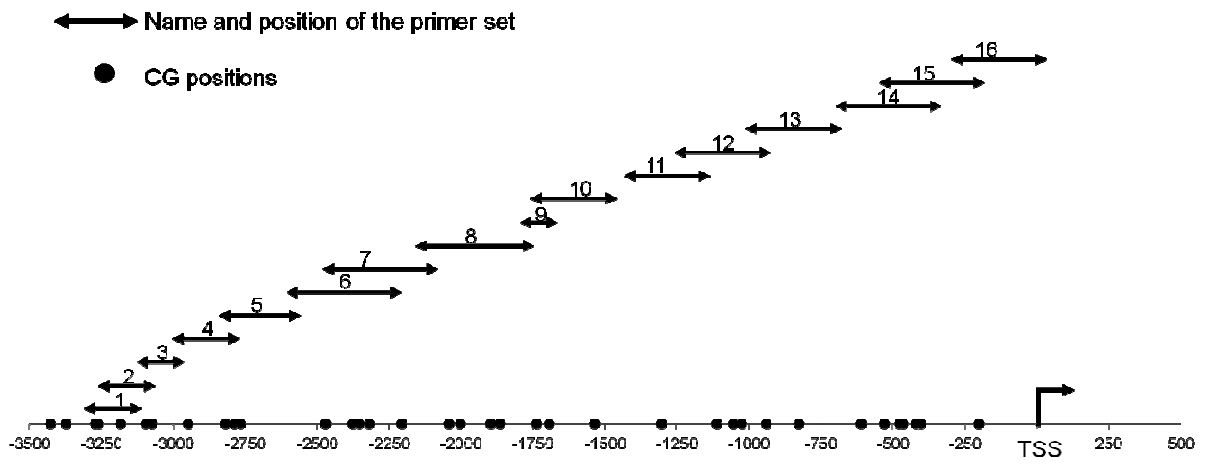


Figure 5



Supplementary figure 1



Tables

Table 1.1:

Primers used for real-time PCR	
MMP-1	F : 5'-GAGCAAACACATCTGACC TACAGGA-3' R : 5'-TTGTCCCGATGATCTCCCCTGACA-3'
MMP-2	F : 5'-TCTTCCCCTTCACTTTCCTG-3' R : 5'-ACTTGCGGTCGTCATCGT-3'
MMP-9	F : 5'-TGACAGCGACAAGAAGTGG-3' R : 5'-GGCGAGGACCATAGAGGTG-3'
β_2 microglobulin	F : 5'- ATCTTCAAACCTCCATGATG-3' R : 5'-ACCCCCACTGAAAA AGATGA-3'
F : Forward	
R : Reverse	

Table 1.2:

Primers used for BSP on MMP-1 promoter *	
set 1	F : 5'- TTTGGATTGTAGTGGTATAGAATTGTG-3' R : 5'- CCATTAACCAAACACTTAACAAACA-3'
set 2	F : 5'-TTGTGTGTTTTGGGATTTGG-3' R : 5'-TCTTCCTACATATAACCATACAATAACTTT-3'
set 3	F : 5'-TGTTTGTAAAGTGTGGTAAATGG-3' R : 5'-TTTTTATCCTACAAATCTTAAATAATCT-3'
set 4	F : 5'-TTTGATATTTAAATGGAGTT-3' R : 5'-ACTATTTCAAACAACCAAATTA-3'
set 5	F : 5'-GAAATAATTTGGTTGTTGGAAATAG-3' R : 5'-AAATCATACCAAATCCTAACAATACCT-3'
set 6	F : 5'-GGTATTGTTAGGATTTGGTATGATTT-3' R : 5'-ACTTTTTCTCCCTTCTCTAACAAA-3'
set 7	F : 5'-TTAGGGTTATGTGAATTTAGGTTGG-3' R : 5'-TCCTTACTTAAACAAAATCCCTTT-3'
set 8	F : 5'-AAAAGGGATTTTGTAAAGTAAAGG-3' R : 5'-CCACTAAAATAACTCCAACACAAAC-3'
set 9	F : 5'-TTGTGTTGGAGTTATTTTAGTGGT-3' R : 5'-AAAATCCTCTTCTCAAAAACATCA-3'
set 10	F : 5'-TGATGTTTTTGGAGAAGAGGATTTTT-3' R : 5'-TTTCAACACTTCTCCCTTA-3'
set 11	F : 5'-GGAAGGAGAGTGGGGTATGAG-3' R : 5'-ATTTATACATTCTCCATTCAAAAA-3'
set 12	F : 5'-TTATTTAATGGGTAGGAGATGTTAAA-3' R : 5'-AAAACAAATAAAATCCTTCCCTAAA-3'
set 13	F : 5'-AAGGGGTGGGGAGTTATTT-3' R : 5'-TTAACCAAACATAAAATCATACACCTA-3'
set 14 #	F : 5'-TTTAGGTTGATTTTGAATTTTTGG-3' R : 5'-CACACCTTACTCCCAAAACAAA-3'
set 15	F : 5'-AGTTTAATAAAGGTAGAAGGGAATTTT-3' R : 5'-CAATAATTTTCTCCAATTCATTTCTTT-3'
set 16	F : 5'-GGAAAGAAATGAATTGGAGAAAA-3' R : 5'-TTCCCAACCTCTTACTACTCCA-3'

F : Forward
R : Reverse
* : set positions on supplementary figure 1
† : set positions on figure 4A
set used for CoBRA

Table 1.3:

Primers used for ChIP on MMP-1 promoter †	
- 15 kb	F : 5'- GAAGGATTGCCTTCCAATCA-3' R : 5'- GGTCTCAGAATGTCCCCAAA-3'
- 3.6 kb	F : 5'- TGTAACAATCATTTCAGACACC-3' R : 5'- AATCCTCCCCTTCAAGCTGT-3'
- 2.2 kb	F : 5'- CCAATCCACGTCAGCAACTA-3' R : 5'- ACATCATGCCTGCTTTTTCC-3'
- 1 kb	F : 5'- TTCCAGCCTTTTCATCATCC-3' R : 5'- GAGAAGACCCCTCATCCACA-3'
- 0.5 kb	F : 5'- GGGTACCAGGCAGCTTAACA-3' R : 5'-CGGCACCTGTACTGACTGAA-3'
TSS kb	F : 5'-CTGGAAGGGCAAGGACTCTA-3' R : 5'- AGCTGTGCATACTGGCCTTT-3'
F : Forward	
R : Reverse	

Table 2:

Target	Company	Reference no.	Application
MMP-1	Calbiochem	IM35	WB
c-Fos	Millipore	06-341	WB
c-Jun	Active Motif	39309	WB
PEA3	Santa Cruz	sc-22806	WB
NFκB p50	Santa Cruz	sc-7178	WB
NFκB p65	Santa Cruz	sc-8008	WB
p53	Dako Cytomation	M7001	WB
Sp1	Active Motif	39058	WB, ChIP
Sp3	Santa Cruz	sc-644	WB, ChIP
Lamin-B	Santa Cruz	sc-6216	WB
β-actin	Sigma Aldrich	A1978	WB
H4ac	Millipore	06-866	ChIP
H3K4me2	Millipore	07-030	ChIP
H3K4me3	Millipore	07-473	ChIP
H3K27me3	Millipore	07-449	ChIP
RNA pol II	Millipore	05-623	ChIP
Goat anti-mouse IgG-HRP	Millipore	12-349	WB
Donkey anti-rabbit IgG-HRP	Millipore	12-348	WB
Sheep anti-goat IgG-HRP	Sigma Aldrich	A9452	WB

WB: western blot ; ChIP: chromatin Immunoprecipitation

PUBLICATION N°4

The histone deacetylase inhibitor MS-275 induces MMP-1 gene expression and chromatin dynamics on its promoter

M. Poplineau, M. Schnekenburger, J. Dufer, F. Antonicelli, M. Diederich, A. Trussardi-Régnier.

Manuscrit en cours de finalisation pour publication

Présentation de la PUBLICATION N°4

L'inhibiteur d'HDACs de classe I, MS-275, induit l'expression du gène de la MMP-1 et un remodelage de la chromatine au niveau de son promoteur

Contexte

Au cours du cancer, l'expression de certains gènes est affectée en raison de dérégulations de l'activité de modulateurs transcriptionnels, tels que les histones désacétylases (HDACs). De ce fait, ces enzymes constituent une cible thérapeutique d'intérêt et leur activité peut être réprimée par diverses molécules appelées HDIs (*HDAC inhibitors*). A l'heure actuelle, quelques HDIs sont en essai clinique ou sont déjà utilisés en thérapeutique (Florea, 2011). Ces modulateurs épigénétiques sont regroupés en différentes catégories en fonction de leur spécificité structurale et de substrats. Le SAHA (*suberoylanilide hydroxamic acid*) et la TSA sont des dérivés de l'acide hydroxamique et sont communément appelés pan-HDIs puisqu'ils répriment différentes HDACs de classe I et II. Le MS-275 est un benzamide dont la spécificité se limite aux HDACs de classe I. Nous avons montré précédemment que la TSA, administrée seule, n'affectait, ni l'organisation de la chromatine, ni l'expression de MMP-1 et des gélatinases (Poplineau, 2011a). De ce fait, nous avons voulu savoir si ces résultats étaient uniquement spécifiques de la TSA ou pouvaient être étendus à d'autres HDIs. Pour répondre à cette interrogation, nous avons donc testé l'effet de différents HDIs, le MS-275, le SAHA et la TSA, sur l'expression de MMP-1. Ces travaux préliminaires, ont fait l'objet de la **PUBLICATION N°4** qui est en cours de finalisation.

Matériel et Méthodes

Des cellules HT1080 ont été traitées par les HDIs suivants : le MS-275, le SAHA ou la TSA. De plus, ces cellules ont été traitées simultanément par le MS-275 et la 5-azadC. L'expression génique et protéique de la MMP-1 ont été analysées respectivement par RT-PCR en temps réel et western blot. Les niveaux nucléaires des facteurs de transcription

étudiés dans la PUBLICATION N° 3 ont été également analysés dans cette étude par western blot. Des expériences de CHIP ont été réalisées en vue d'analyser la dynamique transcriptionnelle et le remodelage chromatinien au niveau du promoteur du gène de la MMP-1 induit par le MS-275. Des données supplémentaires concernant les effets du MS-275 sur la viabilité, l'apoptose et le cycle des cellules HT1080 sont développées en ANNEXE 7.

Résultats

Dans cette étude préliminaire, nous montrons que le MS-275 et les pan-HDIs, SAHA et TSA, entraînent une hyperacétylation de l'histone H4 (nettement plus marquée après un traitement par le MS-275) mais présentent des effets différentiels sur l'expression de MMP-1. En effet, seul le MS-275 est capable d'augmenter les niveaux d'ARNm et protéique de MMP-1. Un traitement par les pan-HDIs SAHA et TSA demeure sans effet sur ces deux paramètres étudiés. Un traitement simultané des cellules HT1080 par le MS-275 et la 5-azadC augmente de surcroît l'expression génique de MMP-1. Cet effet correspond à la somme des effets générés par chacun des deux modulateurs épigénétiques. Par ailleurs, nous montrons que le MS-275 augmente les niveaux nucléaires de Sp1, Sp3 et diminue celui de c-Jun. Néanmoins, le MS-275 n'induit aucun recrutement de ces facteurs de transcription au niveau du promoteur du gène de la MMP-1. Le MS-275 induit cependant un recrutement d'ARN polymérase II et des protéines CBP et p300. Ces recrutements s'accompagnent d'un enrichissement en marques actives H4ac, H3K4me2, H3K4me3 et H3S10P au niveau du promoteur du gène de la MMP-1.

Conclusion

Dans cette étude, nous montrons que le MS-275 et les pan-HDIS, SAHA et TSA présentent des effets différentiels sur l'expression de MMP-1. De plus, nous montrons que le MS-275 et la 5-azadC exercent des effets additifs sur l'expression de ce gène, suggérant que ces deux modulateurs agissent sur des voies de régulation génique distinctes. L'établissement d'une chromatine plus permissive caractérisée par un enrichissement en marques actives et en actelytransférases (p300 majoritairement et CBP dans une moindre mesure), semble favoriser l'activation transcriptionnelle (recrutement d'ARN polymérase II) du gène de la MMP-1 induite par le MS-275.

The histone deacetylase inhibitor MS-275 induces MMP-1 gene expression and chromatin dynamics on its promoter.

Mathilde Poplineau¹, Michael Schnekenburger², Frank Antonicelli³, Marc Diederich², Jean Dufer¹, Aurélie Trussardi-Régnier¹ *.

¹Unité MEDyC, URCA-CNRS FRE 3481, SFR Cap Santé, Faculté de Pharmacie, Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

²Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, L-2540 Luxembourg, Luxembourg

³Laboratoire de Dermatologie, UFR Médecine, 1 avenue du Maréchal Juin, 51096 Reims, France

* corresponding author: Tel: +33 326918016; Fax: +33 326913550; E-mail address: aurelie.regnier@univ-reims.fr; address: Unité MEDyC, URCA-CNRS FRE 3481, SFR Cap Santé, Faculté de Pharmacie, Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

Abstract

Matrix metalloproteinases (MMPs) are potentially targets for molecular therapies in patients with aggressive cancer, and histone deacetylase inhibitors (HDIs) represent promising drugs for such a cancer treatment. Within the MMP family, MMP-1 is an important member which plays critical role in cancer progression and metastasis. In a previous work, we demonstrated that, when used alone, trichostatin A (TSA), a broad-spectrum HDI, was unable to modulate MMP-1 gene expression in human HT1080 fibrosarcoma cells. In the present study, we extended this analysis and examined the effects of the HDAC Class I-specific benzamide MS-275 on MMP-1 expression in HT1080 cells. We found that this HDI could markedly induce MMP-1 mRNA and protein production. This was associated with a chromatin remodeling at the MMP-1 gene promoter level, characterized by a local histone H4 hyperacetylation through the recruitment of p300. Furthermore, MS-275 displayed additive effects to the hypomethylating agent 5-aza-deoxycytidine (5-azadC) in the induction of MMP-1 expression.

1. Introduction

Epigenetics refers to a variety of processes which have long-term effects on gene expression without changes in DNA sequence. Key players in the epigenetic control are histone post-translational modifications and DNA methylation which act in concert with chromatin remodeling complexes to establish the chromatin structure around a gene and regulate its transcriptional activity [1]. Among the covalent modifications representing a chromatin-level regulation, the most studied are acetylation of the N terminal tails of histones H3 and H4. The transfers of acetyl groups are catalyzed by histone acetyl transferases (HATs) and histone deacetylases (HDACs). These enzymes act in concert with other regulators to induce chromatin remodeling leading to the transcriptional gene activation or repression [2]. Acetylation of histones is known to turn on gene expression by masking the positive charge on lysine residues thereby antagonizing chromatin folding [3]. It was previously reported that transcriptional regulators such as HDACs could be deregulated in cancer at the level of their expression or their activity resulting in changes in gene expression [4]. This observation prompted the cancer research field to elaborate new class of compound targeting HDAC activity. HDAC inhibitors (HDIs) have emerged as new promising tools [5] for the pharmacological gene regulation in cancer due to their ability to induce cell cycle arrest, differentiation and cell death [6]. Some of these have been approved for the treatment of hematological malignancies [1] and are available for clinical investigation in solid tumors [7, 8]. Several molecules, belonging to different classes have then been identified as potential anticancer drugs: e.g fatty acids such as sodium butyrate or valproic acid, hydroxamates such as SAHA (suberoylanilide hydroxamic acid), cyclic tetrapeptides such as depsipeptide, or benzamides such as MS-275 [9]. These drugs have been shown to modulate the activity of a myriad of

genes in various tumor types [10]. For instance, it has been reported that MS-275 could promote differentiation and apoptosis of leukemia cells [11], sensitize glioblastoma cells for chemotherapy-induced apoptosis [12] and inhibit the growth of tumor cell lines implanted into a mouse model [13]. However, the impact of this drug on genes implicated in tumor invasion and progression is unclear. Among these, genes encoding matrix metalloproteinases appear particularly important [14].

Modulation of cell-matrix interactions in cancer occurs through proteolytic systems responsible for the aberrant degradation of extracellular matrix (ECM) components. By regulating the composition and the dynamic structure of the ECM, these systems play a pivotal role in the control of the proliferation, the migration and the invasion of cancer cells. Mounting evidences supports the view that among these proteolytic systems, matrix metalloproteinases (MMPs) appeared as key regulators of the tumor microenvironment [15]. In cancer, these enzymes regulate, through the matrix breakdown and the release of bioactive molecules, a variety of processes and signaling events such as inflammation, angiogenesis, cell growth and survival, invasion and metastasis [16]. An interesting mechanism of MMP-mediated signal linked with increased metastasis is observed in the presence of MMP-1 (type I collagenase) which is able to degrade main components of the basal membrane (*i-e*: type I and III collagens) [17] but also non-matrix substrate such as PAR-1 [18] or EGF-like ligands [19]. MMP-1 is considered as an inducible gene targeted by upstream signaling pathways, and whose expression involved a series of transcriptional and posttranslational events [20]. Moreover, recent reports highlighted the important role of epigenetic mechanisms in the regulation of this gene expression [21, 22]. Histone acetylation *per se* seemed to be insufficient to induce MMP-1 expression in different cell lines since broad spectrum HDIs (pan-HDIs) such as

trichostatin A (TSA) had no effect on MMP-1 gene expression despite global or local histone hyperacetylation [21, 22]. Pan-HDI seemed to have effects only on stimulated level of MMP-1 suggesting that these HDI effects could target the signaling pathways induced by exogenous stimuli (*e.g.*, inflammatory cytokines, growth factors and phorbol esters) rather than the MMP-1 gene itself [21]. Currently, little is known about the absence of effects of pan-HDIs on MMP-1 expression, and no study using more specific HDIs have been performed. For this purpose, in the present study, we analyzed the effects of three HDIs presenting different specificity degrees: TSA and SAHA (pan-HDIs) and MS-275 (Class I HDAC inhibitor) on MMP-1 expression in the human HT1080 fibrosarcoma cell line.

2. Material and Methods

2.1. Chemicals

Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and 5-aza-2'deoxycytidine (5-azadC) were purchased from Sigma-Aldrich (France). MS-275 was purchased from Alexis (San-Diego, CA, USA).

2.2. Cell culture

Human fibrosarcoma HT1080 cells (ATCC, CCL-121) were grown at 37 °C in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified 95% air, 5% CO₂ atmosphere.

2.3. MMP-1 gene expression

After treatment, cells were washed twice in ice-cold PBS and total RNAs were isolated using NucleoSpin® RNA II kit (Macherey Nagel, France). For real time RT-PCR, 1 µg of RNA was reverse transcribed using MML-V reverse transcriptase (Fisher Scientific, France), and each cDNA sample was analyzed by real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, France) on the LightCycler system (Roche Diagnostics, France). MMP-1 transcripts were amplified using primers represented in table 1. Relative quantification was performed by using the housekeeping gene β_2 microglobulin as internal control.

2.4. Western blot analyses

For MMP-1 western blot analysis, serum-free media from HT1080 cell cultures were harvested and proteins concentrated using Amicon Ultra centrifugal filters (Millipore, France). For transcription factor western blot analyses, total nuclear proteins were extracted using the Nuclear Extract kit (Active Motif, Belgium). Total proteins (35 µg) were heated 5 min at 100 °C in Laemmli sample buffer, separated on 10% SDS PAGE under reducing conditions and transferred to PVDF membrane by using I-Blot

system (Invitrogen, France). The membranes were immersed in blocking solution for 2 h at room temperature, and incubated with the primary antibodies of interest (table 2). Proteins were revealed using a peroxidase-conjugated secondary antibody (table 2) and the ECL Plus detection kit (Amersham, France). Cells culture media were harvested and concentrated using Amicon Ultra centrifugal filters (Millipore, France).

2.5. Histone extraction and immunoblotting

Histones were acid extracted with HCl and precipitated with acetone. Total histones (15 µg) were separated on 15% SDS PAGE and transferred to PVDF membrane. The membranes were blocked during 2 h and incubated overnight with a primary antibody against acetylated total histone H4 (table 2). Histone acetylation was revealed using a peroxidase-conjugated anti rabbit secondary antibody (table 2) and the ECL Plus detection kit (Amersham, France).

2.6. Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [23]. In brief, $5 \cdot 10^6$ cells were cross-linked with 1% formaldehyde (MP biomedical, France) for 8 min at room temperature. After cross-linking, the reaction was quenched with 0.125 M of glycine for 10 min at room temperature. Cells were washed twice with ice cold D- PBS, pelleted by centrifugation, resuspended in 1 ml of lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Igepal) containing 1X protease inhibitor cocktail (Complete® minus EDTA) and incubated 30 min at 4 °C under agitation. After centrifugation, nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing 1X protease inhibitor cocktail and incubated for 10 min on ice. Chromatin suspensions were sonicated on ice using a Bioruptor (Diagenode, France). After centrifugation, to remove cell debris, chromatin was pre-cleared 1 h at 4 °C with 50% gel slurry of protein A/G-agarose beads saturated with salmon sperm DNA and

bovine serum albumin (Millipore, France), and diluted in the IP dilution buffer (0.01% SDS, 0.5% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 100 mM NaCl and 1X protease inhibitor cocktail). 10% of the diluted supernatant was used as input. The diluted chromatin was incubated overnight at 4 °C with the antibodies of interest (table 2), and the immune complexes were recovered by 2 h incubation at 4 °C with gel slurry of protein A/G-agarose beads. The precipitated immune complexes (DNA-proteins-antibodies-beads) were washed sequentially with the ice-cold buffers of the kit: once wash with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA and 20 mM Tris-HCl pH 8.1, 150 mM NaCl and 1X protease inhibitor cocktail), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA and 20 mM Tris-HCl pH 8.1, 500 mM NaCl and 1X protease inhibitor cocktail), LiCl buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1% Igepal, 1% deoxycholic acid and 1X protease inhibitor cocktail), twice washes with the TE buffer (1 mM EDTA and 20 mM Tris-HCl pH 8.1). Then, DNA was extracted twice with freshly prepared elution buffer (100 mM NaHCO₃ and 1% SDS) with mild homogenization. The cross-linking between DNA and proteins was reversed by an overnight incubation at 67 °C with 0.3 M NaCl with proteinase K (Roche, France). DNA was purified using spin columns of the QIAquick PCR purification kit (Qiagen, France). Purified DNA samples were analyzed by real-time PCR using the Power SYBR® Green PCR Master Mix (Life Technologies, France). PCRs were performed using primers that covered different regions of the MMP-1 promoter.

2.7. Statistical analyses

All experiments were performed at least in triplicate. Results are expressed as mean ± SEM. Statistical analysis was performed using Kruskal-Wallis test. The results were considered significantly different when $p < 0.05$.

3. Results

3.1. MS-275 increases MMP-1 expression

In order to know whether global histone hyperacetylation could be related to MMP-1 expression, fibrosarcoma HT1080 cells were treated with the following HDIs: MS-275 (10 μ M), SAHA (2 μ M) or TSA (100 ng/ml) for 24 h. As shown on Figure 1A, all HDIs induced global histone H4 acetylation, with a more pronounced effect for MS-275 as compared to SAHA and TSA. Real-time RT-PCR revealed that MS-275 significantly increased MMP-1 mRNA level but SAHA and TSA were ineffective on this gene expression (figure 1B). Analyses of extracellular secretion of MMP-1 by western blot confirmed that this MS-275-enhanced MMP-1 gene expression was correlated with an increase of the protein level (figure 1C).

3.2. MS-275 and the hypomethylating agent 5-azadC have additive effects

As a cross talk between DNA methylation and histone acetylation could occur, the effects of a simultaneous treatment with MS-275 and the hypomethylating agent 5-azadC were analyzed. For this purpose, HT1080 cells were treated for 72 h with 5-azadC (5 μ M) and with MS-275 (10 μ M) added during the last 24 h of incubation. Results showed that treatments with 5-azadC alone and MS-275 alone increased MMP-1 gene expression (figure 2A). The simultaneous exposure to both drugs induced a further increase in MMP-1 gene expression (figure 2A). This increase (60 fold) seemed to be equivalent to the sum of the effects induced by MS-275 alone and 5-azadC (15 fold and 45 fold, respectively). The MMP-1 secretion pattern analyzed by western blot was correlated to the gene expression profile (figure 2B).

3.3. MS-275 induces chromatin dynamics on MMP-1 promoter

The potential chromatin remodeling induced by MS-275 on MMP-1 promoter was analyzed by chromatin immunoprecipitation (ChIP). For this purpose, RNA pol II,

histone acetyl transferase (CBP and p300) recruitments and the deposition of the active marks H3ac, H4ac, H3S10P, H3K4me2 and H3K4me3 were evaluated by real-time PCR using specific primers sets targeting MMP-1 promoter (figure 3A). MS-275 increased RNA pol II and CBP enrichments on the regions close to the TSS (-0.5 kb and TSS for RNA pol II, and -0.5 kb and + 0.75 kb for CBP) whereas p300 was recruited to several regions within MMP-1 promoter (figure 3B). Moreover, cells treated with MS-275 exhibited important changes in histone modification marks which globally correspond to a shift to a more permissive chromatin state (figure 3B). This changes affected mainly H4ac (globally increased along MMP-1 promoter), H3S10P (increased at -3.6 kb and - 1 kb), H3K4me2 and H3K4me3 (increased at +0.75 kb) depositions (figure 3B). However, H3ac deposition remained unaffected by MS-275 treatment (figure 3B).

In order to know if transcription factor(s) could be involved in the MS-275-enhanced MMP-1 gene expression, the nuclear levels of transcription factors known to be implicated in MMP-1 gene regulation were evaluated by western blot (Figure 4). The treatment with MS-275 did not affect nuclear level of c-Fos, p50, p65, p53 and PEA3. It increased slightly the nuclear level of the Sp-family members Sp1 and Sp3, but decreased the nuclear level of c-Jun.

Subsequently, the potential recruitments of these transcription factors on the MMP-1 promoter were analysed by ChIP (Figure 5). Binding sites of these transcription factors and specific primers sets targeting MMP-1 promoter are represented on the figure 5A. As shown on figure 5B, MS-275 did not induce any significant recruitment of these transcription factors to the MMP-1 promoter.

4. Discussion

A link between chromatin hyperacetylation and tumorigenesis seems to emerge from studies indicating that many HDIs are able to induce cell growth arrest and differentiation in a variety of cancer cells [6]. However, elucidating the roles of the individual HDAC isoforms in cancer requires new molecular tools, including isoform-selective inhibitors. Studies assessing HDAC enzymatic selectivity seem to agree that most of hydroxamic acids such as TSA or SAHA are not selective [24]. MS-275 is a novel agent with HDAC-inhibiting activity that is structurally dissimilar from other HDIs [9]. In this study, we focused on the effects of this HDI on the expression of MMP-1 and compared its effects to those induced by the non-selective HDIs, TSA and SAHA.

The role of histone acetylation in the expression of MMPs has been studied for a number of genes such as MMP-7, gelatinases or MMP-3 [25-29] but only few data pointed the role of this epigenetic event in the regulation of MMP-1 expression [21, 22]. It was previously reported that the pan-HDI TSA repress $IL-1\beta$ or $TNF\alpha$ -mediated induction of MMP-1, both at the mRNA and protein levels [29] and that this HDI does not display any effect on unstimulated levels of MMP-1 [21, 22, 29]. Consistent with this observation, we showed that the pan-HDIs TSA and SAHA, despite induction of global histone H4 hyperacetylation, did not modulate MMP-1 expression. In contrast, MS-275 strongly acetylated histone H4, and significantly increased MMP-1 expression. It was reported that MS-275 preferentially inhibits class I HDACs (HDAC1 over HDAC3) [9]. This could suggest that HDAC1, -2, -3 might potentially contribute to the repression of MMP-1 gene expression under basal condition. However, TSA and SAHA also efficiently inhibit HDAC1, -2, -3 and class II HDACs (HDAC4, -6, -7 -9), but were not able to modulate MMP-1 gene expression. Further

experiments are then needed to evaluate whether Class I HDACs might contribute to repress MMP-1 gene expression and that Class II HDACs might rather participate to enhance its expression. This could fit well with previous data showing that the class I HDAC, HDAC1, led to the transcriptional repression of MMP-1 in rheumatoid arthritis synovial fibroblast (RA-SF) [30], but conversely, the class II HDAC, HDAC4, was also reported as a repressor of MMP-1 expression in the same cellular RA-SF model [31]. Concerning the role of the other class II HDACs inhibited here by the pan-HDIs (HDAC6, -7, -9), to the best of our knowledge, no data could bring understanding about their role in the regulation of MMP-1 expression. Accumulation of acetylated histones induced by HDIs associated with changes in gene expression remains a complex mechanism, since it could result from a decrease in HDAC activity, an increase of HAT activity, or both. In addition to histones, HDACs target a huge number of non-histone proteins, including many transcription factors (*e.g.*, p53), α -tubulin, Hsp90, and various signaling proteins [32], which further complicates the understanding of HDI actions.

We previously reported that TSA was able to induce MMP-1 expression only after DNA hypomethylation by 5-azadC and that both drugs displayed synergistic effects on either gene expression or nuclear architecture alterations [22]. These ordered re-expression and synergy have already been reported for other genes [33]. In contrast, MS-275 does not display any synergistic effect with 5-azadC but presents rather additive effects on the MMP-1 expression. This could suggest that MS-275, unlike to TSA, does not require DNA methylation to be efficient on MMP1 gene expression and that it might act on a different pathway than those targeted by 5-azadC.

Screening nuclear levels of transcription factors, known to be involved in MMP-1 expression reveals that MS-275 increases the Sp family members Sp1 and Sp3 as it

was previously reported for 5-azadC (Poplineau 2012, manuscript in preparation). However, mapping transcription factor binding to the MMP-1 promoter showed that MS-275 was not able to induce Sp1 recruitment, contrary to 5-azadC (Poplineau 2012, manuscript in preparation). Despite the fact that none of the transcription factors (p50, p65, c-Fos, c-Jun, PEA3 and p53) analyzed in the present study was recruited to MMP-1 promoter, MS-275 was able to induce chromatin remodeling at this level. This remodeling globally corresponded to a shift to a more permissive chromatin state (increase in H4ac, H3K4me2, H3K4me3 and H3S10P depositions) and is associated with an increase of transcription (increase in RNA pol II recruitment at the TSS). MS-275 seemed to induce local histone H4 hyperacetylation through the recruitment of p300 (whereas CBP binding remain weakly affected), a process already described in MMP-1 gene regulation [34].

Taken together, our data indicate that MS-275 is able to induce MMP-1 expression in human fibrosarcoma cells. This could have therapeutic relevance as this MMP play a significant role in tumor cell metastasis and modulating its expression in HT1080 cells results in increased cell invasion (Poplineau submitted). Indeed, the results obtained with MS-275 may be specific for the HT1080 cells and the conditions of the experiment, as this drug has been shown to suppress cell invasion in thyroid cancer cells [35]. MS-275 (entinostat) is an HDI that demonstrates promising therapeutic potential in both solid and hematologic malignancies [36]. In non small cell lung cancer (NSCLC) patients, it has shown promising results when combined with 5-azadC [37], but did not improve patient outcomes in combination with erlotinib [38]. Therefore, more relevant biomarkers are needed to adequately assess its activity before clinical applications in new types of solid neoplasms.

5. Acknowledgements

Michael Schnekenburger is supported by a “Waxweiler grant for cancer prevention research” from the Action Lions “Vaincre le Cancer”. Research in Marc Diederich’s laboratory is supported by the “Recherche Cancer et Sang” foundation, the “Recherches Scientifiques Luxembourg” association, by the “Een Häerz fir kribbskrank Kanner” association, by the Action LIONS “Vaincre le Cancer” association and by Télévie Luxembourg. Research in Unité MEDyc’s laboratory is supported by grants from the Comité Départemental de l’Aube de la Ligue Nationale Contre le Cancer.

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Legends of figures

Figure 1: HDI effect on MMP-1 expression. HT1080 cells were treated or not for 24 h with MS-275 (10 μ M), SAHA (2 μ M) or TSA (100 ng/ml). (A) Histones were acid extracted and western blotted using total acetylated H4 histone antibody. (B) Endogenous MMP-1 mRNA level was monitored by real-time RT-PCR. Results are expressed as a mean \pm SEM of MMP-1/ β_2 m ratio.* $p < 0.05$ compared to untreated cells. (C) MMP-1 western blot analysis of cell concentrated media. β -actin was used as loading control.

Figure 2: Combined effects of MS-275 and 5-azadC on MMP-1 expression.

HT1080 cells were treated or not with MS-275 (10 μ M for 24 h) and/or 5-azadC (5 μ M for 72 h). (A) Endogenous MMP-1 mRNA level was monitored by real time RT-PCR. Results are expressed as a mean \pm SEM of MMP-1/ β_2 m ratio.* $p < 0.05$. (B) MMP-1 western blot analysis of cell concentrated conditioned media. β -actin was used as loading control.

Figure 3: Chromatin remodeling on MMP-1 promoter. HT1080 cells were treated or not for 24 h with MS-275 (10 μ M). (A) Schematic representation of the 5' upstream region of MMP-1 gene with the position of specific PCR primer sets. (B) ChIP experiments. ChIPs were performed as mentioned in material and methods. Enrichments were measured by real-time PCR and results are expressed as recovery (% of total input) = $((Ct_{IP} - Ct_{IG})/Ct_{input})$. Data are the mean \pm SEM of 3 independent experiments. Grey columns: untreated cells; White columns: MS-275-treated cells.

Figure 4: Nuclear levels of transcription factors. HT1080 cells were treated or not for 24 h with MS-275 (10 μ M). Nuclear proteins were extracted as mentioned in material and methods. c-Fos, c-Jun, p50, p65, p53, PEA3, Sp1 and Sp3 nuclear levels were analyzed by western blot. Lamin-B was used as loading control.

Figure 5: Transcription factor recruitments on MMP-1 promoter. HT1080 cells were treated or not for 24 h with MS-275 (10 μ M). (A) Schematic representation of the 5' upstream region of MMP-1 gene with the position of specific PCR primer sets and the potential binding sites for the transcription factors studied (B) ChIP experiments. ChIPs were performed as mentioned in material and methods. Transcription factor enrichment was measured by real-time PCR and results are expressed as recovery (% of total input) = $((Ct_{IP} - Ct_{IgG})/Ct_{input})$. Data are the mean \pm SEM of 3 independent experiments. Grey columns: untreated cells; White columns: MS-275-treated cells.

Figures

Figure 1

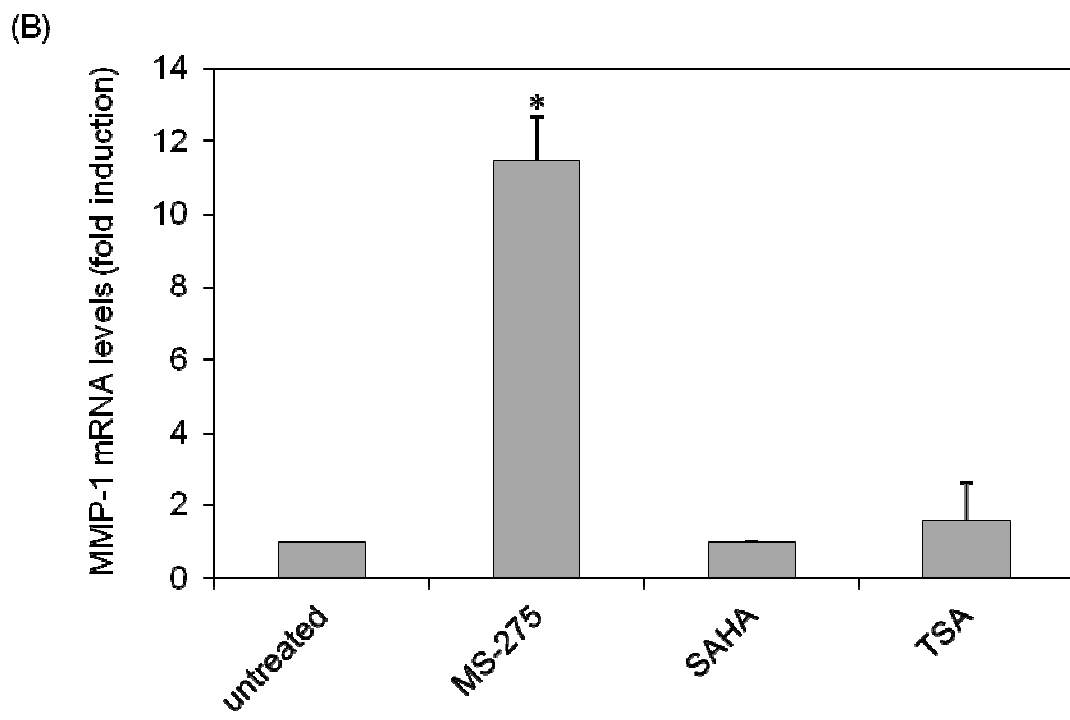
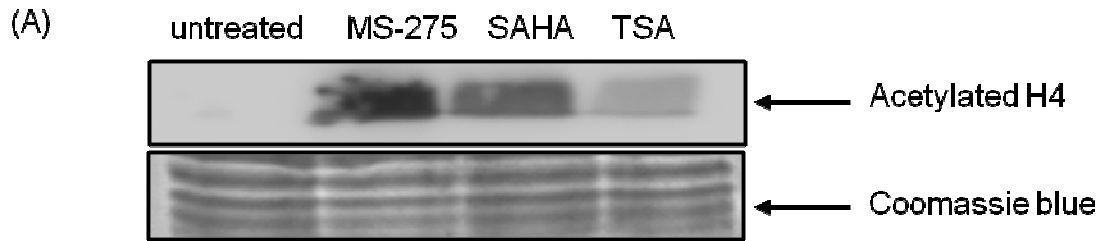


Figure 1 (to be continued)

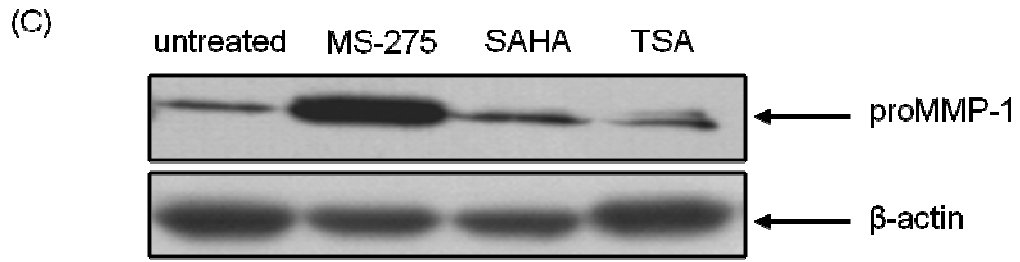
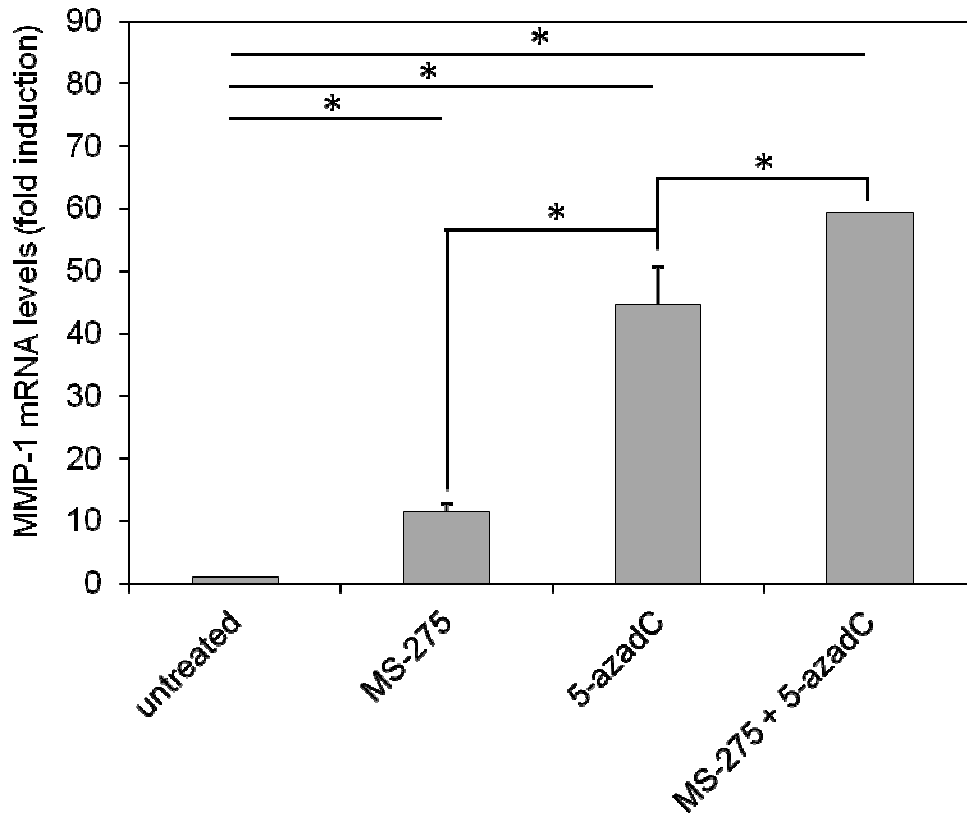


Figure 2

(A)



(B)

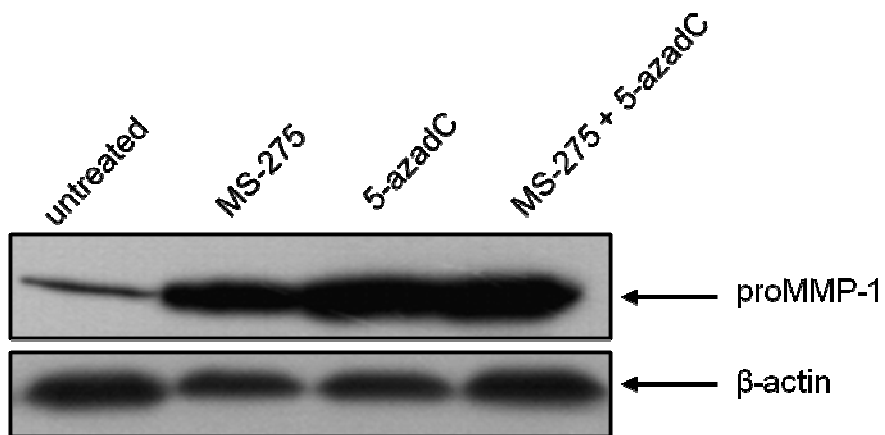


Figure 3

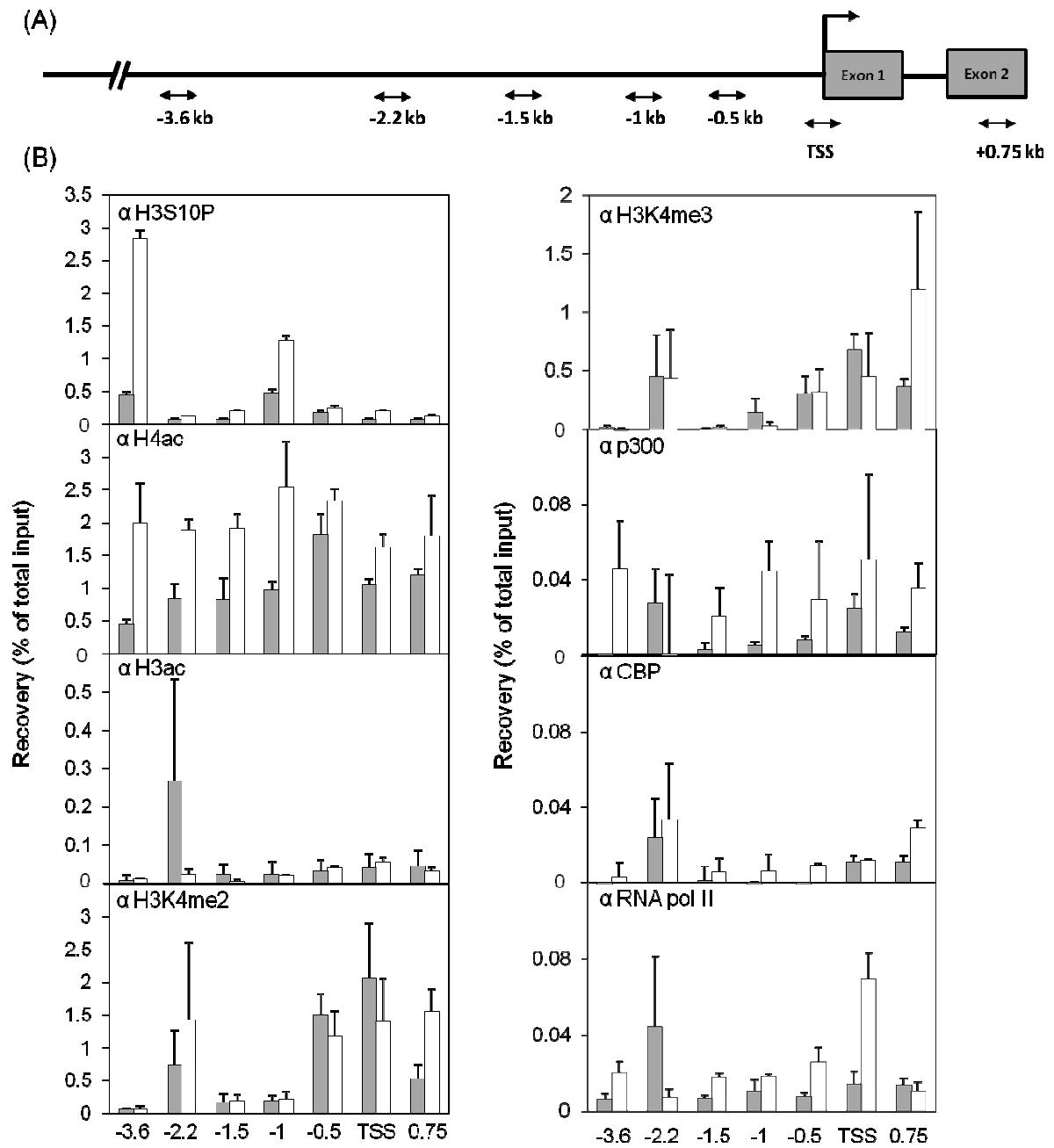


Figure 4

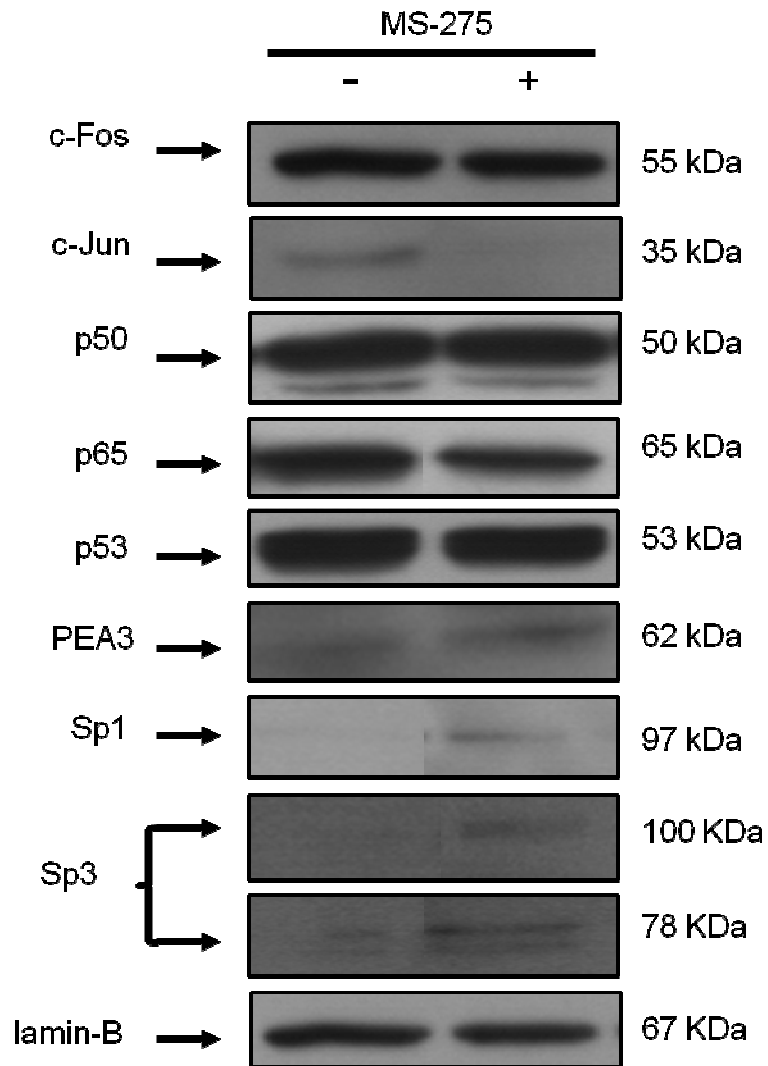
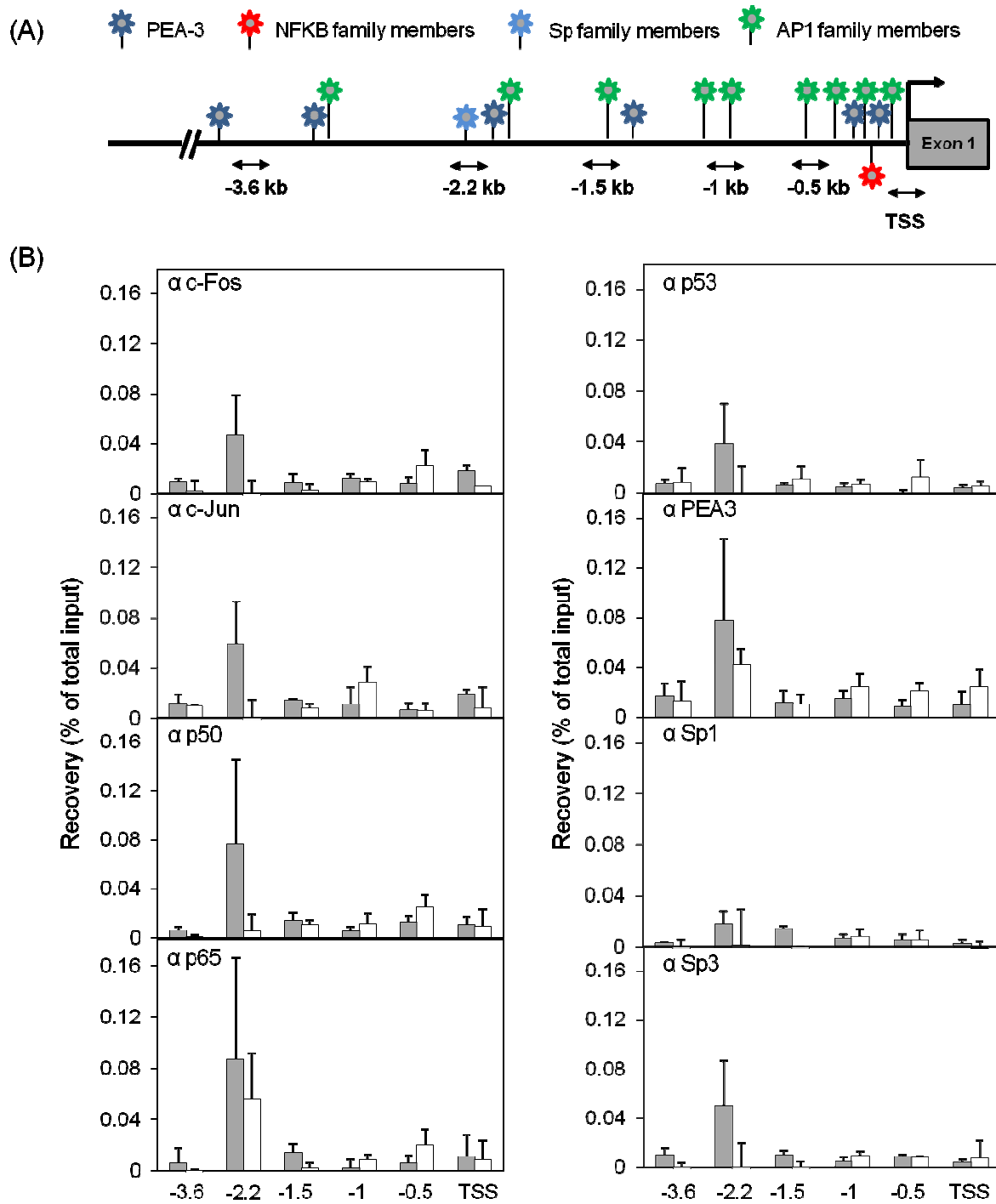


Figure 5



Tables

Table 1.1: primers for real-time PCR

Primers used for real-time PCR	
MMP-1	F : 5'-GAGCAAACACATCTGACC TACAGGA-3' R : 5'-TTGTCCCGATGATCTCCCCTGACA-3'
β_2 microglobulin	F : 5'- ATCTTCAAACCTCCATGATG-3' R : 5'-ACCCCCACTGAAAA AGATGA-3'
F : Forward; R : Reverse	

Table 1.2: primers for ChIP

Primers used for ChIP on MMP-1 promoter	
- 3.6 kb	F : 5'- TGTAACAATCATTTCAGACACC-3' R : 5'- AATCCTCCCCTTCAAGCTGT-3'
- 2.2 kb	F : 5'- CCAATCCACGTCAGCAACTA-3' R : 5'- ACATCATGCCTGCTTTTTCC-3'
- 1.5 kb	F : 5'- TGACGCAGAAAGAAAACAGG-3' R : 5'- CTCCCACCTTTCCCCTGTA-3'
- 1 kb	F : 5'- TTCCAGCCTTTTCATCATCC-3' R : 5'- GAGAAGACCCCTCATCCACA-3'
- 0.5 kb	F : 5'- GGGTACCAGGCAGCTTAACA-3' R : 5'-CGGCACCTGTAAGTACTGAA-3'
TSS	F : 5'-CTGGAAGGGCAAGGACTCTA-3' R : 5'- AGCTGTGCATACTGGCCTTT-3'
+ 0.75 kb	F : 5'- GATGGGAGGCAAGTTGAAAA-3' R : 5'- CAGGGTTTCAGCATCTGGTT-3'
F : Forward; R : Reverse	

Table 2: antibodies for western blot and ChIP

Target	Company	Reference no.	Application
MMP-1	Calbiochem	IM35	WB
c-Fos	Millipore	06-341	WB, ChIP
c-Jun	Active Motif	39309	WB, ChIP
PEA3	Santa Cruz	sc-22806	WB, ChIP
NFκB p50	Santa Cruz	sc-7178	WB, ChIP
NFκB p65	Santa Cruz	sc-8008	WB, ChIP
p53	Dako Cytomation	M7001	WB
p53	Active Motif	39334	ChIP
Sp1	Active Motif	39058	WB, ChIP
Sp3	Santa Cruz	sc-644	WB, ChIP
Lamin-B	Santa Cruz	sc-6216	WB
β-actin	Sigma Aldrich	A1978	WB
H3ac	Millipore	06-599	ChIP
H4ac	Millipore	06-866	WB, ChIP
H3K4me2	Millipore	07-030	ChIP
H3K4me3	Millipore	07-473	ChIP
H3S10P	Millipore	04-817	ChIP
p300	Millipore	05-257	ChIP
CBP	Abcam	ab-2832	ChIP
RNA pol II	Millipore	05-623	ChIP
Goat anti-mouse IgG-HRP	Millipore	12-349	WB
Donkey anti-rabbit IgG-HRP	Millipore	12-348	WB
Sheep anti-goat IgG-HRP	Sigma Aldrich	A9452	WB

WB : western blot; ChIP : chromatin Immunoprecipitation

Conclusion générale

L'issue fatale de la grande majorité des cancers est liée à l'envahissement des tissus sains environnants par la tumeur et sa dissémination vers des sites plus distants. Ces dernières années, de nombreux travaux ont tenté de décrypter le concept de « microenvironnement tumoral », afin de décrire et prédire les caractéristiques phénotypiques des cellules cancéreuses. De plus, une meilleure compréhension de l'interaction entre ces cellules et l'environnement extracellulaire pourrait se révéler utile pour la découverte de nouveaux marqueurs moléculaires du cancer. L'action protéolytique des MMPs jouant un rôle primordial dans les processus d'invasion tumorale et de formation des métastases, l'analyse des niveaux d'expression de ces gènes pourrait, notamment, servir de base pour la caractérisation phénotypique de tumeurs. Par exemple, l'axe de signalisation MMP-1/PAR-1 a été récemment décrit comme un nouveau marqueur permettant d'évaluer le potentiel métastatique de carcinomes hépatocellulaires et, ainsi servir de marqueur pronostic indépendant chez des patients souffrant de ce type de cancer (Liao, 2012; Peng, 2012). L'élaboration d'une « signature épigénétique » de gènes codants les MMPs pourrait, ainsi, permettre une meilleure compréhension des phénomènes participant au potentiel agressif des tumeurs et pourrait également faciliter l'élaboration de critères diagnostiques et de pronostiques. De plus, ces analyses permettraient de mieux appréhender le comportement de cellules tumorales face à des agents chimiothérapeutiques.

En l'occurrence, un des points importants de nos travaux de thèse a été la mise en évidence d'effets antagonistes, d'agents utilisés comme médicaments anticancéreux, sur le comportement invasif de cellules de fibrosarcome humain (cellules HT1080). En effet, il est apparu qu'un modulateur épigénétique, la 5-azadC, agent utilisé à l'heure actuelle en pratique clinique (decitabine ou Dacogen®) (Florea, 2011; Silverman, 2005), induit la surexpression de certaines MMPs (MMP-1, -2 et -9) et augmente l'invasion des cellules HT1080 *in vitro*.

Dans la mesure où les modifications épigénétiques provoquent des remaniements de la structure chromatinienne, leur impact sur la texture nucléaire pourrait refléter des modifications précoces du fonctionnement cellulaire. Ainsi, nous avons montré que l'augmentation de l'invasion cellulaire associée à des variations de l'expression génique des MMPs, est corrélée à des modifications significatives de l'architecture nucléaire. En effet, l'analyse des paramètres de texture nucléaire nous a permis de définir une « signature

nucléaire » des cellules, qui varie en fonction du profil de méthylation de l'ADN. Les mesures des différents paramètres de texture nucléaire sont des éléments importants de diagnostic qui, entre autres, ont déjà permis la caractérisation de changements induits par des agents thérapeutiques (Poplineau, 2011b) et permettraient à l'heure actuelle d'établir une empreinte épigénétique des cellules tumorales permettant, indirectement, de refléter leurs propriétés invasives.

La modulation des propriétés invasives des cellules HT1080, induite par la 5-azadC, semble impliquer l'activité protéolytique de la MMP-1. L'identification de médiateurs, ainsi que l'étude du profil de méthylation du promoteur du gène de la MMP-1, nous a permis d'approfondir le mécanisme de régulation épigénétique de ce gène. La modulation de l'expression de ce gène par la 5-azadC ne semble pas requérir une déméthylation totale de son promoteur. Néanmoins, l'expression de ce gène implique un remodelage de la chromatine et la fixation du facteur de transcription Sp1 au niveau de son promoteur, induisant ainsi sa transcription. Après traitement par la 5-azadC, Sp1 est recruté au niveau de deux sites distincts (-2,2 kb et -0,5 kb) sur le promoteur du gène de la MMP-1. La fixation de Sp1 en -2,2 kb été décrite dans la littérature (Rutter, 1998) , mais aucune étude, à l'heure actuelle, n'a relaté la fixation éventuelle de Sp1 en -0,5 kb. De ce fait, deux hypothèses s'offrent à nous : (i) il existerait un site supplémentaire Sp1 non décrit dans la littérature en -0,5 kb ou (ii) la 5-azadC induit la formation d'une boucle (« chromatin looping ») entre -2,2 kb et -0,5 kb expliquant la présence de Sp1 en -0,5kb. Des analyses complémentaires de mutagenèse dirigée ou de technique 3C (chromosome conformation capture), par exemple, sont nécessaires en vue de déterminer laquelle des deux hypothèses serait la plus plausible.

D'autre part, nous avons montré que le MS-275 et les pan-HDIs, SAHA et TSA présentent des effets différentiels sur l'expression de la MMP-1. De plus, le MS-275 et la 5-azadC semblent exercer des effets additifs sur l'expression de ce gène, alors que la TSA et 5-azadC agissent de manière synergique. Ces résultats suggèrent que la 5-azadC et le MS-275 agissent sur des voies de régulation génique distinctes. L'établissement d'un état chromatinien permissif semble participer à l'activation transcriptionnelle du gène de la MMP-1 induite par le MS-275, mais la voie de signalisation et le(s) facteur(s) de transcription impliqué(s) dans cette activation demeurent, à l'heure actuelle, encore inconnus et restent à définir. Notamment, l'implication du facteur de transcription STAT3 dans la régulation de

l'expression du gène de la MMP-1 reste à explorer. La régulation de l'activité de STAT3 semble être modulée par des processus d'acétylation et de désacétylation sous l'action antagoniste de l'HDAC1 et de p300 (Buchwald, 2009). Au vu de nos résultats montrant que le MS-275 induit le recrutement de p300 au niveau du promoteur du gène de la MMP-1 et compte tenu de l'implication de STAT3 dans la régulation transcriptionnelle du gène de la MMP-1 (Itoh, 2006; O'Kane, 2010; Zugowski, 2011), l'analyse, après traitement par le MS-275, des niveaux nucléaires de STAT3 ainsi que son potentiel recrutement au niveau du promoteur du gène de la MMP-1 restent à étudier. De plus, l'introduction d'une stratégie antisens dirigées contre différentes isoformes d'HDACs, permettraient d'évaluer de manière précise l'implication de ces différentes isoformes dans la régulation de l'expression du gène de la MMP-1.

L'ensemble des résultats obtenus au cours de cette thèse sont issus d'expériences *in vitro* menées sur une seule lignée cellulaire. Les effets observés de la 5-azadC sur l'invasion tumorale peuvent paraître inattendus compte tenu de l'utilisation de cette substance en pratique clinique anticancéreuse. Cependant, des résultats similaires ont déjà été observés dans d'autres lignées cellulaires (lignées de carcinomes pancréatiques) (Sato, 2003). Il apparaît ainsi que l'utilisation d'agents hypométhylants peut, au moins dans certains cas, stimuler l'invasion de cellules cancéreuses. Cet effet indésirable potentiel de la 5-azadC sur la progression tumorale devra donc être évalué *in vivo* avant toute utilisation de cet agent pour le traitement de nouveaux types de cancers.

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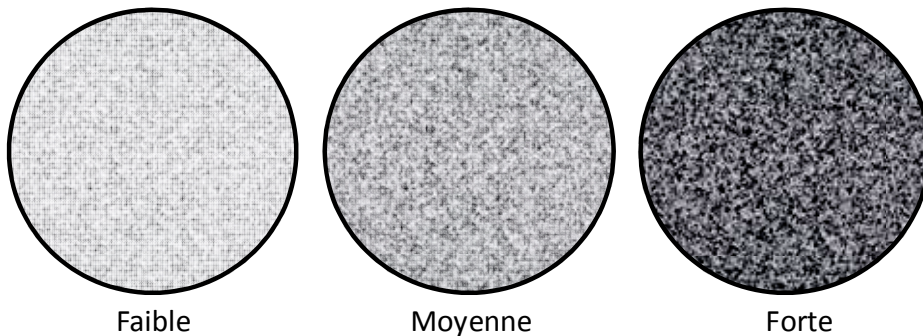
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Annexes

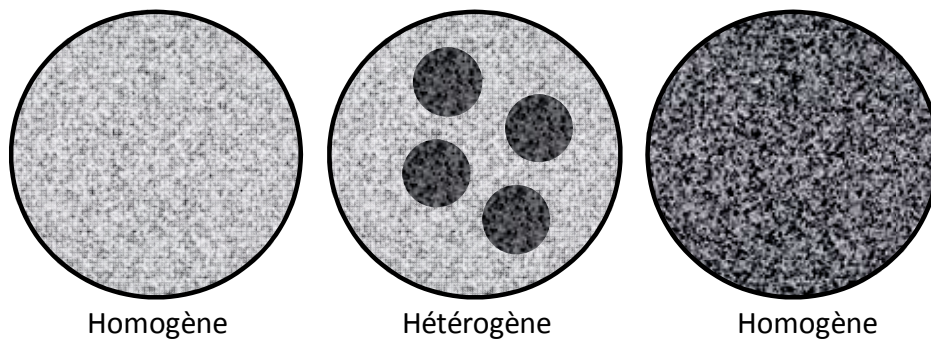
ANNEXE 1 : Analyse de la texture nucléaire

La texture du noyau décrit les faciès de la chromatine avec trois attributs différents : sa condensation, sa distribution et son organisation dans noyau (thèse de Giroud Françoise, approches cytométriques de la prolifération et de la différenciation : notion de faciès chromatinien, Université de Grenoble, 1987).

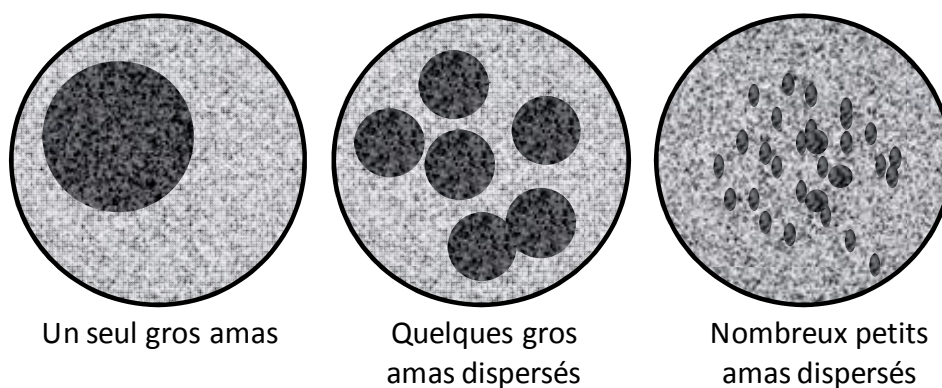
- La **condensation** de la chromatine concerne le degré de compaction des segments de la chromatine



- La **distribution** de la chromatine concerne la proportion relative des différents degrés de compaction de la chromatine.



- L'**organisation** de la chromatine concerne l'arrangement topographique des divers degrés de condensation de la chromatine



La densité optique intégrée (DOI) correspond à la somme de tous les pixels de l'image du noyau. Dans le cas de la coloration stoechiométrique de Feulgen, sa valeur est directement proportionnelle au contenu en ADN du noyau, ce qui permet de mesurer la distribution des cellules dans les différentes phases du cycle cellulaire. Les valeurs des paramètres de texture sont liées à la phase du cycle cellulaire, c'est pourquoi une sélection préalable d'une population cellulaire a été réalisée. Pour chaque expérience et chaque paramètre de texture, les mesures ont été exclusivement réalisées sur des noyaux de cellules en phase G_0/G_1 , sélectionnés sur la base de la valeur de leur DOI. Les différents paramètres de texture analysés figurent dans le tableau suivant :

Paramètres de texture		Interprétations
<i>Matrice de co-occurrence</i>		
LM	Moyenne locale (Local Mean)	⇒ LM donne une estimation globale de la condensation de la chromatine. Elle augmente avec la condensation.
E	Energie (Energy)	⇒ E reflète plutôt la distribution de la chromatine que sa condensation. Elle diminue avec une distribution plus compacte et augmente avec une distribution plus homogène.
ENT	Entropie (Entropy)	⇒ ENT et I mesurent le contraste dans une image.
I	Contraste (Inertia)	
<i>Matrice des longueurs de section</i>		
SRE	Importance des courtes sections (Short Run-length Emphasis)	⇒ SRE et LRE mesurent la distribution des longueurs de sections qui dépendent de l'organisation de la chromatine. SRE diminue et LRE augmente quand la texture chromatiniennne est finement granulaire et homogène. Les variations sont inversées lorsque la texture est hétérogène avec de gros amas.
LRE	Importance des longues sections (Long Run-length Emphasis)	
GLD	Distribution des niveaux de gris (Grey-Level Distribution)	⇒ GLD mesure l'uniformité de la distribution des niveaux de gris parmi les longueurs de section
RLD	Distribution des longueurs de section (Run-Length Distribution)	⇒ RLD augmente avec l'homogénéité des longueurs de section
RPC	Pourcentage des longueurs de section (Run-Length Percentage)	⇒ RPC donne des interprétations similaires à celles de la SRE et LRE

Ces paramètres de texture sont calculés sur l'image nucléaire après réduction à 16 niveaux de gris. Pour chaque population cellulaire, la moyenne et l'écart-type de chaque paramètre sont calculés. La significativité des différences observées entre les cellules non traitées et traitées a été estimée par un test *t* de Student après une correction de Bonferroni ou de Kolmogorov-Smirnov sur les fréquences cumulées. En vue d'obtenir des résultats plus informatifs, un index de déviation (ID) a été calculé pour chaque paramètre selon la formule suivante :

$$ID = \frac{\text{valeur cellule traitée} - \text{valeur moyenne cellules contrôles}}{\text{écart-type valeurs cellules contrôles}}$$

Pour chaque population cellulaire, l'index d'anormalité nucléaire (NAI ou *nuclear abnormality index*) est calculé à partir de la somme des valeurs absolues des ID de tous les paramètres selon la formule suivante :

$$NAI = \frac{\sum_1^n |ID|}{n}$$

n= nombre des ID calculés à partir de tous les paramètres

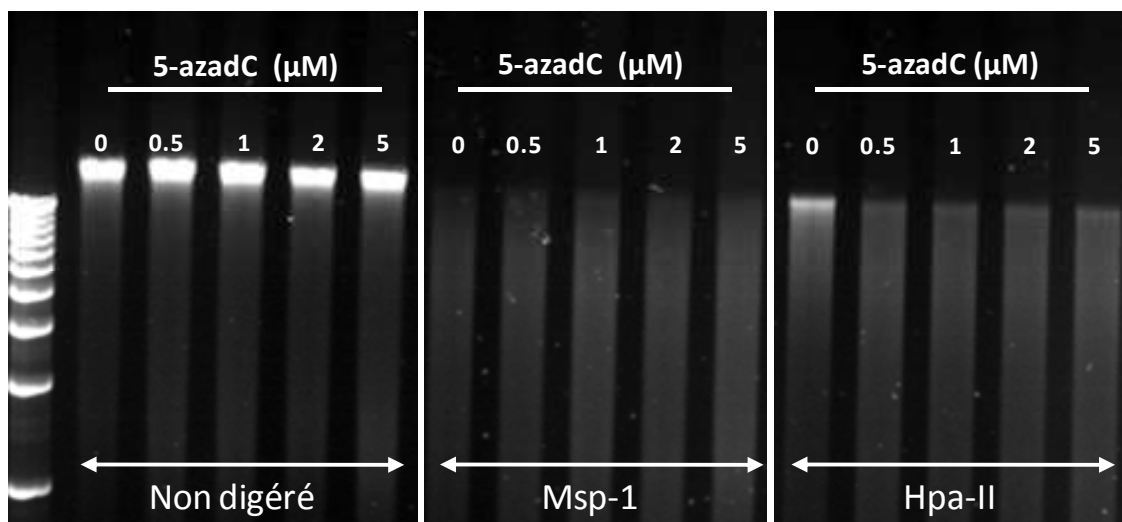
ANNEXE 2 : Analyse de la méthylation de l'ADN par MSRA

(methylation-sensitive restriction assay)

Après traitement des cellules par la 5-azadC, l'ADN génomique des cellules est extrait puis dosé. 0,5 µg d'ADN génomique sont alors incubés en présence ou non de l'une des deux enzymes suivantes :

- **Msp1** : cette enzyme est **insensible** au **degré de méthylation** de l'ADN. Elle est donc capable de digérer aussi bien l'ADN méthylé que l'ADN non méthylé
- **HpaII** : cette enzyme est **sensible** au **degré de méthylation** de l'ADN. Elle est donc capable de digérer uniquement l'ADN déméthylé et est entièrement inefficace sur l'ADN méthylé.

L'efficacité de la digestion enzymatique est évaluée par électrophorèse des ADN génomiques sur gel d'agarose à 0,8 %. Les résultats sont représentés sur la figure suivante :



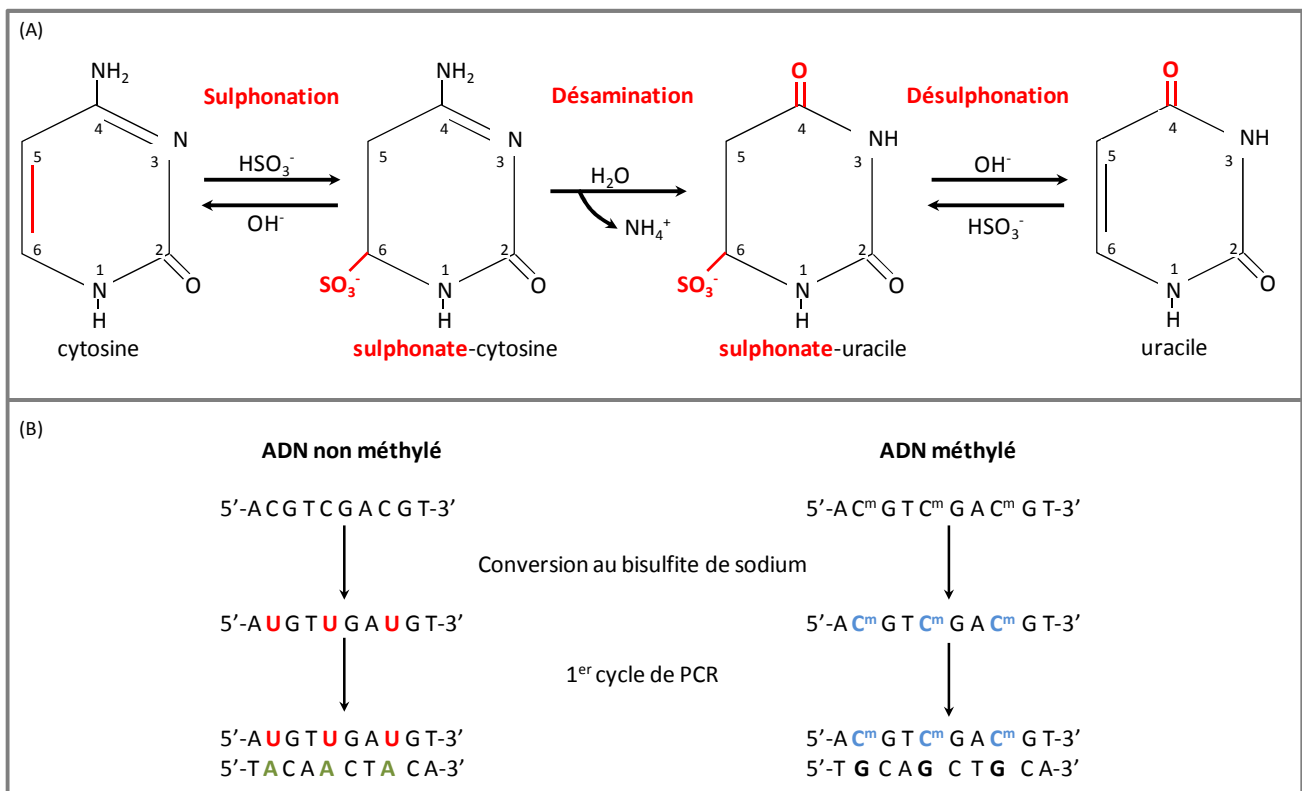
A gauche de l'image, figurent les fractions d'ADN génomiques non digérées. Ces fractions d'ADN correspondent au contrôle négatif de l'expérience.

Au milieu de l'image, figurent les fractions d'ADN génomiques digérées par *Msp1*. Ces fractions d'ADN correspondent au contrôle positif de l'expérience.

A droite de l'image, figurent les fractions d'ADN génomiques digérées par *HpaII*. Ces fractions d'ADN analysées rendent compte du résultat de l'expérience : la 5-azadC induit une déméthylation globale de l'ADN.

ANNEXE 3 : Principe de la conversion au bisulfite de sodium de sodium

La conversion au bisulfite de sodium permet de convertir les cytosines non méthylées en uraciles (A), détectés comme des thymines après amplification de l'ADN par PCR (B). Les cytosines méthylées ne sont pas affectées par un tel traitement et vont être détectées comme cytosines après PCR de l'ADN (B).

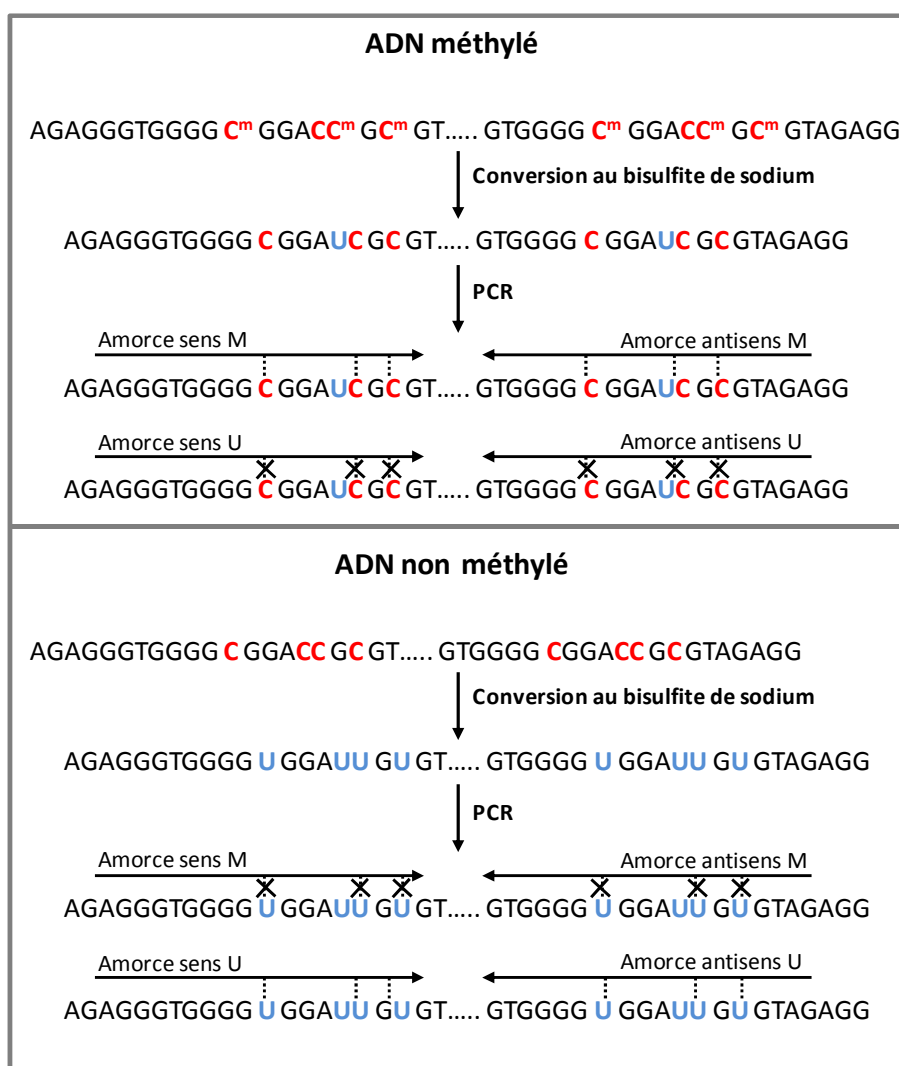


(A) : Réactions chimiques générées par la conversion au bisulfite de sodium

(B) : Séquence des ADN méthylés et non méthylés après une conversion au bisulfite de sodium

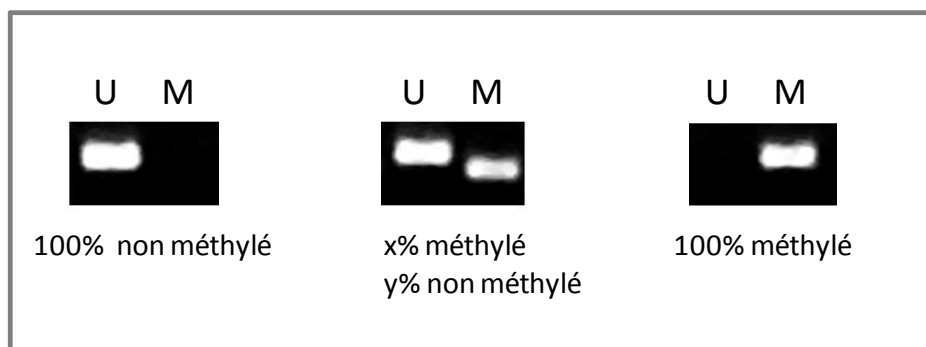
ANNEXE 4 : Analyse de la méthylation de l'ADN par MSP (*methylation-specific PCR*)

La PCR spécifique de la méthylation (MSP) est une technique rapide et très sensible pour l'analyse de la méthylation des cytosines contenues au niveau d'îlots CpG. Elle est basée sur l'utilisation de deux paires distinctes d'amorces au niveau de la région d'intérêt, destinées à amplifier les brins méthylés (amorces M pour *methylated*) ou non méthylés (amorces U pour *unmethylated*) après conversion de l'ADN par le bisulfite de sodium (**ANNEXE 2**). Les amorces M et U ont été élaborées à l'aide du logiciel *Methyl Primer Express* (créé par Fraga, Ferrero et Esteller pour la compagnie Applied Biosystem).



Spécificité des amorces M et U pour les brins méthylés et non méthylés

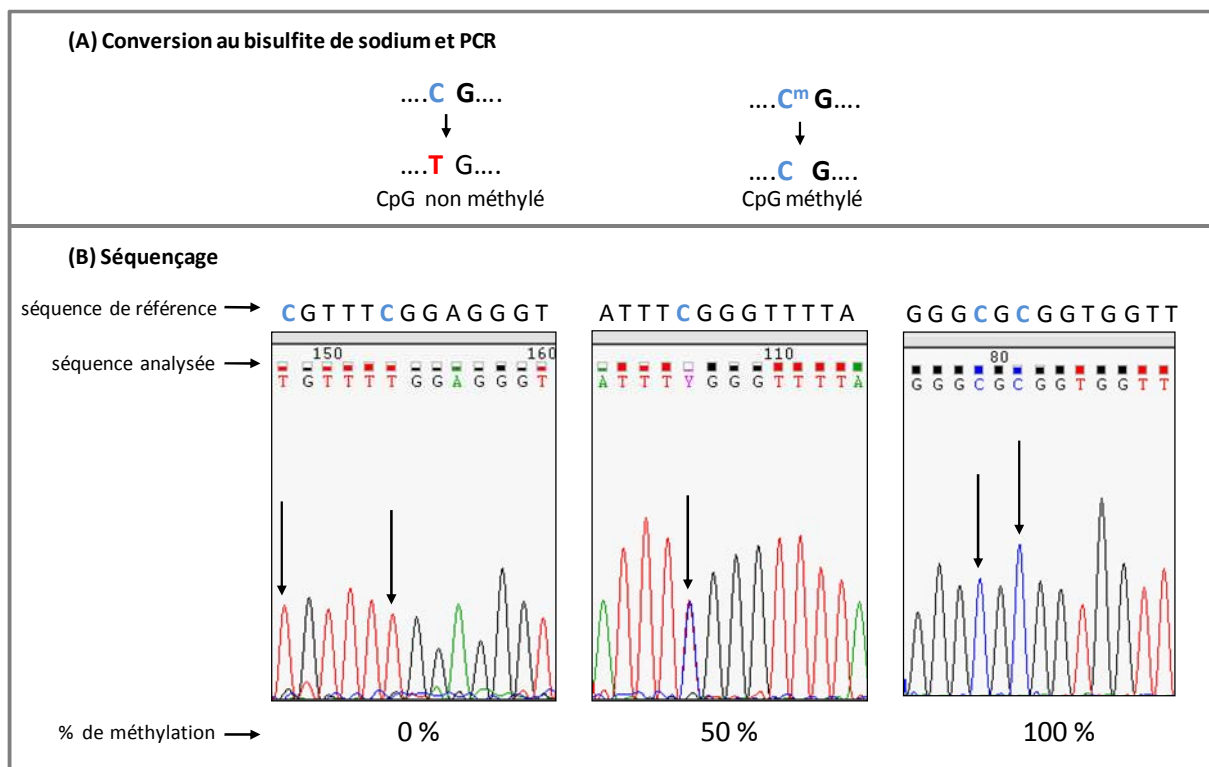
Après PCR, les résultats sont visualisés sur un gel d'agarose à 2 % comme représenté ci-dessous :



Profils de méthylation obtenus par MSP

ANNEXE 5 : Analyse de la méthylation de l'ADN par séquençage au bisulfite de sodium

Cette technique est utilisée pour déterminer de manière quantitative le pourcentage de méthylation des dinucléotides CpG contenus dans une séquence d'ADN. La première étape de cette technique est la conversion au bisulfite de sodium de l'ADN génomique. Elle est suivie d'une PCR d'amplification de ces ADN convertis, puis d'une PCR de séquence des produits purifiés. Les résultats du séquençage sont représentés sous forme de chromatogramme, sur lequel chaque pic correspond à un nucléotide (adénine (A), guanine (G), thymine (T) ou cytosine (C)). L'intensité du pic fournit des informations quantitatives et qualitatives sur le pourcentage de méthylation des cytosines, comme indiquées sur la figure ci-dessous.

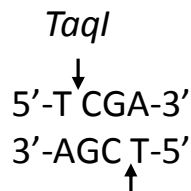


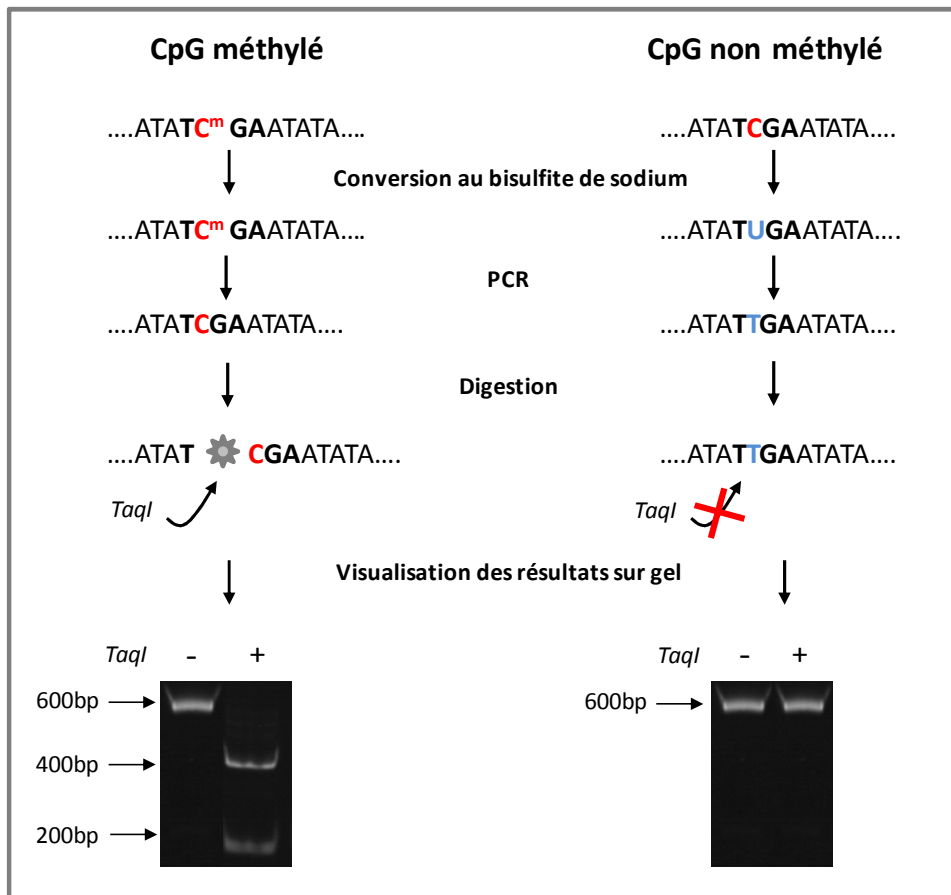
Interprétation des chromatogrammes obtenus après séquençage

ANNEXE 6 : Analyse de la méthylation de l'ADN par CoBRA (*combined bisulfite restriction analysis*)

Cette technique est utilisée pour déterminer de façon quantitative le taux de méthylation de l'ADN sur un (ou plusieurs) dinucléotide(s) CpG précis. Elle combine l'amplification de l'ADN converti au bisulfite de sodium et la digestion des produits post-PCR par une enzyme de restriction. La conversion des cytosines non méthylées en uraciles (puis en thymines après PCR) et des cytosines méthylées en cytosines permet la création de nouveaux sites de restriction enzymatique ou la préservation de sites préexistants. Le rapport entre la fraction d'ADN digéré et la fraction totale reflète le pourcentage de méthylation de l'ADN du site étudié dans l'ADN génomique d'origine.

Dans la PUBLICATION N°3 les produits post-PCR, préalablement convertis au bisulfite de sodium, ont été digérés par l'enzyme de restriction *TaqI* qui reconnaît la séquence palindromique TCGA :



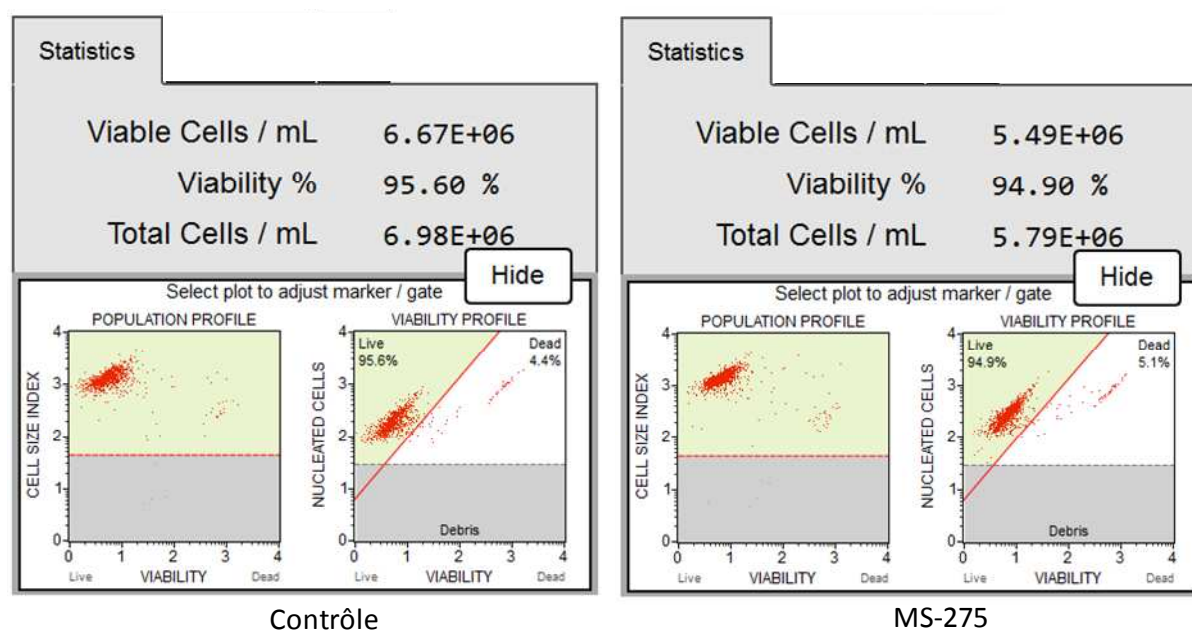


Principe et interprétation de l'analyse CoBRA

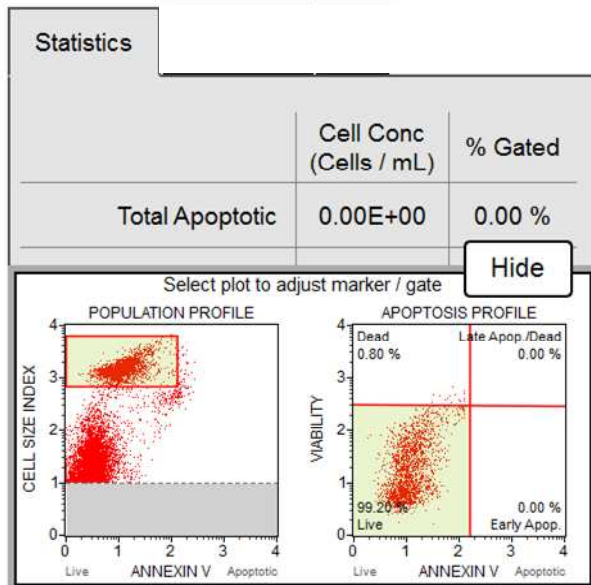
ANNEXE 7 : Effet du MS-275 sur la viabilité, l'apoptose et le cycle des cellules HT1080

Les inhibiteurs d'HDAC (HDIs) sont capables d'affecter la viabilité cellulaire, d'induire l'apoptose et de provoquer un arrêt du cycle cellulaire. Ainsi, nous avons testé l'effet du MS-275 sur ces différents paramètres cellulaires. Pour cela, les cellules HT1080 ont été traitées par 10 μM de MS-275 pendant 24 h et la viabilité, l'apoptose et le cycle cellulaire ont été analysées par la technologie MuseTM (Millipore) basée sur le principe de la cytométrie en flux. Les échantillons cellulaires ont été préparés selon les consignes du fabricant et les résultats obtenus sont représentés ci-dessous. Le MS-275 n'affecte pas la viabilité des cellules HT1080 et n'induit pas leur apoptose, mais entraîne un blocage de ces cellules en phase G₀/G₁, avec une diminution du pourcentage de cellules en phase S.

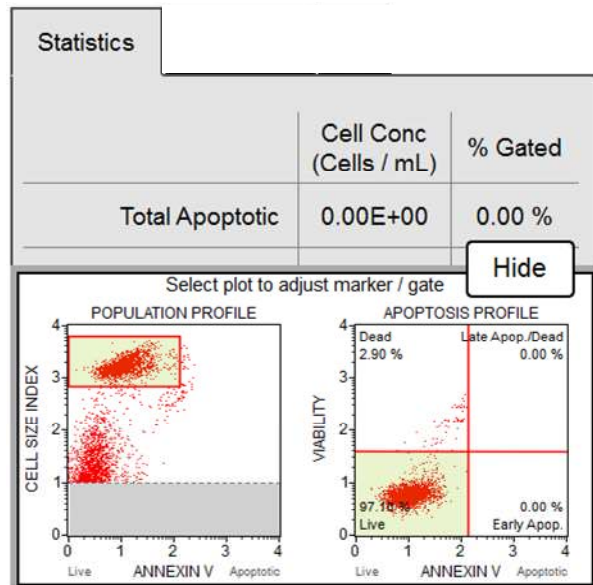
Analyse de la viabilité cellulaire :



Analyse de l'apoptose cellulaire :

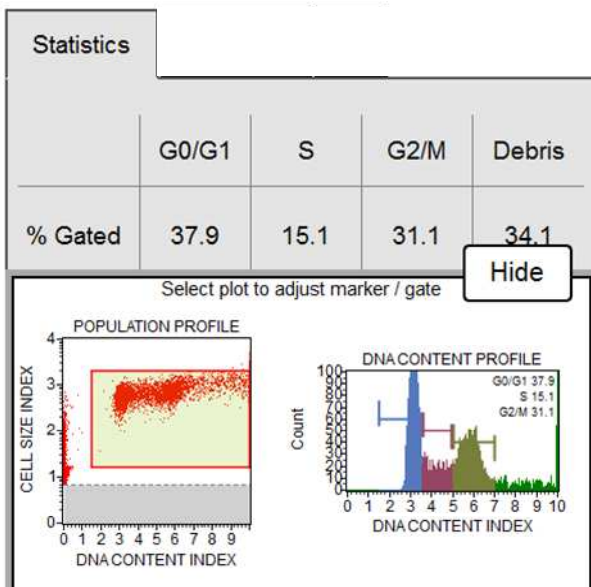


Contrôle

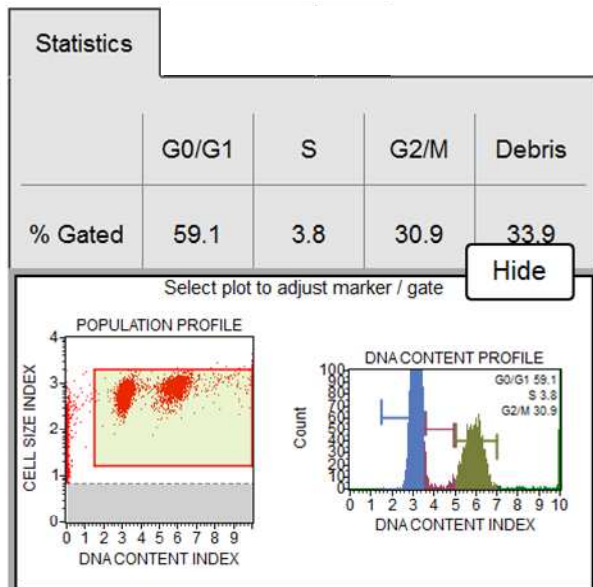


MS-275

Analyse du cycle cellulaire :



Contrôle



MS-275

Publications & Communications

1. **POPLINEAU M**, DUFER J, ANTONICELLI F, TRUSSARDI-REGNIER A.
Epigenetic regulation of proMMP-1 expression in the HT1080 human fibrosarcoma cell line.
Int J Oncol. 2011 Jun;38(6):1713-8.
2. **POPLINEAU M**, TRUSSARDI-REGNIER A, HAPPILLON T, DUFER J, MANFAIT M, BERNARD P, PIOT O, ANTONICELLI F.
Raman microspectroscopy detects epigenetic modifications in living Jurkat leukemic cells.
Epigenomics. 2011 Dec;3(6):785-94.
3. **POPLINEAU M**, DOLIWA C, SCHNEKENBURGER M, ANTONICELLI F, DIEDERICH M, TRUSSARDI-REGNIER A, DUFER J.
Epigenetically-induced changes in nuclear textural patterns and gelatinase expression in human fibrosarcoma cells.
Article en révision pour publication dans Cell Proliferation
4. **POPLINEAU M**, SCHNEKENBURGER M, KOŚCIARZ M, DUFER J, ANTONICELLI F, DIEDERICH M, TRUSSARDI-REGNIER A.
The DNA hypomethylating agent 5-aza-2'-deoxycytidine enhances tumor cell invasion through MMP-1 transcription-dependent modulation in human fibrosarcoma cells.
Manuscrit soumis pour publication dans Molecular Oncology
5. **POPLINEAU M.**, M. SCHNEKENBURGER, J. DUFER, F. ANTONICELLI, M. DIEDERICH, A. TRUSSARDI-RÉGNIER.
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HEV stimulates innate immunity through activation of the non-canonical NF- κ B pathway via NF- κ B-inducing Kinase (NIK) in infected human airway epithelial cells.

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1. **POPLINEAU M**, RIPAMONTI J, DUFER J, TRUSSARDI-REGNIER A.

Epigenetic regulation of proMMP-1 gene expression.

Journée des Jeunes Chercheurs de l'IFR53, 29 Avril 2010, Reims, France.

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Raman microspectroscopy detects epigenetic modifications in living Jurkat leukemic cells

Aims: Classical biochemical and molecular methods for discerning cells with epigenetic modifications are often biologically perturbing or even destructive. We wondered whether the noninvasive laser tweezer Raman spectroscopy technique allowed the discrimination of single living human cells undergoing epigenetic modifications. **Materials & methods:** Human Jurkat leukemic cells were treated with inhibitors of histone deacetylases (trichostatin A and MS-275). Epigenetic changes were monitored through histone electrophoresis, nuclear image cytometry and laser tweezer Raman spectroscopy. **Results:** Treatment of Jurkat cells with histone deacetylase inhibitors increased histone acetylation and induced chromatin organization changes. Characteristic vibrations, issued from laser tweezer Raman spectroscopy analyses, mostly assigned to DNA and proteins allowed discerning histone deacetylase inhibitor-treated cells from control with high confidence. Statistical processing of laser tweezer Raman spectroscopy data led to the definition of specific biomolecular fingerprints of each cell group. **Conclusion:** This original study shows that laser tweezer Raman spectroscopy is a label-free rapid tool to identify living cells that underwent epigenetic changes.

KEYWORDS: acetylation ■ epigenetics ■ histone ■ MS-275 ■ Raman microspectroscopy ■ trichostatin A

Most of current biochemical and molecular techniques used to detect, identify and quantify epigenetic modifications are destructive. In order to avoid these shortfalls, new technologies based on light scattering methods are used to measure cell alterations, which are associated with changes in cell physical properties. Image cytometry study of pathological specimens or cell lines, through nuclear texture analysis, revealed that epigenetic mechanisms play a major role in determining chromatin patterns [1,2]. Therefore, this type of methodology was applied to evaluate the effects of epigenetic events on the chromatin higher order spatial organization. Several studies revealed variations in chromatin condensation and modifications in the spatial arrangement of chromatin packing states, either in response to histone acetylation [1-3] and/or DNA methylation [4,5] in various cell lines. However, information arising from such methodologies are only related to gross genomic changes observed on 2D preparations of fixed cells and do not take into account the finely tuned and highly dynamic molecular modifications induced by these epigenetic modulations in cell suspension. Alternative methodologies such as confocal imaging of fluorescent probes enable more integrated approaches to study the nuclear organization [6]. However, this less invasive

method is only applicable to characterize fixed cells, and often requires confirmatory biological evaluation, which is subject to the aforementioned shortfalls. Therefore, there is still a need for new techniques that overcome such limitations to provide an easily automated and accurate method for the detection of epigenetic alterations in both research and clinical domains. Thus, research development has now focused on new noninvasive biophysical approaches able to detect these molecular changes on living cells. Amongst these new technologies, Raman microspectroscopy could offer a promising way to analyze the chromatin structure [7].

Raman microspectroscopy is an optical laser-based technique which relies on the inelastic interaction between light (a monochromatic source) and matter. In complex samples such as cells or tissues, this tool provides information about the vibrational energies of the biomolecular constituents of the sample. Raman microspectroscopy does not require any sample labeling and appears to be noninvasive and nondestructive when suitable laser wavelengths and intensities are used. Recently, this method has been used in combination with microscopy and optical 'traps' or 'tweezers' to study isolated living cells [8,9]. This approach, known as laser tweezers Raman spectroscopy

Mathilde Poplineau^{1†},
Aurélie
Trussardi-Régnier^{*1‡},
Teddy Happillon¹,
Jean Dufer¹,
Michel Manfait¹,
Philippe Bernard²,
Olivier Piot¹
& Frank Antonicelli²

¹Unité MEDyC, UMR URCA CNRS 6237, IFR53, Faculté de Pharmacie, Université de Reims, 1 avenue du Maréchal Juin, 51096 Reims, France

²Université de Reims Champagne-Ardenne, Laboratoire de Dermatologie, UMR URCA CNRS 6237, IFR53, Faculté de médecine, Reims, France

*Author for correspondence:

Tel.: +33 32 691 8016

Fax: +33 32 691 3550

aurelie.trussardi@univ-reims.fr

[†]These authors contributed equally

(LTRS), permits probing of a single cell over an extended period of time by 'trapping' the cell in suspension, provided that the cell is nonadherent and the trapping forces can overcome the size and weight of this cell. Previous studies have shown the potential of LTRS to sort living microorganisms [10], to monitor protein expression in living bacteria [11], to detect hyperosmotic stress in optically trapped yeast cells [12] and also to study dynamic cellular processes such as cell bacterial lysis [13]. The originality and efficiency of this physical noninvasive technique prompted us to investigate its potential to evaluate biological changes induced by epigenetic modulators in living cells.

Aberrant transcriptional gene regulations, related to genetic and epigenetic alterations, are observed during tumorigenesis and cancer progression. Amongst histone post-translational modifications associated with epigenetic regulations, the balance of histone acetylation/deacetylation has received a critical focus for transcriptional regulation since it controls chromatin accessibility [14–16]. Histone deacetylases (HDACs) play a key role in the regulation of gene expression by catalyzing the removal of acetyl groups on the N-terminal tail of histones, triggering chromatin condensation and promoting gene silencing [16–18]. Since aberrant epigenetic changes are nowadays a hallmark of cancer, drugs targeting these mechanisms are promising tools for pharmacological gene regulation. HDAC inhibitors (HDIs) can induce cell cycle arrest, promote differentiation and stimulate tumor cell death. In leukemia models, HDIs used either alone or in combination with other drugs, have demonstrated their ability to modulate gene expression, and for instance, it was shown that the HDI MS-275 could promote differentiation and apoptosis of human leukemic cells [19].

Thus, the purpose of our study was to evaluate the potential of LTRS to detect cellular changes induced by HDI treatment. To our knowledge, no study using Raman microscopy has so far been performed to evaluate the effects of epigenetic drugs in individual living leukemic cells. In this study, we demonstrated that LTRS can be used to monitor epigenetic changes induced by the HDIs MS-275 and trichostatin A (TSA) in living unfixed Jurkat T-lymphocyte-derived cells. Statistical analysis of spectra revealed changes in the spectral fingerprint of HDI-treated cells that displayed distinct clusters between HDI-treated and -untreated cells.

Materials & methods

■ Cell culture & treatments

Human Jurkat cell line (American Type Culture Collection, TIB-152) was obtained by Weiss *et al.* [20] from leukemic T cells. Cells were grown at 37°C in Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in 95% humidified air, 5% CO₂ atmosphere. Cells were treated or untreated for 24 h with the following HDIs: 165 nM (50 ng/ml) of TSA (Sigma Aldrich, France) or 26.6 µM (10 µg/ml) of MS-275 (Alexis, San Diego, CA, USA). These concentrations were defined according to their Jurkat cell toxicity, and with respect to their known EC₅₀ ranges [21].

■ Histone extraction & acid-urea-TritonX100 polyacrylamide gel electrophoresis

Details of the procedure are previously described in El-Khoury *et al.* [1]. Briefly, 1×10^7 cells were kept in 500 µl of ice cold phosphate-buffered saline. 2 ml of cytoplasmic lysis buffer (320 mM saccharose, 5 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 1% Triton™ X100, 10 mM butyric acid, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride) was added and incubated for 10 min on ice. After centrifugation, the nuclear pellet was washed with cytoplasmic lysis buffer and resuspended in 500 µl of nuclear lysis buffer (10 mM Tris-HCl pH7.4, 4 mM MgCl₂, 10 mM butyric acid, 0.1 mM phenylmethylsulfonylfluoride and 0.1 mM benzamidine). Histones were acid-extracted for 2 h with 500 µl of ice cold 0.8 M HCl. Acid-insoluble material was removed by centrifugation at 12,000 g for 10 min at 4°C. Supernatants were harvested, precipitated with eight volumes of acetone, kept at -20°C overnight, and histones were collected by centrifugation at 2000 g for 10 min at 4°C. Pellets were air-dried, suspended in distilled water and protein content evaluated using the Quant-it Assay Kit (Invitrogen, France).

The level of acetylation was analyzed by polyacrylamide gel electrophoresis (PAGE) using acid-urea-Triton X100 (AUT). The migration system consisted of a separating gel containing 13.1% acrylamide, 0.09% bisacrylamide, 4.5% glacial acetic acid, 3.2% Triton X100 and 5.2 M urea. Histones (10 µg) were separated for 16 h at 4°C at 150 V in a running buffer (17.4 M acetic acid). Histones were revealed using a staining solution (50% methanol, 10% acetic acid, 40% distilled water and 0.1% Coomassie blue R250) and a destaining

solution (50% methanol, 10% acetic acid and 40% distilled water).

■ Conventional image cytometry

Cells were smeared by cytocentrifugation, air-dried, fixed in alcohol-formalin and stained by the Feulgen method. Image cytometry was performed with an image analysis system (Samba Technologies, Meylan, France) as previously reported [1]. In brief, at least 300 interphase cell nuclei were analyzed on each microscopic slide. Eleven parameters were computed from each nuclear image: one geometric feature (nuclear area), one densitometric parameter (the DNA integrated optical density [DNA-IOD]) and nine textural parameters. Geometric and textural features estimated by image cytometry are closely related to the location of a given cell in the cell cycle. G0/G1 cells were therefore isolated on the basis of their DNA-IOD value as previously reported and analyzed for morphometry and chromatin texture.

Nine textural parameters were calculated on the nuclear image. Four features were calculated on the gray-levels co-occurrence matrix: local mean, energy, entropy and inertia. Five parameters were calculated on the run-length matrix: short run-length emphasis, long run-length emphasis, gray levels distribution, run-length distribution and run-length percentage.

The distribution, mean and standard deviation of the nuclear parameters were calculated for each cell population. Significance of the differences between parameter values were estimated by student T-test after Bonferroni correction for multiple variables.

To compare multiple cell groups, a deviation index was computed and an average nuclear abnormality was then calculated for each cell line as the arithmetic mean of the absolute values of the deviation indexes (z-scores) observed over all included features for all nuclei in a data set as previously reported [22–24].

■ Raman microspectroscopy

In order to avoid spectral interference with the cell culture medium, 1×10^7 cells were washed twice with phosphate buffered saline and resuspended in physiological serum. More than 20 living cells were randomly analyzed for each sample and the acquisition procedure was repeated three times. Two spectra per cell were collected consecutively, thus leading to approximately 120 spectra per sample. These spectra were obtained using a Labram microspectrometer (Horiba Scientific, Villeneuve

d'Ascq, France) coupled to a charge-coupled device camera (Andor, Belfast, Northern Ireland) and a right microscope. The excitation light was provided by a titanium-sapphire laser tuned at 785 nm. A 100× water immersion objective (numerical aperture = 1) was used to focus the laser and to trap the probed cell. The laser beam waist was evaluated to 4 μm, and the laser power at the objective output was adjusted to 35 mW, resulting in an irradiance of approximately 2.8×10^8 mW/cm² [25]. Spectra were collected with an integration time of 30 s and the acquisition was performed in a temperature-controlled room. Observations of the sample before and just after the acquisition allowed for the checking that only one cell was trapped at once and that the cell stayed trapped all along the measurement. Rayleigh elastic scattering and reflected laser were rejected by placing an edge filter before the spectroscopic part of the instrument. The Raman Stokes scattering was spectrally analyzed in the 590–1750 cm⁻¹ spectral region with a 950 grooves/mm holographic grating, assuring a spectral resolution of 4 cm⁻¹.

■ Data processing & statistical analysis

A Raman spectrum is composed of 1024 points along the wavenumber axis. A value of the Raman scattering intensity is measured to each wavenumber. Raman spectra were first corrected for the apparatus response by dividing the raw spectra by a National Institute of Standard spectrum. The National Institute of Standard was used to mimic a black body in order to control and correct the dependency of the instrument efficiency with the light frequency. After this first step, the spectra were slightly smoothed and baseline corrected using toolboxes of Labspec software (Horiba Scientific, Villeneuve d'Ascq, France). Parameters used to treat spectra were: three points and average mode for filtering and line segment baseline using manually selected wavenumbers (17 minima points were used). Spectra were afterwards vector normalized on the whole 590–1750 cm⁻¹ spectral range.

After this correction step, spectra were processed by a random features method for selection of discriminant wavenumbers, and by hierarchical cluster analysis (HCA) for spectra classification. Random features algorithm permitted to identify in a non-supervised way the most relevant wavenumbers in order to discriminate two groups of spectra. For this processing, the MatLab rand-features function (MathWorks, Inc., Natick, MA,

USA) was used with standard parameters. A score plot allowed visualizing, in a decreasing order, the discriminant weight of the wavenumbers.

HCA of spectra was then performed on the wavenumbers selected by the random features algorithm, using a customized-software developed in MatLab. Grouping of spectra was carried out using the Euclidean distance and the Ward's method for linkage. This method proved to be efficient to reveal distinctive classes of Raman spectra, with relevance to biological properties of samples [26].

Results

■ Biomolecular effects of TSA & MS-275 on histone post-translational modifications

The first step to analyze the ability of LTRS to detect cellular changes induced by HDIs has been to validate a cellular model displaying different levels of histone acetylation. For that purpose, Jurkat cells were treated or not with nontoxic doses of either, TSA or MS-275 (data not shown). Separation of histones H1, H2B, H3 and H4 was performed using AUT-PAGE (FIGURE 1). In these stimulatory conditions, treatment of Jurkat cells with MS-275 markedly increased acetylation of histones H4 and H3, whereas the effects of TSA was mainly observed on histone H3, and weakly on histone H4.

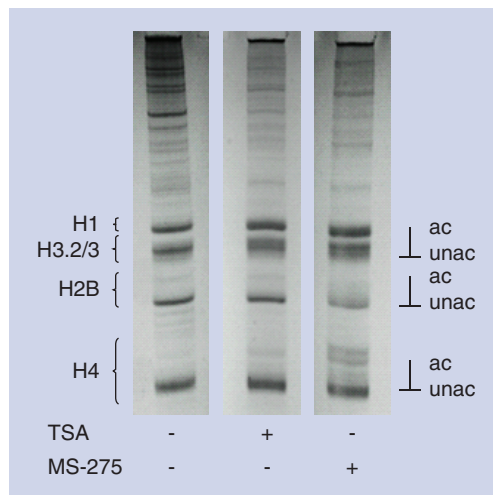


Figure 1. Histone deacetylase inhibitor treatment modulates histone acetylation in Jurkat cancer cells. Jurkat cancer cells were treated either with TSA 165 nM (50 ng/ml) or MS-275 26.6 μ M (10 μ g/ml) for 24 h. Total histones were acid extracted and analyzed by acid-urea-TritonX100 polyacrylamide gel electrophoresis. ac: Acetylated; TSA: Trichostatin A; unac: Unacetylated.

■ Effects of TSA & MS-275 on nuclear morphometry & texture

The effects of these two HDIs on the nuclear organization of fixed Jurkat cells were analyzed using conventional image cytometry. Values of the analyzed features were estimated in G0/G1 cells and are listed in TABLE 1. Control and HDI-treated cell populations exhibited the same nuclear areas. Values of the textural features showed significant variations in MS-275-treated cells only. These changes corresponded to a slight, but significant, chromatin global decondensation (decrease in local mean by 7% compared with the control). Besides, the main chromatin alteration concerned spatial arrangements with a less compact (increase in energy, whilst decreases in entropy and inertia by 73, 20 and 23% compared with the control, respectively) and more homogeneously and finely distributed chromatin (decrease in short run-length emphasis [4%] and run-length percentage [10%], while increases in long run-length emphasis and gray level distribution by 35 and 38% with respect to controls). Meanwhile, TSA did not induce any significant modification of this chromatin supraorganization compared with untreated cells. Lack of effects of TSA on the structural organization relatively to MS-275 treatments was visualized in the integrative average nuclear abnormality representation (FIGURE 2).

■ HDI treatment induces Raman spectral differences in Jurkat cells

Effects of HDI treatment on living Jurkat cells in suspension was then investigated using the LTRS technique. First, aberrant spectra were eliminated. Then, spectra issued from MS-275- (n = 107) and TSA- (n = 119) treated cells were merged to form one group (HDI-treated cells). These 226 Raman spectra from HDI-treated cells and 107 Raman spectra from untreated cells were processed by random features selection of discriminant wavenumbers and HCA. This processing resulted in the generation of a score plot (SUPPLEMENTARY FIGURE 1; WWW.futuremedicine.com/doi/suppl/10.2217/epi.11.102) and in the identification of 16 discriminant wavenumbers (TABLE 2) leading to a classification in two separate clusters (FIGURE 3). 77.5% of spectra from control cells were localized in cluster 1 and 76.1% of spectra from HDI-treated cells appeared in cluster 2 (TABLE 3). Adding more wavenumbers in the discriminant analysis did not result in a better classification of HDI-treated and control cells (data not shown). The most discriminant wavenumbers belonged mainly to one spectral

Table 1. Nuclear geometric and textural parameter values in G0/G1 Jurkat cells.

Parameter	Treatment		
	Control	TSA	MS-275
A	404.6 ± 82.6	405.1 ± 83.9	404.7 ± 70.0
LM	199.1 ± 47.6	192.3 ± 45.3	185.4 ± 39.5 [†]
E	8.6 ± 2.6	8.0 ± 2.4	14.9 ± 4.3 [†]
ENT	279.2 ± 31.2	285.8 ± 30.5	224.2 ± 30.2 [†]
I	302.0 ± 52.4	310.9 ± 52.9	233.6 ± 20.9 [†]
SRE	61.6 ± 2.8	62.2 ± 2.9	59.2 ± 1.1 [†]
LRE	5.6 ± 0.9	5.4 ± 0.9	7.6 ± 0.8 [†]
GLD	20.3 ± 4.2	19.3 ± 3.9	28.1 ± 5.3 [†]
RLD	36.4 ± 3.1	37.2 ± 3.1	33.5 ± 1.1 [†]
RPC	104.8 ± 6.9	106.7 ± 6.9	94.4 ± 3.6 [†]

Values are mean ± standard error of mean, and data are expressed in arbitrary units.
[†]Significantly different from control cells (T-test after Bonferroni correction for multiple variables).
A: Nuclear area; E: Energy; ENT: Entropy; GLD: Gray level distribution; I: Inertia; LM: Local mean of gray levels; LRE: Long run-length emphasis; RLD: Run-length distribution; RPC: Run-length percentage; SRE: Short run-length emphasis; TSA: Trichostatin A.

range, located between 978 and 989 cm^{-1} , as shown in FIGURE 4 & TABLE 2. Other wavenumbers (i.e., 839, 846, 848, 1355, 1358 or 1413) have also been identified by the selection algorithm, but with lower discriminant scores (TABLE 2). Besides these wavenumbers defined by random features selection with a very high discriminant power ($p < 1 \times 10^{-8}$), we also observed a significant decrease in the intensity of several peaks (720–725; 785; 1093–1094; 1315–1320; and 1575 cm^{-1} ; $p < 0.001$; Kruskal–Wallis test).

The potential of LTRS to discriminate TSA-treated cells from control cells and MS-275-treated cells from control cells was then assessed using the 16 wavenumbers previously retained by random features and shown in TABLE 2. This analysis showed that 73.1 and 80.4% of spectra from TSA- and MS-275-treated cells, respectively, were localized in cluster 2 (TABLE 3).

This type of analysis was applied in order to discriminate TSA- from MS-275-treated cells. Random features selection followed by HCA was unable to efficiently separate these two cell groups (data not shown).

Discussion

In this study, we focused our investigation on the histone acetylation/deacetylation balance, which is kept under control in the cell by the expression of the histone acetylase and HDAC enzymes. Accordingly, we found that the biological consequence of HDAC inhibition by TSA and MS-275 was an accumulation of acetylated histones in the cell nucleus. Histone H1 remained unaffected by both treatments. Accordingly, this histone form is not acetylated through post-translational processes

in vivo [27]. Nevertheless, modification of histone acetylation upon MS-275 treatment appeared more pronounced compared with that induced by TSA. Several matters could be implicated in these differences. MS-275 was used at higher concentration than TSA. However, both HDI concentrations were adjusted at sublethal levels and within their EC_{50} ranges [21]. Therefore, differences observed could be mainly due to their substrate specificity. In that line, use of other HDIs such as suberoylanilide hydroxamic acid

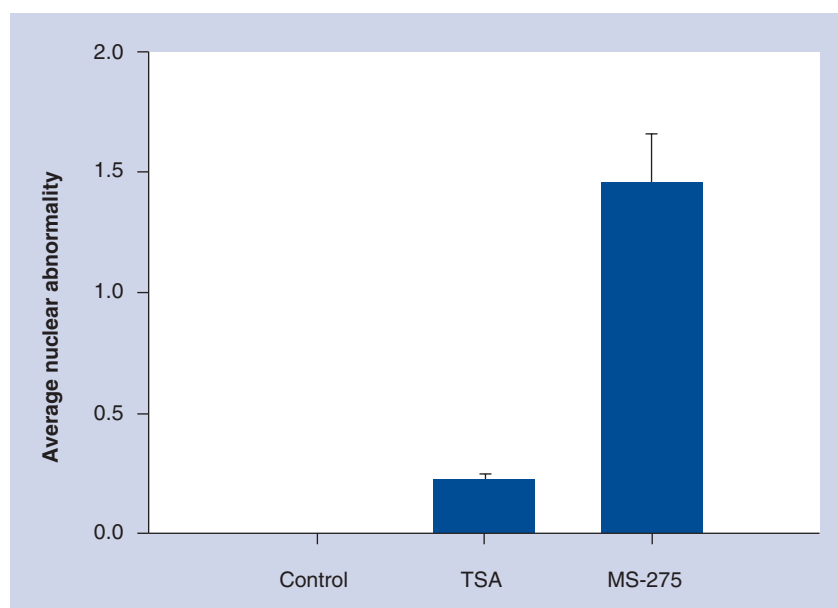


Figure 2. Average nuclear abnormality index extracted from conventional cytometry analysis. Deviation indices signatures for nuclei from Jurkat cells treated either with TSA or MS-275 were calculated according to Bartels *et al.* [22] and the average nuclear abnormality was computed as described by Montironi *et al.* [23]. Indices (mean ± standard error of mean) are expressed as units of standard deviation in the control untreated Jurkat data set. TSA: Trichostatin A.

Table 2. Classification of wavenumbers according to their score obtained after random features analysis.

Wavenumbers	Score [†]
984	184
986	157
983	134
987	124
981	93
982	93
988	85
979	76
978	64
848	50
839	43
989	43
846	38
1358	37
1355	35
1413	35

The more discriminant wavenumbers are represented in the table.

[†]The values of the score reflect the discriminating power of the corresponding wavenumber.

and sodium butyrate also displayed specific patterns of histone acetylation (data not shown). Concomitantly to these histone acetylation changes, we showed that these HDI treatments were associated with modifications of the nuclear morphometry and texture, and with Raman spectral differences.

The introduction of epigenetic strategies in the therapeutic arsenal against cancer led

to the need of techniques able to evaluate the efficiency of these therapies *in vivo*. Besides molecular biological approaches, these drug effects were also monitored through morphological methods such as nuclear texture analysis. For instance, conventional image cytometry showed significant morphometric signature changes after cell treatments with epigenetic modulators, and similar phenomena have already been observed in human fibrosarcoma [5] or HeLa cells [28]. Interestingly, MS-275 only induced strong variations in chromatin distribution, whereas TSA seemed ineffective on these parameters. In previous studies, we showed that TSA could induce modulation of cytometric features in cells whose DNA was hypomethylated such as OV1 tumor cells [29]. In HT1080, whose DNA was less hypomethylated, TSA appeared effective only after previous treatment with the demethylating agent 5-azadC [5]. This suggests different mechanisms involved by this HDI treatment in chromatin supraorganization changes evaluated by image cytometry. However, this technique could only be applied to fixed-cell specimens and could hardly be correlated with specific molecular events. Moreover, this technique requires cell staining. Raman microspectroscopy could offer a new noninvasive way to follow dynamic epigenetic changes in living, unstained and unfixed tumor cells. We previously reported the use of Raman microspectroscopy to discriminate healthy epidermis from normal and peritumoral stroma in basal cell carcinoma [30].

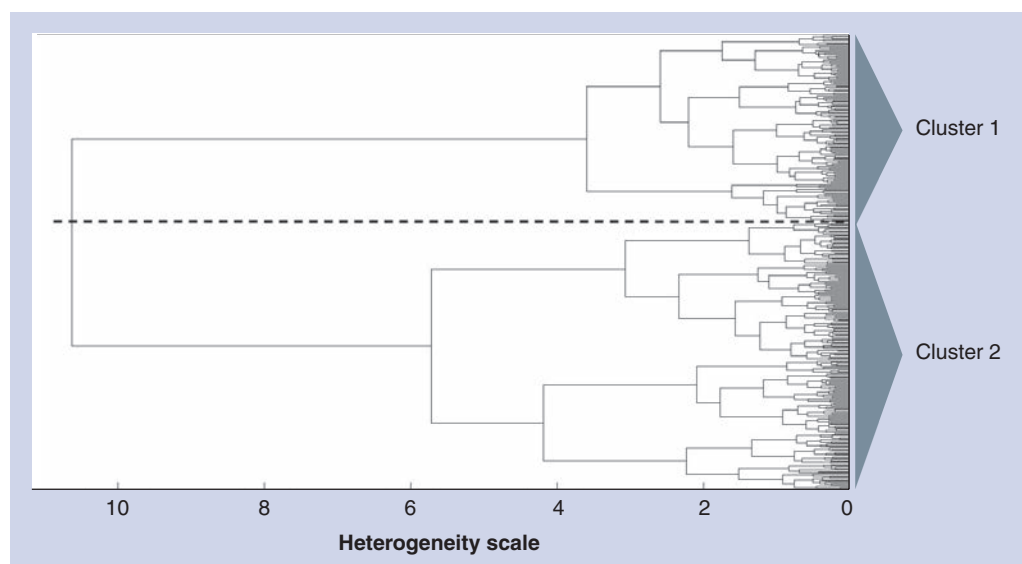


Figure 3. Hierarchical cluster analysis of spectra issued from control and histone deacetylase inhibitor-treated cells. Hierarchical cluster analysis was performed using the discriminant wavenumbers selected by random features analysis listed in TABLE 2.

Table 3. Distribution of spectra from the different cell groups among the clusters defined after random feature analysis and hierarchical cluster analysis of control and histone deacetylase inhibitor-treated cell populations.

Cell group	n [†]	Spectra classified in clusters (%)	
		Cluster 1	Cluster 2
Control	107	77.5	22.5
HDI	226	23.9	76.1
TSA	119	26.9	73.1
MS-275	107	19.6	80.4

Overall classification accuracy: 76.8.
[†]Number of spectra analyzed in each experimental group.
HDI: Histone deacetylase inhibitor; TSA: Trichostatin A.

Moreover, such an approach has been recently applied to the identification of stem cell states [8,31] and to the follow-up of their differentiation pathways by the detection of intracellular molecular markers [32]. A previous study from Chan *et al.* [33] also revealed the beneficial use of LTRS to discriminate human T lymphocytes from Jurkat cells, hence demonstrating the potential of this technique to discriminate malignant from normal lymphoid cells. Concerning the study of epigenetic phenomena, Raman-based techniques seemed to be restricted, up to now, to the analysis of acellular *in vitro* molecular changes observed on DNA or histones [9,34] and on miRNA identification [35], and to our knowledge no study at the level of the whole-living cell has been performed. We demonstrated here that LTRS, coupled to a statistical data processing, allowed detecting epigenetic changes in whole-living cells.

Epigenetic modulators induce finely tuned molecular changes that can be monitored by Raman microspectroscopy. Using Jurkat cells, we demonstrated that HDI treatments induced changes in the spectral signatures issued from single-cell LTRS. Before treatment, Jurkat cells displayed spectral signatures similar to those previously described by Chan and colleagues [33,36]. Compared with these control cells, HDI-treated cells displayed spectral changes mostly located in the 978–989 cm⁻¹ range and around 845 cm⁻¹. With respect to epigenetic changes, these spectral signals have been associated with phosphate bonds and deoxyribose ring vibrations [37]. These changes could be related to modifications of nucleosome conformation induced by the addition of acetyl residues at the N-terminal tail of histones. This was in line with the significant decrease in the intensity of several peaks (720–725; 785; 1093–1094; 1315–1320; and 1575 cm⁻¹), which could be attributed to a decrease of DNA signal [38]. Indeed, these peaks have been attributed to

ring breathing modes in the DNA bases, (725, 785, 1318 and 1575 cm⁻¹) or to the symmetric PO₂ stretching vibration of the DNA backbone (1093 cm⁻¹) [38]. Since Raman signal intensity of these vibrations is linked to the concentration of molecular bonds [39] or structural modifications of the related compounds, this decrease in intensity of DNA specific peaks could reflect the chromatin decondensation induced by HDI treatment and already observed by image cytometry in MS-275-treated cells.

Interestingly, some of the DNA relative features allowing discrimination between Jurkat cells and healthy T-lymphocytes (785, 1093 and 1575 cm⁻¹) [33] were also observed in our study when we compared spectra of HDI-treated

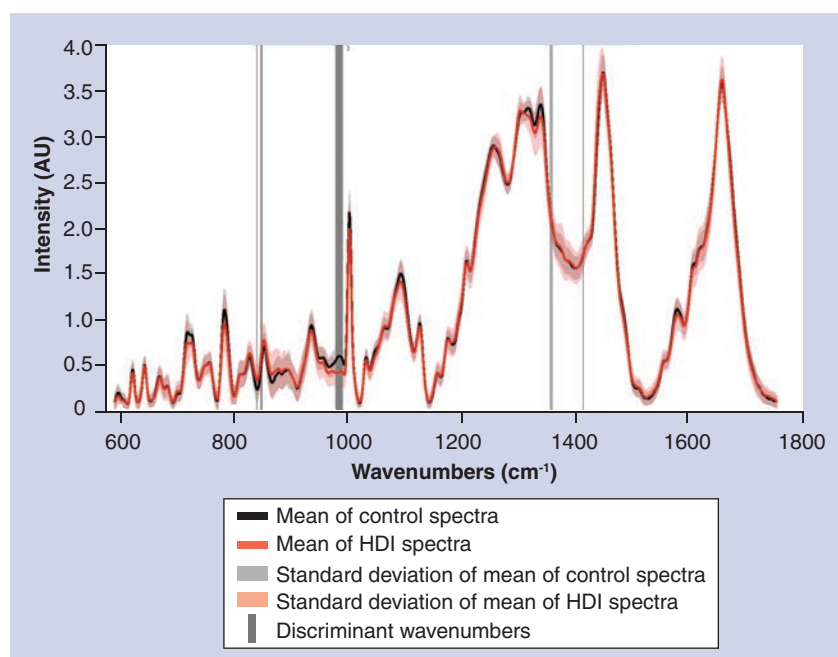


Figure 4. Raman spectra representative of control and histone deacetylase inhibitor-treated cells. Data correspond to 107 individual spectra from control cells and 226 spectra from histone deacetylase inhibitor-treated cells (107 MS-275- and 119 trichostatin A-treated cells). Discriminant wavenumbers were selected after random features analysis. AU: Arbitrary units; HDI: HDAC inhibitor.

Jurkat cells to spectra of control cells. As stated above, these spectral modifications suggested an overall DNA decondensation in HDI-treated cells as it was observed in T-lymphocytes compared with their leukemic malignant counterpart. Nevertheless, the differences observed between TSA- and MS-275-treated cells both at the AUT-PAGE and the image cytometry levels were not detected by Raman microspectroscopy analysis. However, the enlarging of the number of discriminant wavenumbers in the random features analysis improved the spectral discrimination of MS-275-treated cells from control cells. This improvement could not be observed for TSA-treated cells (data not shown), suggesting differential biological changes induced by these two drugs as already observed.

Thus, LTRS seems to be a noninvasive powerful technique to assess epigenetic changes in living cells. More advanced LTRS developments could be set up to probe more selectively cellular compartments. In the results described above, the Raman analysis was performed with the simplest configuration in that the same laser beam was used as optical tweezers and simultaneously as excitation source for Raman scattering. However, different experimental schemes could be set up, and improve in the future, the quality of interpretation of the biological alterations observed either in pathological conditions or upon treatment [40–44]. In more advanced configurations, dual wavelength optical tweezers could be coupled to confocal Raman microscopes; when one of the beams excites the Raman spectrum, a second beam for tweezing

enables manipulation of the environment of the trapped objects, for example, a single living cell. Raman microspectroscopy could also be combined with dual beam optical traps which allow distinguishing Raman spectra from the nucleus, cytoplasm and cell membrane. In forthcoming studies, these technological improvements could allow for deeper investigations of the molecular mechanisms associated with the observed epigenetic-induced spectral changes.

Acknowledgements

The authors wish to thank V Untereiner for her expert technical advice.

Financial & competing interests disclosure

This work was supported by grants from the Comité Départemental de l'Aube de la Ligue Nationale Contre le Cancer. The authors would like to thank CNRS Projets Exploratoires Pluridisciplinaires for financial support. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Histone acetylation in human leukemic Jurkat cells can be modulated by histone deacetylase inhibitors

- Using biochemical methods, we showed the modulation of histone acetylation level in Jurkat cells treated or not by two different histone deacetylase inhibitors (HDIs).
- The MS-275 effects were more pronounced than those of trichostatin A in terms of histone acetylation.
- Biochemical variations of histone acetylation were confirmed by conventional image cytometry on fixed cells.

Laser tweezer Raman spectroscopy can be used to perceive epigenetic modifications in living Jurkat cells

- Molecular and biochemical conventional approaches of epigenetic alteration are biologically perturbing or even destructive.
- Up to now and concerning epigenetics, Raman microspectroscopy was restricted to the analysis of the acellular *in vitro* modeling of the molecular changes observed on DNA or histones.
- We demonstrated here that Raman microspectroscopy, thanks to its trapping capability of the excitation laser, could also be used to probe isolated living cells and allow epigenetic discrimination.

Laser tweezer Raman spectroscopy detects cellular biological modifications following HDI treatment

- The random features selection associated with hierarchical cluster analysis discriminated HDI-treated cells from control cells on the basis of their Raman spectral signatures.

Conclusion

- Laser tweezer Raman spectroscopy is a rapid label-free tool to identify living cells that underwent epigenetic changes.
- In the future, laser tweezer Raman spectroscopy technique improvements should allow for the fine detection of the molecular mechanisms associated with the observed epigenetic-induced changes in living cells.

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▪ of interest

▪▪ of considerable interest

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Auteur Mathilde POPLINEAU
Diplôme Thèse de Docteur de l'Université de Reims Champagne-Ardenne
Discipline Biologie Cellulaire – Biologie Moléculaire
Adresse Laboratoire MEDyC, unité CNRS FRE 3481, UFR Pharmacie, 51 rue Cognacq Jay, 51096 Reims

Rôles des mécanismes épigénétiques dans la régulation de l'expression de gènes impliqués dans l'invasion de cellules tumorales

Mots clés : *Epigénétique, 5-azadC, inhibiteurs d'HDACs, MMPs, invasion tumorale, fibrosarcome humain*

Les propriétés invasives des cellules cancéreuses sont liées à des modulations importantes de l'expression de gènes. Des protéases doivent être exprimées afin de permettre la dégradation de la matrice extracellulaire (MEC), l'activation protéolytique de protéines matricielles et la libération de facteurs de croissance, de cytokines, de récepteurs et de molécules d'adhérence. Parmi ces protéases, les métalloprotéinases matricielles (MMPs) jouent un rôle crucial dans la dégradation de la MEC et dans le remodelage tissulaire observé au cours de l'invasion tumorale. L'émergence de thérapies anticancéreuses basées sur des stratégies épigénétiques nécessitent d'évaluer leurs effets sur les propriétés des cellules tumorales. Ce travail a pour objectif d'analyser les effets de modulateurs épigénétiques (un agent hypométhylant de l'ADN et des inhibiteurs d'histone désacétylases (inhibiteurs d'HDACs ou HDIs)) sur l'expression des MMP-1, -2 et -9 dans la lignée cellulaire de fibrosarcome humain HT1080. Dans un premier temps, il apparaît que l'agent hypométhylant de l'ADN, la 5-aza-2'-désoxycytidine (5-azadC), augmente l'expression génique et protéique des MMP-1, -2 et -9. Ces modifications de l'expression sont associées à (i) une déméthylation globale de l'ADN et (ii) des modifications de la supra-organisation chromatiniennne correspondant globalement à une chromatine moins condensée. De plus, la 5-azadC est capable d'accroître les propriétés invasives des cellules par l'intermédiaire, notamment, d'une augmentation de l'expression de la MMP-1 par un mécanisme transcriptionnel. Cette augmentation de la transcription implique le recrutement du facteur Sp1 et un remodelage chromatinien au niveau du promoteur du gène de la MMP-1. Néanmoins, une déméthylation totale de ce promoteur n'est pas nécessaire à cette induction. De manière complémentaire, le traitement des cellules HT1080 par différents HDIs révèle le rôle potentiel d'HDACs dans la régulation de l'expression de la MMP-1. Un HDI à large spectre, la trichostatine A (TSA), est capable de moduler l'expression de la MMP-1 et la texture nucléaire, mais uniquement après déméthylation préalable de l'ADN par la 5-azadC. Par contre, l'HDI spécifique des HDACs de classe I, le MS-275, est capable d'induire, à lui seul, l'expression génique et protéique de la MMP-1. Cette expression génique requiert un remodelage de la chromatine et le recrutement de l'histone acétyltransférase p300 au niveau du promoteur du gène de la MMP-1. L'ensemble de ces résultats suggèrent que des mécanismes épigénétiques jouent un rôle crucial dans le contrôle de l'expression de la MMP-1 dans les cellules HT1080, influençant ainsi les propriétés invasives de ces cellules.

Roles of epigenetic mechanisms in the regulation of the expression of genes involved in tumor cell invasion

Key words : *Epigenetics, 5-azadC, HDAC inhibitors, MMPs, tumor invasion, human fibrosarcoma*

Invasive properties of cancer cells require critical changes in gene expression. Proteases must be expressed for the degradation of the extracellular matrix (ECM), the proteolytic activation of matrix proteins and the release of bioactive molecules such as growth factors, cytokines, receptors and adhesion molecules. Among these proteases, the matrix metalloproteinase (MMP) family members play a crucial role in the ECM breakdown and remodeling of tissues during tumor invasion. The introduction of epigenetic strategies in the therapeutic arsenal against cancer led to the need to evaluate the effects of such therapeutic approaches on cell behavior. Here we focused our attention on the effects of epigenetic modulators, a DNA hypomethylating agent and histone deacetylase inhibitors (HDAC inhibitors or HDI), on the expressions of MMP-1, -2, and -9 in the human HT1080 fibrosarcoma cell line. First, we showed that the DNA hypomethylating drug 5-aza-2'-deoxycytidine (5-azadC) increases MMP-1, -2, -9 expressions both at the mRNA and protein levels. These changes in gene expression are associated with (i) a global DNA demethylation and with (ii) modifications in chromatin supra-organization which globally correspond to a more decondensed chromatin. Moreover, 5-azadC is able to increase the invasive properties capability of the HT1080 cells mainly *via* MMP-1 transcription-dependent expression. This enhancement of transcription occurs through (i) Sp1 recruitment, (ii) chromatin remodeling and (iii) in absence of full demethylation on the MMP-1 gene promoter. Using different HDIs reveals that HDACs could potentially play a role in MMP-1 expression. The pan-HDI trichostatin A (TSA) act in synergy with 5-azadC and is able to modulate MMP-1 expression and nuclear texture, but only after DNA demethylation. In contrast, the HDAC class I inhibitor, MS-275, which display additive effect with 5-azadC, is able to induce, alone, MMP-1 gene expression through chromatin remodeling and p300 recruitment to its promoter. These data suggest that epigenetic mechanisms play a crucial role in MMP-1 expression control in HT1080 cells thus influencing the invasive potential of these cells.