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

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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. 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

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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% $C0_2$ 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 Platinium 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 *$\beta_2$macroglobulin* as a reference: *β_2m* forward 5'-ATCTTCAA ACCTCCATGATG-3'; *β_2m* reverse 5'-ACCCCCACTGAAAA AGATGA-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 peroxydase-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 aceton. 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 peroxydase-conjugated anti rabbit secondary antibody (GE Healthcore, 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). G_0/G_1 nuclei were extracted as previously reported (23). Nine texture parameters were computed from G_0/G_1 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 ± 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

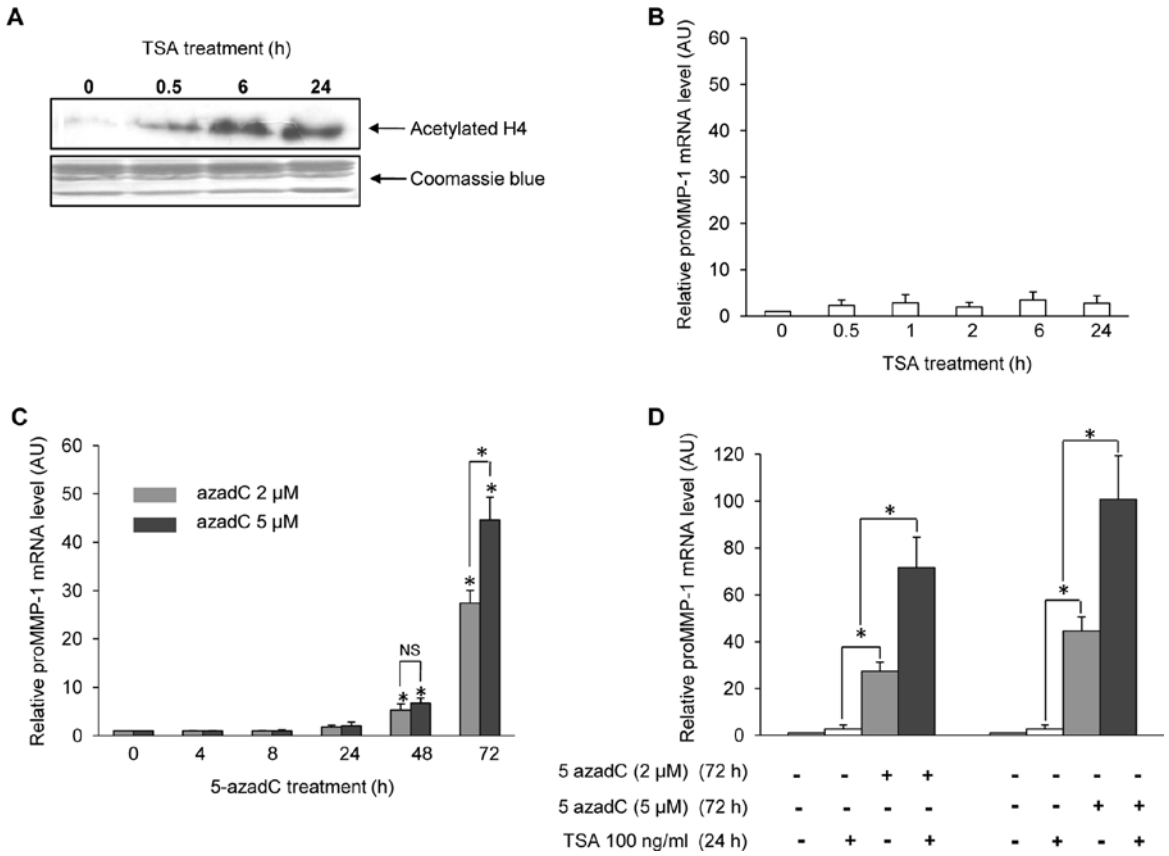

Figure 1. Effects of 5-azadC and TSA on *proMMP-1* gene expression. HT1080 cells were treated or not with TSA (100 ng/ml), 5-azadC (2 or 5 μM), or both drugs for various times as indicated on the figures. (A) Histones were acid extracted and Western blotting using total acetylated H4 histone antibody was performed. (B-D) Levels of endogenous mRNA were monitored by real-time RT-PCR. *$\beta_2$microglobulin* (β_2m) was used as internal control. Results are expressed as the mean ± SEM of normalized ratio *proMMP-1*/β_2m.*p<0.05 (Kruskal Wallis statistical test).

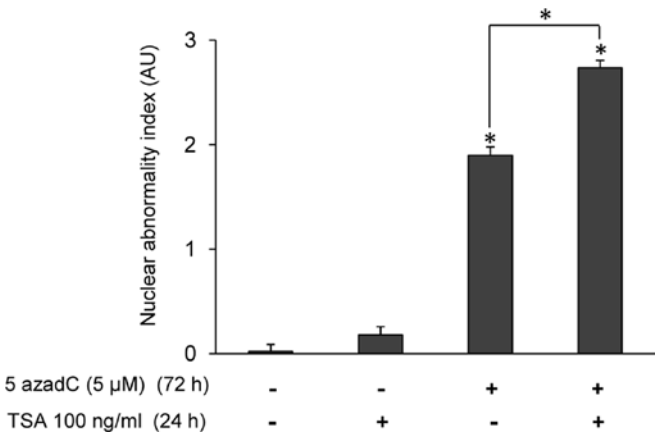

Figure 2. Effects of 5-azadC and TSA on chromatin supraorganization. HT1080 cells were treated or not with TSA (100 ng/ml 24 h) and/or 5-azadC (5 μM 72 h). After Feulgen staining, 9 nuclear texture features were analyzed on G_0/G_1 nuclei using image cytometry. Results are expressed as nuclear abnormality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

5 μM 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 μM p<0.05, 45-fold increased with 5 μM p<0.05) (Fig. 1C). When cells were simultaneously treated with TSA (100 ng/ml 24 h) and 5-azadC (2 or 5 μM 72 h), *proMMP-1* was strongly expressed (70-fold increased with 2 μM, 100-fold increased with 5 μM) 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 μM 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.

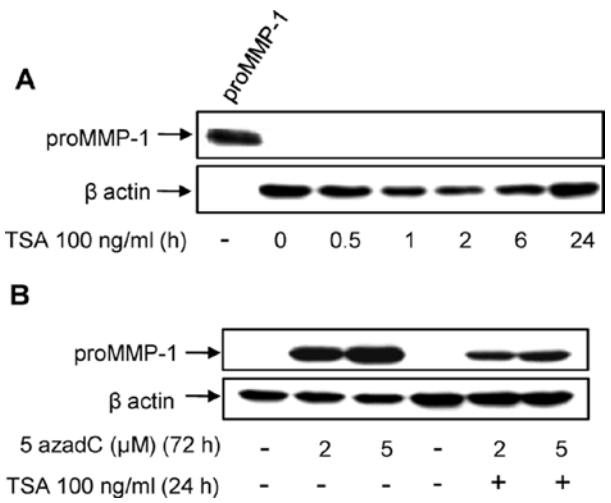

Figure 3. Effects of 5-azadC and TSA on proMMP-1 secretion. HT1080 cells were treated or not with TSA and/or 5-azadC as indicated on the figures. (A and B) Western blot analyses of cell concentrated conditioned media: 35 μg of total proteins were analyzed. Panel A (lane 1), recombinant proMMP-1 was used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

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

	Treatments		
Parameters	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 ± SEM. [a]p<0.05 as compared to control untreated cells (t-test after Bonferroni correction). [b]p<0.05 as compared to 5-azadC alone (t-test after Bonferroni correction).

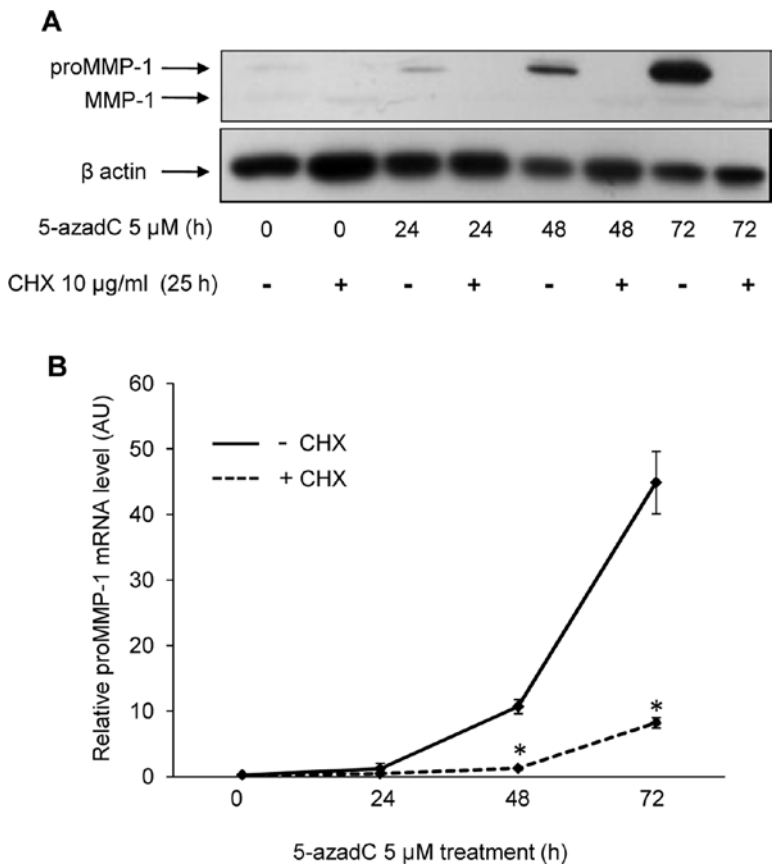

Figure 4. Effect of protein neosynthesis inhibition on proMMP-1 mRNA and protein levels. HT1080 cells were treated or not with 5-azadC and/or CHX as indicated in the figures. (A) Western blot analysis of cell-concentrated conditioned media: 35 μg of total proteins were analyzed. (B) Real-time RT-PCR of *proMMP-1* mRNA. *$\beta_2$microglobulin* (β_2m) was used as internal control. Results are expressed as the mean ± SEM of normalized ratio *proMMP-1*/β_2m. *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 μM 72 h) and CHX (10 μg/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-1β, 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-1β (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 hyper-acetylation 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.

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