MMP Inhibitors Augment Fibroblast Adhesion through Stabilization of Focal Adhesion Contacts and Upregulation of Cadherin Function

Andrew T. Ho,* Evelyn B. Voura,* Paul D. Soloway,† Katrina L. M. Watson* and Rama Khokha*‡

*Department of Medical Biophysics and Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario Cancer Institute, Toronto Health Network, Toronto, Ontario, M5G 2M9, Canada. †Roswell Park Cancer Institute, Department of Molecular and Cellular Biology, Buffalo, New York 16263

Running Title: MMP inhibitors promote cell adhesion.

‡Correspondence to Rama Khokha, Department of Medical Biophysics, Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada. Tel: (416) 946-2051, fax: (416) 946-2984, E-mail: rkhokha@uhnres.utoronto.ca

Key Words: TIMP-1, MMP inhibitors, cell-cell adhesion, p125FAK, cadherin, β-catenin.
SUMMARY

Increased pericellular proteolysis due to an imbalance between MMPs (matrix metalloproteinases) and TIMPs (tissue inhibitors of metalloproteinases) promotes early stages of tumorigenesis. We have reported that TIMP-1 downregulation confers tumorigenicity on immortal Swiss 3T3 fibroblasts. In pursuit of the mechanism involved in this transformation, we asked whether MMP inhibitors modulate contact inhibition and cell adhesion, since the dysregulation of these events is essential for cellular transformation. Using both genetic and biochemical means, we demonstrate that MMP inhibitors regulate fibroblast cell adhesion. TIMP-1 downregulated cells formed dense, multi-layered colonies, suggesting a loss of contact inhibition. Recombinant TIMP-1 and synthetic MMP inhibitors (MMPi) restored normal cell contact and density of these cells in a dose dependent manner. Consequently, the effect of MMPi on both cell-extracellular matrix (ECM) and cell-cell adhesion were investigated. Upon MMPi treatment, p125\(^{\text{FAK}}\) was redistributed, together with vinculin, to points of cell-ECM contact. Further, phosphorylation of p125\(^{\text{FAK}}\) was restored to levels similar to that of wildtype. In parallel, MMPi treatment increased cadherin levels and stabilized cadherin-mediated cell-cell contacts. Moreover, enhanced cadherin function was evident as increased calcium dependent cell-cell aggregation and co-localization of cadherin and \(\beta\)-catenin at the cell membrane. We also obtained independent evidence of altered cadherin function using \(\text{timp-1}^{-/-}\) mouse embryonic fibroblasts. Our data provide provocative evidence that increased pericellular proteolysis impacts cell adhesion systems to offset normal contact inhibition, with subsequent effects on cell transformation and tumorigenesis.
INTRODUCTION

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) constitute a key system of pericellular proteolysis within the cell microenvironment. Our understanding of the role of this proteolytic system in cancer has evolved over the past decade. Initially linked to tumor invasion and metastasis, an MMP/TIMP imbalance is now thought to function in promoting early events of tumor development (1). The current emphasis is on identifying the mechanisms underlying these early effects. A better understanding of the relationship between MMP/TIMP activity and cell-extracellular matrix (ECM) and cell-cell communication is fundamental to this effort.

We had reported that downregulation of TIMP-1 expression caused an immortal fibroblast cell line to become tumorigenic (2). Extensive literature has since led to the knowledge that cancer involves a disrupted balance between MMPs and TIMPs. Both TIMPs and MMPs have been manipulated through genetic and biochemical approaches in tissue culture systems to demonstrate that, in general, TIMPs inhibit tumor cell invasion, angiogenesis, metastasis as well as tumor formation (3-9), while MMPs promote these events (10-13). Transgenic and knockout animals have further supported the role of this proteolytic system in early tumorigenesis (14-19). In addition to the classical MMP substrates (the structural ECM proteins), soluble and cell surface proteins have been recognized as novel MMP substrates. These include growth factor binding proteins (20), growth factor receptors (21) and cell adhesion proteins (22). These molecules are involved in cell signaling and thus expand the possible roles of pericellular proteolysis in early events of tumorigenesis.

Non-transformed cells exhibit reduced proliferation at high density, and this phenomenon has been termed contact inhibition (23,24). Loss of contact inhibition is
MMP inhibitors promote cell adhesion

one of the early events associated with cellular transformation and cancer. Physical contacts that include both cell-ECM and cell-cell adhesion are known to influence cell fate through apoptosis, proliferation and differentiation (25,26). The cell interacts with ECM through an establishment of focal adhesion contacts, typically composed of integrins and various cytoplasmic proteins that link the cytoskeleton to the ECM (27). Cell-cell interactions, on the other hand, primarily involve cadherins (28). The function of integrin and cadherin in cell contact and adhesion has been extensively studied, while the role of pericellular proteolysis in influencing physical contact has just begun to unfold.

One potential mechanism by which pericellular proteolysis may influence physical contact is through the modification of integrins and cadherins. Integrins are the primary cell surface receptors for ECM molecules, and the latter are the principal substrates for MMPs. Therefore, MMP degradation of ECM molecules can indirectly affect integrin-mediated focal contacts. Although direct associations of specific MMPs and cell adhesion molecules, such as integrins, have been reported (29,30), MMP-mediated cleavage of these proteins is not known to occur. In contrast to integrins, MMPs have been suggested to target cadherin cleavage. For example, overexpression of stromelysin-1 (MMP-3) leads to the release of E-cadherin ectodomain in mammary epithelial cells, while a synthetic MMP inhibitor or transgenic TIMP-1 inhibits this event (22,31,32). Similarly, MMPi has been reported to reduce VE-cadherin shedding in endothelial cells (33). Together, these studies suggest that MMP proteolysis may process cell adhesion molecules to modify cell-cell adhesion and consequently affect cell fate.
The purpose of this study was to determine whether lack of cellular contacts underlies the acquisition of tumorigenic potential of TIMP-1 downregulated fibroblasts. Our investigation of the effects of MMP inhibitors on cell interactions with the matrix and adjacent cells revealed substantial effects on both adhesion systems. In TIMP-1 modulated fibroblast cell lines, we observed a relationship between TIMP-1 and cell density that suggested decreased TIMP-1 reduces contact inhibition. A biochemical approach using recombinant and synthetic MMP inhibitors showed that MMP inhibitors restored cell-ECM and cell-cell contacts. Moreover, laser scanning confocal microscopy identified that this rescue was due to an upregulation of focal adhesion contacts and cadherin localization at cell-cell contacts. Subsequently, the molecules downstream of these key adhesion proteins were functionally altered, including increased FAK activation and facilitation of β-catenin localization to the cell membrane. We propose that MMP:TIMP proteolytic axis may impact tumorigenesis through dysregulation of cell adhesion systems.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture

Swiss 3T3 (S3T3), MC2, TIMP-1 downregulated (LA1), and TIMP-1 upregulated (16S1) fibroblast cell lines have been previously reported (2,34-36). Briefly, Swiss 3T3 and MC2 clones are the parental and mock-transfected lines, respectively. Transfection of Swiss 3T3 cells with an antisense TIMP-1 RNA construct, or a sense TIMP-1 cDNA expression construct, followed by G418 (Gibco BRL, Ontario, Canada) selection, led to the generation of stable clonal cell lines called LA1 and 16S1 respectively (2, 35, 36). The mRNA and MMP inhibitory profiles were previously described (36). Compared to
parental and control cell lines, LA1 had half the MMP inhibitory activity, while 16S1 had four-fold higher activity when measured by collagenase-mediated degradation of $^{14}\text{C}]$glycine–labeled type I collagen. The 1CN cell lines was established from a LA1-induced tumor isolated from a nude mouse (34). All clones were cultured in DMEM (Gibco BRL, Ontario, Canada) supplemented with 10% (v/v) fetal bovine serum and maintained in humidified incubators at 37°C in 5% CO$_2$ and 95% air. The primary mouse embryonic fibroblast (MEF) cell lines were established from TIMP-1 wildtype and TIMP-1 null ($\text{timp-1}^{-/-}$) embryos at day 16.5 of gestation.

Subconfluent monolayer cells were trypsinized and plated at low cell density (300 cells per 35mm$^2$ dish) to allow formation of isolated colonies. At 4 hours post-plating, the cells were treated with either the synthetic MMP inhibitor (MMPI; N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpantanoyl]-L-tryptophan methylamide; GM6001 AMS Scientific, Pleasant Hill, CA) or recombinant TIMP-1 (rTIMP-1, kindly provided by Dr. D. R. Edwards, University of East Anglia, UK). Cells were grown for 7-10 days until isolated colonies formed. Colonies were visualized by fixing the cultures with 70% (v/v) ethanol in PBS and staining with 0.1% (w/v) methylene blue for 30 minutes at room temperature. The density of cells within a colony was determined by counting the number of nuclei that intersected the five lines of an E11-19 1.0-mm grid (Graticules Ltd., Tonbridge, UK) per microscopic field at 400×magnification. The density was scored from the center of the colony to avoid the bias contributed from the periphery of the colony. At least 6 colonies were counted for each treatment. One-way analysis of variance (ANOVA) and the Fisher-test were used to assess statistical significance. Difference was deemed significant when $p \leq 0.05$. 
**Reverse Transcription and PCR**

RNA from sub-confluent cultures was isolated with an RNA kit (Pharmingen, Ontario, Canada). Two microgram of RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco BRL, Ontario, Canada). A forward TIMP-1 sense-specific primer with a unique 5' tag, as indicated in lowercase, (5' gggcagctggagctACTGATAGCTTCCACTAAGGCC 3') was used to generate a complementary sequence of the first strand cDNA via primer extension reaction at 60°C for 2 minutes. Subsequent PCR was performed with tag-specific forward primer (5' ggcgctggagctACTGATA 3') and TIMP-1 reverse primer (5' CAAATTTCCGTTCCTTAGCG 3') in 25 cycles using an annealing temperature of 70°C for 1 minute and extension for 40 seconds at 72°C. The final 317bp PCR product was subsequently resolved on a 1.8% agarose gel, transferred to Hybond membrane and hybridized to [32 P-dCTP] labeled TIMP-1 cDNA probe (16). Signals were quantified using an ImageQuant program (Molecular Dynamics, Sunnyvale, CA)

**Gelatin Zymography**

Serum-free, 48 hour conditioned media was harvested from sub confluent cultures. Volumes representing equivalent cell number were separated on a 10% SDS-PAGE containing 0.1% gelatin. The gel was incubated in substrate buffer (50mM Tris, pH 7.5, 5mM CaCl₂, 40mM NaN₃) containing 2.5% Triton X-100 at room temperature for 1 hour, followed by further incubation in substrate buffer at 37°C for 20 hours. Gelatinolytic activity was visualized following Coomassie blue staining. Equivalent loading was confirmed by silver staining of a gel performed in parallel.
Confocal Microscopic Imaging

Swiss 3T3 and LA1 fibroblasts were grown on poly-L-lysine (Sigma, Saint Louis, MO) coated round glass coverslips. These were then fixed and used for specific staining. Specimens were washed 3 times for 3 minutes after staining and mounted on glass slides. The mounting medium was composed of 72% glycerol in PBS and contained 2.5% (w/v) of the antioxidant 1,4-diazabicyclo-[2,2,2]-octane (DABCO) (Sigma, St. Louis, MO) as an anti-bleaching agent. Preparations were then sealed with nail enamel and stored in the dark at 4°C. Confocal images were captured using a Zeiss Axiovert 100M inverted microscope equipped with a 63× c-apochromat objective lens and a LSM 510 confocal attachment.

Fluorogenic Substrate Digestion

Matrigel (BD Biosciences, Bedford, MA) was diluted 1:8 and combined with a 1:10 dilution of FITC conjugated gelatin (Molecular Probes, Eugene, OR). 100 µl of the mixture was used to coat coverslips deposited in a 24 well plate. The combined substrate was air-dried overnight and reconstituted the next day in HBSS. The coverslips were then transferred to fresh wells containing serum-free DMEM. Fibroblasts were labeled with 10 nM orange cell tracker (Molecular Probes, Eugene, OR) for 1 hour then washed 3 times in HBSS. 6x10^4 cells were added to 400 µl media containing none or 4.5 µM MMPi for 3 hours. Cultures were fixed in 3.5 % w/v paraformaldehyde for 10 minutes, washed and mounted as described above.
**Immunofluorescence**

For pan-cadherin or N-cadherin staining, cells were fixed and extracted with 100% methanol at \(-20^\circ\text{C}\) for 5 minutes and then washed three times (3 minutes each) in PBS (Gibco BRL, Ontario, Canada). The coverslips were blocked for 5 minutes in 1% (w/v) bovine serum albumin at room temperature. Cells were incubated with a 1:100 dilution of a polyclonal anti-pan cadherin antibody or a monoclonal anti-N-cadherin antibody (Sigma, Saint Louis, MO) in blocking solution for 45 minutes at room temperature. Coverslips were washed as described above and incubated for 45 minutes further in a 1:300 dilution of Alexa 488 (Molecular Probes, Eugene, OR) goat anti-rabbit secondary antibody in blocking solution. The samples were then washed 3 times in PBS and mounted and sealed on glass slides using single plastic spacers as described above.

For multiple immunofluorescent labeling, samples were fixed using 3.5% (w/v) paraformaldehyde in PBS at room temperature for 5 minutes and then washed three times (3 minutes each) in PBS. Cells were then extracted for 5 minutes in a cytoskeleton-stabilizing buffer (1 mM ethyleneglycoltetraacetic acid (EGTA), 4% (w/v) polyethylene glycol 8000, 0.1 M 1,4-piperazine-bis(ethanesulfonic acid) (Aldrich, Milwaukee, WI) and 0.1% Triton X-100, pH 6.9). This was followed by three more washes and a 5-minute blocking step in 1% (w/v) bovine serum albumin. For focal adhesion kinase/vinculin co-localization studies, primary mouse monoclonal anti-focal adhesion kinase (Transduction Laboratories, Lexington, KY) and polyclonal rabbit anti-vinculin (kindly given by Dr. C.-H. Siu, Banting and Best Institute, University of Toronto, Toronto, ON, Canada) antibodies were diluted 1:100 in blocking solution. Samples with β-catenin/pan-cadherin labeling were incubated with anti-β-catenin monoclonal antibody and rabbit anti-pan-cadherin polyclonal antibody diluted 1:50-fold and 1:200-fold in
blocking solution respectively. These were then left at room temperature for 45 minutes. After 3 washes with PBS, the samples were incubated with solution containing 1:300 dilutions of both Alexa 568 (red)-conjugated goat anti-mouse secondary antibodies and Alexa 488 (green)-conjugated goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR) in blocking solution for 45 minutes at room temperature. The coverslips labeled with β-catenin/pan-cadherin antibodies were further subjected to Hoechst staining (10 µg/ml; Sigma, Saint Louis, MO) for 15 minutes at room temperature. The coverslips were subsequently washed and mounted as described above.

**Fluorescent Image Quantification**

A series of 0.5 µm confocal images were captured starting from the bottom of each cell. The middle and the base section from each stack of images were chosen for further analysis. These digital images were subjected to the Northern Eclipse computer program to quantify the co-localization signals arising from focal adhesion kinase and vinculin, represented by the yellow pixels. The relative level of co-localization was calculated by dividing the area represented with yellow pixels by that of total pixel area.

**Cell Aggregation Assay**

Cell lines were initially grown as monolayers in the presence or absence of 4.5 µM MMPi for 24 hours. After trypsinization, trypan blue exclusion assay was performed to ensure more than 95% viability. 1×10⁵ cells were plated onto each well of a 24-well-plate pre-coated with 1% (w/v) Seaplaque TGT agarose (FMC BioProducts, Rockland ME). Cells were immediately incubated at 37°C with constant agitation of 100 rpm,
MMP inhibitors promote cell adhesion

using the corresponding media collected from each line. The cultures were fixed with 5% (v/v) formalin after 1 hour of incubation.

**Western Blotting**

Exponentially growing cells were lysed with ice-cold RIPA buffer (PBS, 1% (v/v) NP 40, 0.5% (w/v) Sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 200µg/ml phenylmethylsulfonyl fluoride, 60 µg/ml Aprotinin, 1 mM Na₃VO₄). The protein concentrations were determined using BioRad protein assay (BioRad, Hercules, CA) according to the instructions of the manufacturer. Total cellular proteins from the lysate were resolved by 8 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond ECL nitrocellulose membrane (Amersham) by electrotransfer. The blot was subsequently blocked with TBST (Tris-buffered saline containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20) and 5% (w/v) skim milk at room temperature for 1 hour, and then incubated at 4°C overnight with primary mouse anti-focal adhesion kinase (1:1000-fold dilution) or anti-β-catenin (1:500-fold dilution) monoclonal antibody (Transduction Laboratories, Lexington, KY). For phospho-p125FAK signals, the membrane was incubated with 1:500 dilution of phospho-p125FAK specific antibody (BD Transduction Laboratories, Ontario, Canada). After 3 washes with TBST (10 minutes each), the membrane was incubated with 1:3000 dilution of peroxidase-labeled anti-mouse IgG secondary antibody (Gibco BRL, Ontario, Canada) at room temperature for 1 hour. The blot was processed with the ECL chemiluminescence detection system (Amersham) and visualized by autoradiography.

For pan-cadherin western analysis, the same western blot was stripped with 20 ml of stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS and 150 µl β-
MMP inhibitors promote cell adhesion

mercaptoethanol) at 50°C for 20 minutes and incubated with blocking buffer described above at 4°C overnight. The blot was subsequently incubated with a rabbit anti-pan-cadherin polyclonal antibody (Sigma, Saint Louis, MO) diluted 1:2000-fold in TBST containing 2.5% (w/v) skim milk at room temperature for 1 hour. After 2 washes in TBST (10 minutes each), the blot was incubated with 1:3000 dilution of peroxidase-conjugated anti-rabbit IgG secondary antibodies (Gibco BRL, Ontario, Canada) in 2.5% (w/v) skim milk in TBST for 1 hour at room temperature. The signals were enhanced and detected as described above. The signals for both focal adhesion kinase and pan-cadherin western blotting were quantified with a densitometer and ImageQuant computer software (Molecular Dynamics, Arlington Heights, Il). The signals were normalized against the corresponding total proteins in a Coomassie-stained SDS-PAGE gel that was performed in parallel. Additionally, Amido black (Bio-Rad) or anti-α-tubulin monoclonal antibodies (1:4000 dilution; Sigma) staining of the nitrocellulose confirmed or the equivalent loading and transfer of each sample.

**Immunoprecipitation**

Total cell lysates (100 µg) were subjected to immunoprecipitation by incubating with 5 µg of monoclonal anti-β-catenin antibody in binding buffer (1 mM PMSF, 100 µM NaVanadate, 50 mM NaF, 50 µg/ml leupeptin, 300 mM NaCl, 20 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP40 and 0.5% Deoxycholate) under gentle rocking for 2 h at 4°C. Immunocomplexes were then absorbed to Gamma-bind-plus (20 µl of 50% slurry) at 4°C overnight with constant rocking. After 3 washes with binding buffer, immunoprecipitated proteins were separated in a 10% SDS-PAGE gel, transferred to
Hybond ECL nitrocellulose membrane (Amersham) and probed with anti-pan-cadherin antibody as described above.

RESULTS

Natural and Synthetic MMP Inhibitors Alter Cell Density.

Previously we showed that TIMP-1 downregulation confers tumorigenicity on immortal, non-tumorigenic Swiss 3T3 fibroblasts. Further, TIMP-1 downregulated cells displayed a higher saturation density and had the ability to form colonies in soft agar (2,34,36). Such phenotypes are typically associated with cell transformation.

Here we investigated whether these cell lines showed morphological distinction, or a lack of contact inhibition by plating the cells at a low number and growing isolated colonies. Figure 1A shows the clonal morphology of parental Swiss 3T3 cells (S3T3), mock-transfected control (MC2), TIMP-1 sense, upregulated control (16S1), a representative of TIMP-1 downregulated (LA1), and the highly tumorigenic cells (1CN). Swiss 3T3 and MC2 lines formed an organized monolayer. 16S1 cells showed a remarkable organization and contact inhibition, where cells remained as an ordered monolayer even at the core of the colony. Conversely, LA1 cells showed a tendency to overgrow. These were more tightly packed and grew at a higher cell density per microscopic field. Highly tumorigenic lines such as 1CN, which were derived from independent LA1-induced tumors, were used as positive controls for contact inhibition experiments. These exhibited the most disorganized morphology and grew in multi-layer colonies. Differences in colony density between the these cell lines were not due to a difference in their rate of cell doubling, since the doubling time of the two cell line were comparable (data not shown). In addition, the plating efficiency of these cell lines was
not different (data not shown). Thus, genetically modulated fibroblasts with reduced TIMP-1 expression showed an intrinsic difference in cell growth and colony morphology.

Cell density was used as readout for colony compactness and a marker for contact inhibition (37). Swiss 3T3 and MC2 grew to a similar cell density, while a significant increase of 1.3-fold and 2.1-fold was observed for LA1 and 1CN respectively when compared to the parental cell line (Figure 1B). Particular attention was given to use colonies of comparable size and quantification was limited to the core of the colony. These data suggested that contact inhibition of these fibroblast colonies may be related to their level of TIMP-1 expression. We then determined the effect of MMP inhibitor (MMPi) on cell density. Upon culturing in presence of 4.5µM MMPi, both S3T3 and LA1 lines showed a reduction in cell density, with the latter being statistically significant (Figure 1C). Therefore, LA1 cells were cultured in the presence of increasing concentrations of either a synthetic MMPi (0.5-4.5 µM) or recombinant TIMP-1 (rTIMP-1; 25-100 ng/ml). We found that these treatments exerted a dual effect: first, they altered the LA1 cell morphology such that cells appeared elongated, resembling the parental fibroblasts (Figure 1D), and second, they decreased cell density. Both MMPi and rTIMP-1 supplemented LA1 cultures showed a dose-dependent reduction in cell density. The decrease of 43% following 4.5 µM MMPi and of 33% in colony density upon addition of 100 ng/ml rTIMP1, were statistically significant (Figure 1E, P ≤ 0.05). MMPi concentrations used in our studies were below the LD50 obtained from the MTT assay (data not shown). Overall, these analyses indicated that natural and synthetic MMP inhibitors directly influence cells, leading them to become more organized with a reduced cell density.
Next, we confirmed the altered TIMP-1 mRNA levels of these previously generated cell lines (2,34-36). RT-PCR analysis with sense strand-specific primers revealed an upregulation (at least two-fold) of TIMP-1 mRNA in 16S1 cells and a downregulation (one-fold) in LA1 cells (Figure 1F). LA1 cells still produced antisense TIMP-1 mRNA (data not shown). Zymography showed that these cells primarily produced MMP-9, while MMP-2 was detectable upon prolonged incubation. The levels of MMP-9 were apparently reduced in serum-free conditioned media of 16S1 cells and conversely increased in LA1 cells (Figure 1G). In a functional assay, increased MMP activity of LA1 cells was consistent with enhanced digestion of the fluorogenic gelatin (green signal), compared to Swiss 3T3 cells. MMPi was able to inhibit the digestion of this substrate. Thus, the TIMP-1 downregulated LA1 cells had increased functional MMP activity, which was inhibitable by MMPi treatment.

**MMP Inhibitors Influence Cell-ECM Interactions**

It is known that proteins constituting ECM are the primary substrates of MMPs and the MMP-TIMP axis regulates ECM integrity (38). Therefore, we next tested whether altered cell-ECM contact contributed to the lack of contact inhibition. The sites of cell-ECM contact are established by the assembly of various proteins, including integrins, focal adhesion kinase p125FAK and vinculin, which together form complexes known as focal adhesions (27,39). Confocal microscopy, following double immunostaining with antibodies against vinculin (green) and p125FAK (red), allowed the visualization of focal contacts. For these experiments, we studied a stack of 0.5 µm optical sections (approximately 15 sections) starting from the base of the cell that was in contact with the substratum. Parental Swiss 3T3 cells had prominent p125FAK and
vinculin expression, and their co-localization resulted in a yellow signal, which was uniformly distributed at the base of Swiss 3T3 cells (Figure 2A). In contrast, LA1 cells had greatly reduced signals for both p125$^{\text{FAK}}$ and vinculin in the basal 0.5 µm section. Next, we treated these cell lines with MMPi, which did not alter the localization of these molecules in Swiss 3T3 cells. In LA1 cells, however, co-localization of signals specific to p125$^{\text{FAK}}$ and vinculin became upregulated comparable to that of Swiss 3T3 cells (Figure 2A). We quantified the yellow signal, representing co-localized p125$^{\text{FAK}}$/vinculin, in the middle and basal sections using Northern Eclipse imaging software, as shown in Figure 2B. Co-localization was predominantly seen at the base of the Swiss 3T3 cells in the presence or absence of MMPi. This characteristic distribution was shifted in LA1 cells with an aberrant level of co-localization above the cell/ECM base. MMPi treatment of LA1 cells restored the distribution pattern to that resembling Swiss 3T3 cells. These results suggested that focal contact assembly may be compromised in the TIMP-1 reduced cells, and that MMPi could rescue the ability of LA1 cells to assemble these structures. Further, MMPi alters the subcellular distribution of p125$^{\text{FAK}}$ and vinculin proteins at the cell-ECM contact.

**MMP Inhibitors Promote p125$^{\text{FAK}}$ Phosphorylation**

Recruitment of p125$^{\text{FAK}}$ to focal contacts results in its activation through the phosphorylation of tyrosine residues (40). We compared the level of p125$^{\text{FAK}}$ protein as well as its activity between Swiss 3T3 and LA1 cells. Densitometric analysis of the p125$^{\text{FAK}}$–specific band by western blotting showed similar protein levels in the two cell lines, and these levels did not change upon treatment with 4.5 µM MMPi (Figure 2C). However, the phosphorylation level of p125$^{\text{FAK}}$ was remarkably reduced in LA1 cells,
indicative of reduced p125\textsuperscript{FAK} activity. This phosphorylation status could be restored upon MMPI treatment (Figure 2C). Immunoblotting with \(\alpha\)-tubulin confirmed equivalent loading on this membrane. These findings were consistent with our observations from confocal studies. Overall, MMP inhibitors did not affect the levels of p125\textsuperscript{FAK}, but did affect the co-localization of p125\textsuperscript{FAK} with vinculin, implying that the MMPI effect is on the assembly and stability of focal contacts rather than on the degradation of proteins constituting the focal contacts. A secondary effect on LA1 cells is the increased function of p125\textsuperscript{FAK}, as suggested by its increased phosphorylation.

**MMP Inhibitors Up-regulate Cadherin-mediated Cell-Cell Adhesion**

Since homotypic cell-cell adhesion is largely coordinated by cadherins, we used a pan-cadherin antibody and confocal microscopy to analyze the expression of members of the classical cadherins in these contacts. The overall morphology of monolayer Swiss 3T3 cultures was flat with pan-cadherin expression in the cell-cell contacts. In contrast, the limited numbers of cell contacts that did form between LA1 cells had minimal cadherin expression (Figure 3A, compare top panels). This suggested that the mechanism required for the assembly of cadherin-mediated cell-cell adhesion was defective in the LA1 cells resulting in the loss of cell-cell adhesion. This phenomenon occurred independent of the level of cell confluence in the culture (data not shown). Upon MMPI treatment, we observed an upregulation of pan-cadherin signals at cell-cell contact (Figure 3A, compare bottom panels). Next, we quantified the effects of MMPI on cell-cell adhesion in monolayer cultures represented in Figures 3A by scoring the number of cells showing cadherin-positive junctions. These data showed that cell-cell contact was significantly upregulated in MMPI-treated LA1 cells (Figure 3B).
Among the classical cadherins, N-cadherin is expressed by fibroblasts (41). Therefore, we reasoned that N-cadherin might be involved in the observed cell-cell adhesion, while P- and E-cadherins were the less-likely candidates. Confocal microscopy of N-cadherin stained cells showed defined expression of N-cadherin in Swiss 3T3 cells at the sites of cell-cell contact (Figure 3C). On the other hand, LA1 cells showed little N-cadherin localization at the cell boundary (Figure 3C). Further, unlike our observations on pan-cadherin localization in LA1, MMPi treatment failed to upregulate N-cadherin localization in either cell lines (data not shown). Thus, other cadherins, and not N-cadherin, are likely involved in MMPi-modulated cell-cell adhesion.

Next, we performed western blotting using antibodies against pan-cadherin, as well as N-, P- and E-cadherins. LA1 cells had an average 40% reduction in pan-cadherin level compared to the parental line (Figure 3D). In two independent experiments, MMPi treatment resulted in a substantial upregulation of pan-cadherin levels, especially in LA1 cells. With respect to N-cadherin, we also observed a lower basal level in LA1 compared to Swiss 3T3 cells. This did not alter upon MMPi treatment, a finding consistent with our confocal data. Furthermore, western blotting showed these fibroblasts did not express detectable levels of P- or E-cadherin (data not shown). Collectively, our data indicate that biological and synthetic MMP inhibitors promote cell-cell adhesion through an upregulation of members of the cadherin family, other than N-, P- or E-cadherins.

**MMP Inhibitors Increase Cadherin-mediated Cell Aggregation**

The formation of cadherin complex is a calcium-dependent process that results in direct cell-cell aggregation (42). Thus, cell aggregation provides a means of assessing
functional cadherin activity. We investigated whether MMPi-mediated cadherin upregulation facilitated cell aggregation. Figure 4A shows typical cell aggregates that formed under the indicated conditions. First, we found that LA1 cells formed aggregates less frequently than Swiss 3T3 cells. This activity was calcium dependent in both cell lines since EGTA, a calcium chelator, abrogated the aggregation. Second, LA1 cells responded by forming aggregates upon MMPi treatment. This treatment effectively restored the adhesive properties of LA1 cells to a level comparable to the parental line. Third, MMPi treatment did not facilitate cell aggregation in the presence of EGTA, indicating that MMPi facilitated adhesion is calcium dependent. Significantly, these data implicate the involvement of cadherins in cell aggregation that is promoted by MMP inhibitors.

To obtain independent evidence in support of the above concept, we utilized a genetically distinct pair of fibroblast cell lines. We generated primary mouse embryonic fibroblasts (MEFs) from wildtype and timp-1 knockout mice (43) and used them to perform the cell aggregation assays. Early passage timp-1\(^{-/-}\) MEFs showed a complete failure to aggregate compared to wildtype MEFs, and the latter also did not aggregate in the presence of EGTA (Figure 4B). Next, we subjected timp-1\(^{-/-}\) MEFs to treatments with rTIMP-1, MMPi or both. Both individual treatments promoted cell aggregation, and this effect was further enhanced by the combined treatment (Figure 4C). Thus, these data reveal that cellular aggregation of these fibroblasts is facilitated by both calcium and TIMP-1.
MMP Inhibitors Recruit β-Catenin to Cadherin-mediated Cell Junctions

The clustering of cadherins leads to the recruitment of β-catenin to the cadherin-associated complex. This complex is linked to the cytoskeleton and stabilizes cell architecture (44-45). We investigated whether MMPi-mediated clustering of cadherins directly altered intracellular activity such as the sub-cellular distribution of β-catenin. As shown in Figure 5A, the level of β-catenin that co-localized with pan-cadherin was strikingly lower in LA1 cells compared to Swiss 3T3 cells. MMPi treatment resulted in increased co-localization of these two signals at the cell-cell junctions. This effect was more pronounced for LA1 versus Swiss 3T3 cells. Western blotting showed that the level of total intracellular β-catenin protein remained unchanged upon MMPi treatment (Figure 5B), indicating that the increased co-localization resulted from the translocation of β-catenin to the complex rather than an increase in β-catenin protein levels.

Next, we verified the increased association of β-catenin with cadherin biochemically. Immunoprecipitation with an anti-β-catenin antibody and subsequent immunoblottings, first with a pan-cadherin and second with β-catenin antibodies demonstrated a higher pan-cadherin signal upon MMPi treatment in LA1 cells (Figure 5B). Collectively, these data suggest that MMPi stabilize the cadherin-associated complex by promoting the recruitment of β-catenin to the cell membrane.

DISCUSSION

Here we demonstrate that MMP:TIMP proteolytic axis regulates cell adhesion systems and has an important influence on cell characteristics that are linked to cell transformation and tumorigenesis. Tumor cells lack contact inhibition (46), typically observed as anchorage independent growth and continued proliferation in confluent
MMP inhibitors promote cell adhesion
cultures. Effects of MMP inhibitors on cell adhesion were explored using TIMP-1
downregulated cells, which displayed higher saturation density, growth in soft agar, and
tumorigenic and metastatic potential (2,34,36). Physiological and synthetic MMP
inhibitors altered cell morphology, cell contact, and restored cell density in a dose
dependent manner, thus re-establishing the adhesion characteristics of non-
transformed cells. We tracked the effects of MMPi on cell-ECM adhesion through
analysis of focal contacts, and on cadherin-mediated cell-cell adhesion. Even though
p125FAK protein levels were unaffected, increased co-localization of vinculin and
p125FAK were apparent in focal-contacts. Subsequently, the phosphorylation of p125FAK
was substantially increased in LA1 cells upon MMPi treatment. In parallel, MMPi
treatment also affected cell-cell adhesion. Cadherin protein levels and localization at
cell-cell contacts were increased. We further demonstrated enhanced cadherin function
as increased calcium dependent cell-cell aggregation, association of β-catenin with
cadherins, and β-catenin localization at the cell membrane. Moreover, independent
evidence of altered cadherin function was obtained using timp-1−/− MEFs.

We propose (Figure 6) that MMP inhibitors augment cell adhesion by two
methods: First, by preventing cadherin ectodomain cleavage and thus stabilizing
cadherin mediated cell-cell contacts, and their association with the actin cytoskeleton
through β-catenin (44,45). Second, by inhibiting ECM degradation and thereby
maintaining integrin-ECM adhesion, focal contact assembly and activation of p125FAK
via its phosphorylation. Moreover, there is increasing evidence for cross talk between
integrins and cadherins, suggesting that they functionally co-operate during cell
adhesion, migration and developmental processes. For example, the restoration of E-
cadherin-mediated cell-cell adhesion diminishes the motility of ras-transformed MDCK-
MMP inhibitors promote cell adhesion

f3 epithelial cells through ECM-activated rac signaling (47). Also, cell-cell and cell-ECM contacts act together to revert the compact phenotype of the colorectal carcinoma cell line VACO to normal epithelial morphology (48). Furthermore, signaling molecules are shared between N-cadherin and β1-integrins during neurite migration (49,50). Overall, the stabilizing influence of MMP inhibitors on cell-cell and cell-ECM contacts would allow optimal integration of the microenvironmental information.

Following MMPI treatment, we observed enhanced cadherin/β-catenin co-localization at cell-cell contacts by laser scanning confocal microscopy, as well as an enhanced physical association between cadherin/β-catenin proteins by co-immunoprecipitation. The reduced associations between cadherin/β-catenin in TIMP-1 downregulated cells may provide a trigger for tumorigenic potential. This idea stems from literature documenting the involvement of the cadherin/β-catenin pathway in many aspects of tumorigenesis (44,51,52). It is thought that a disruption of adhesion systems can initiate neoplastic transformation and contribute a rate-limiting step to progression (53). Briefly, several studies show that the downregulation of cadherins enhance tumor development, increased cadherins reduce tumorigenic properties (54-56), and a lack of stable E-cadherin-mediated cell-cell contact has even been proposed to initiate genomic instability (53). One reason for the central role of cadherins in tumorigenesis is their ability to bind β-catenin. The latter is a shared target of cadherins and the Wnt signal transduction pathway. Specifically, E-cadherin and LEF-1 that is involved in Wnt signaling form mutually exclusive complexes with β-catenin, and therefore E-cadherin binding prevents β-catenin nuclear localization and transactivation of transcription (57). A modest overexpression of β-catenin has been reported to promote cell cycle progression from G1 to S phase, protect cells from anoikis and lead to cellular
MMP inhibitors promote cell adhesion

transformation (37). Thus, β-catenin signaling through the Wnt pathway may be one mechanistic link between MMP-proteolysis and cell adhesion, contact inhibition and tumorigenesis.

Swiss 3T3 mouse fibroblasts are known to produce ECM components, mainly collagen type I (58). Under adhesive conditions, similar to our studies, collagen production was shown to be independent of the growth state of these fibroblasts (59). ECM components and architecture per se are involved in integrin clustering and integrin-mediated signal transduction through tyrosine phosphorylation of p125FAK (60-62). Thus, it is conceivable that MMPi treatment inhibited the degradation of integrin ligands and promoted the assembly of focal contacts that was observed as altered subcellular distribution of p125FAK and vinculin at cell-ECM contact and p125FAK phosphorylation. Alternatively, it is possible that MMP action modulated the three-dimensional collagen structure that subsequently altered p125FAK phosphorylation, such as that shown by Lauer et al (1998).

Although secreted/transmembrane MMPs are proximal to cell adhesion molecules, the impact of proteolysis on multiple adhesion systems and their downstream effectors remains far from understood. In parallel we examined the influence of MMPi on both, cell-ECM and cell-cell contact. A unique observation is that despite the lack of an effect on the p125FAK protein levels, its phosphorylation status is restored to that of wild type fibroblasts upon MMPi treatment. While the effects of MMPi on p125FAK activity have never been reported, our findings on MMPi-modulation of cadherins are consistent with the other studies that have utilized epithelial or endothelial cells. Overexpression of stromelysin-1 (MMP-3) in mammary epithelial cells altered cell morphology and reduced E-cadherin and β-catenin from cell membrane, which was
inhibited by MMPi (22). These events were proposed to promote cell invasion and epithelial to mesenchymal transition. Similarly, shedding of VE cadherin from endothelial cells was blocked by MMPi treatment during apoptosis (33). In addition to altering cadherin levels in our fibroblasts, we provide novel evidence that MMPi treatment recruits β-catenin to the cell-cell contacts and functionally enhance the calcium-dependent cell adhesion in tumorigenic fibroblasts. Taken together, MMP-based proteolysis of cell adhesion molecules and their downstream pathways may be a universal mechanism for influencing cell fate of various lineages including epithelial, endothelial and fibroblasts.

It is possible that a feedback loop exists between TIMP/MMP and cadherin/β-catenin systems, where increased proteolysis would enhance β-catenin activity, which in turn would induce MMP production. In support of this idea, it has been shown that one of the identified targets of β-catenin/LEF-1 transactivation is MMP-7 (63,64). It has also been reported that E-cadherin transfected prostatic adenocarcinoma cells have reduced MMP-2 activity and display a reversion of an invasive to a stationary phenotype (65), while E-cadherin downregulation results in increased MMP-9 activity, motility and metastasis of skin carcinomas cells (66). Likewise, TIMP-1 downregulated cells, which also have reduced cadherin levels, form tumors in which MMP-3 mRNA is consistently expressed (34). This particular MMP has been suggested to provide a ‘master regulatory switch’ for mammary epithelial tumorigenesis (32). It is conceivable that an integration of proteolytic and adhesion systems is required for optimal cell interaction and function. Once this stable link is breached, a molecular and functional cascade then initiates events conducive to tumorigenesis.
Cadherins and β-catenin have primarily been studied in epithelial cells. At present, the role of cadherins/β-catenin in fibroblasts remains poorly understood. Intriguingly, a recent study showed that mutations in β-catenin occurred with the highest frequency in aggressive infiltrative fibromatosis (67), which are considered to be the most common cause of mortality in familial adenomatous polyposis patients (68). These reports suggest that cadherin/β-catenin dysfunction underlies fibroblast tumorigenesis. Our current investigations focused on tumorigenic fibroblasts and we were able to manipulate the cellular distribution and association of cadherin/β-catenin with MMPi. MMPi may additionally contribute to creating a non-permissive environment for tumor growth, since stromal contribution of MMPs is known to promote tumor progression (69).

We demonstrate that biological and synthetic MMP inhibitors promote the assembly and the stabilization of both focal and cell-cell contacts. Since p125<sup>FAK</sup> and cadherin function were restored by MMPi treatment in TIMP-1 downregulated cells, our data provide compelling evidence for a relationship between pericellular proteolysis and activity of the key signaling molecules downstream of cell adhesion. Our data support the emerging concept that an integration of proteolysis and adhesion influence cell fate and tumorigenesis.

ACKNOWLEDGMENTS

We thank P. Waterhouse and J. E. Fata for helpful discussions and critical reading of the manuscript, and W. A. Cruz for technical help. This work was supported by grants from the Canadian Institutes of Health Research and Human Frontiers of Science.
Program. A. T. Ho was in part supported by a Toronto Open Scholarship and The Princess Margaret Hospital Foundation Fellowship.
REFERENCES


MMP inhibitors promote cell adhesion


MMP inhibitors promote cell adhesion


FIGURE LEGENDS

Figure 1. The clonal morphology and level of contact inhibition in the fibroblast cell lines. Control cell lines, parental Swiss 3T3 (S3T3), mock-transfected MC2, and sense control 16S1, exhibit contact inhibition with cells appearing in a monolayer throughout the colony. TIMP-1 down-regulated LA1 and the tumorigenic 1CN cell lines do not follow contact inhibition and grow in multiple layers (A). Cell organization at the edge of the colony as seen at lower magnification (top panels; bar, 100 µm) and at higher magnification in the center of colony (bottom panel; bar, 20 µm. Cell density measured as cell number per field at the center of the colony (B). A synthetic MMP inhibitor significantly lowered the cell density of LA1 cells (C). Exogenous MMP inhibitors restore cell morphology (D; bar, 5 µm) and cell density in a dose dependent manner (E). A quantitative PCR-based strategy (F) was used to determine the sense TIMP-1 mRNA levels (F, Right panel). Negative control (-) was performed in the absence of the primer during the primer extension reaction, and positive control (+) was done using a plasmid containing full length TIMP-1 cDNA. Beta-actin was used as an internal control. Zymography shows MMP-9 activity in the serum-free conditioned media of the cell lines (G). One micron confocal sections show digestion of fluorogenic gelatin (green signal) in the presence or absence of synthetic MMP inhibitor (MMPi) by the orange cell tracker labeled fibroblasts (red) (H). *p<0.05 in B, C and E.

Figure 2. (A) Reduced co-localization of p125FAK and vinculin in LA1 fibroblasts. Stacks of confocal microscopic images were captured at 0.5 µM intervals following double labeling of cells with anti-vinculin (green) and anti-p125FAK (red) antibodies, and the
lowermost section is shown. Confocal settings normalized on the S3T3 cells remained unchanged during the assessment of LA1 cells. The yellow signal represents the areas where p125^FAK and vinculin co-localized. Co-localization of p125^FAK and vinculin is reduced in LA1 cells compared to S3T3 cells, and is restored to levels comparable to S3T3 cells upon treatment with 4.5µM MMP inhibitor. Bar, 10 µm. (B) Distribution of p125^FAK and vinculin co-localization within Swiss 3T3 and LA1 cells. Treatment with MMPi did not affect the relative co-localization of above molecules either in the middle (M) or the basal (B) section in the S3T3 cells. A redistribution of co-localized signal was evident in LA1 cells upon MMPi treatment. (C) Protein levels of p125^FAK in Swiss 3T3 and LA1 cell lines. Top panel shows p125^FAK levels in incremental amounts of cell lysates analyzed by western blotting. The p125^FAK signal in LA1 cells was marginally lower compared to S3T3 cells. Bottom panel shows p125^FAK in LA1 cells cultured in the absence (-) or presence (+) of MMP inhibitor. The p125^FAK levels were not influenced by MMPi treatment. Anti-α-tubulin staining indicates equivalent protein loading on the nitrocellulose membrane.

**Figure 3.** Synthetic MMP inhibitor restores cadherin localization at cell-cell contacts in LA1 cells (A). S3T3 and LA1 fibroblast cultures at two different densities were stained with an anti pan-cadherin polyclonal antibody. The inset shows the cell pairs found in a sub-confluent culture. Stacks of confocal microscopic images were captured through the cells at 0.5 µm intervals. Typical images of Swiss 3T3 and LA1 cells are shown. Cadherins readily localize to cell contacts between S3T3 cells (arrowheads). In contrast, LA1 fibroblasts rarely form tight contacts and cadherins are not evident in cell junctions (arrowheads), although a diffuse cadherin signal is seen in the vicinity of some
cell junctions. MMPi treatment does not affect cadherin expression in S3T3 cells, while LA1 cells up-regulate cadherin localization at cell-cell contacts following MMPi treatment (arrowheads). Bar, 10 µm. Changes in cadherin positive cell-cell junctions upon MMPi treatment (B). A significant increase in the number of cadherin positive cell-cell contacts is evident in LA1 cells (*p<0.05). N-cadherin localization in Swiss 3T3 and LA1 cell lines (C). S3T3 cells and LA1 cells grown to sub-confluence were stained with N-cadherin monoclonal antibody. A typical image captured from the confocal microscope shows high signal of N-cadherin along the boundaries of the S3T3 cells in contact. In contrast, LA1 cells show very little accumulation of N-cadherin at the boundary between cells in contact. Western blot analysis of cadherins in Swiss 3T3 and LA1 cell lines (D). The protein levels detected by anti pan-cadherin and N-cadherin antibodies are substantially reduced in LA1 cells compared to S3T3 cells. MMPi up-regulates pan-cadherin but not N-cadherin in LA1 cells. Equivalent protein loading was confirmed by Amido black staining of the membrane.

**Figure 4.** Exogenous MMP inhibitors promote cell-cell aggregation of TIMP-1 downregulated and TIMP-1 deficient primary fibroblasts. S3T3 form cell aggregates, while LA1 remain as single cells. MMPi (4.5 µM) treatment leads to cell-cell aggregation of LA1 fibroblasts. The effect of MMPi on cell aggregation is calcium-dependent since it is blocked by the addition of EGTA (A). Mouse embryonic fibroblasts derived from *timp-1*<sup>−/−</sup> mice also fail to form cell aggregates compared to those derived from the *timp-1*<sup>+/+</sup> littermates (B). Addition of MMPi, recombinant human TIMP-1 (rTIMP-1), or both facilitates cell aggregation (C).
**Figure 5.** Enhanced co-localization of β-catenin and cadherins at cell-cell contacts following MMPi treatment (A). Signals specific to pan-cadherin antibodies are visualized as red, β-catenin as green, and Hoechst stains cell nuclei blue. S3T3 cells have strong co-localization of cadherins and β-catenin (arrowheads). In contrast, both signals are reduced in LA1 cells (arrows), and a lack of co-localization at cell junctions in LA1 cells is evident as a lack of intense yellow signal (LA1, merged). The addition of MMPi increases the co-localization of cadherins and β-catenin at sites of cell-cell contacts in LA1 cells (arrowheads). Bar, 10 µm. Synthetic MMP inhibitors increase the association between cadherins and β-catenin (B). Top panel shows that β-catenin levels remain constant in S3T3 and LA1 cells under the conditions indicated. Co-immunoprecipitation assay (immuno-precipitation (IP) with anti β-catenin and subsequent immunoblotting (IB) with anti pan-cadherin antibodies shows increased association of cadherins with β-catenin in LA1 cells. Equivalent amount of immuno-precipitated β-catenin was confirmed by re-blotting with anti-β-catenin antibodies.

**Figure 6.** A model for the impact of MMP inhibitors on fibroblast cell adhesion. Increased MMP activity in the pericellular space abrogates cell-cell adhesion by processing of the cadherin ectodomain. Concurrently, it disrupts the assembly of focal adhesion complexes at the site of cell-ECM contact by degrading ECM components. MMP inhibitor treatment blocks MMP activity, leading to the promotion of focal adhesion contact assembly and the stabilization of cadherin-mediated cell-cell contact. The latter recruits β-catenin to the cadherin complex and reduce the cytoplasmic pools of β-catenin, which subsequently inhibit β-catenin-associated proliferation signals. In
addition, proper assembly of focal contact enhances FAK activity, which provides the appropriate signals to regulate cell growth. These mechanisms may form the basis by which a dysregulated MMP/TIMP proteolytic axis facilitates early events of tumorigenesis.
Figure 1
Figure 1
Figure 2
Figure 2
Figure 3
### Figure 4

#### A

<table>
<thead>
<tr>
<th></th>
<th>MMPi</th>
<th></th>
<th>EGTA</th>
<th></th>
<th>EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3T3</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>LA1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th></th>
<th>EGTA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>timp1+/-</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>timp1/-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### C

<table>
<thead>
<tr>
<th></th>
<th>MMPi</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+TIMP-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5
**Figure 5**
MMPi treatment

 TIMP-1 down-regulation

Figure 6