THE ROLE OF TISSUE INHIBITORS OF METALLOPROTEINASES IN TUMORIGENESIS AND METASTASIS

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Tissue inhibitors of metalloproteinases (TIMPs) are classically known for regulating members of the metzincin protease family and are well recognized for their inhibitory effects in cancer development and progression. Despite their common evolutionary structure, the four TIMP proteins have unique properties and regulation, and produce distinct phenotypes when ablated. A comprehensive assessment of their function during tumorigenesis reveals substantial effects on cell proliferation, apoptosis, angiogenesis, invasion, and metastasis as well as a potential role in genomic instability. The TIMPs universally inhibit angiogenesis, invasion, and metastasis, but their specific effects on cell proliferation and apoptosis are both tissue specific and context dependent. They exert these effects in a metalloproteinase-dependent as well as metalloproteinase-independent manner. Knowledge gained from these biological studies provides a foundation for the full understanding of TIMP function in physiology and various pathologies as well as for the development of the next generation of therapeutic metalloproteinase inhibitors.

Keywords | ADAMs, angiogenesis, apoptosis, invasion, genetic instability, proliferation, MMPs, regulation.

Abbreviations | ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motif; AKT, v-akt murine thymoma viral oncogene homolog 1; ALT, alternative lengthening of telomere; ANG1, angiopoietin-1; ANG2, angiopoietin-2; AoSMC, aortic smooth muscle cells; AP-1, activating protein 1; AP-4, activating enhancer-binding protein 4; APC, adenomatous polyposis coli protein; ATM, ataxia telangiectasia mutated; Bax, BCL2-associated X protein; Bcl2, B-cell CLL/Lymphoma 2; Bid, BH3 interacting domain death agonist; Bim, BCL2 interacting protein; BNIP1, BCL2/adenovirus E1B 19kDa interacting protein 1; BRCA, breast cancer 1, early onset; CAM assay, chorioallantoic membrane assay; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary cell; c-MYC, myc proto-oncogene protein; CXCL12, chemokine (C-X-C motif) ligand 12; DCIS, ductal carcinoma in situ; DMBA, 7, 12-dimethylbenz [a] anthracene; EC, endothelial cells; ECM, extracellular matrix;
EGFR, epidermal growth factor receptor; EMMPRIN, extracellular matrix metalloproteinase inducer; ENU, N-ethyl-N-nitrosourea; EPA, erythroid potentiating activity; ERK, extracellular signal-regulated kinase; ES cells, embryonic stem cells; ETS, E-Twenty-Six family of transcription factors; FAK, focal adhesion kinase; FAS, TNF receptor superfamily, member 6; FGF-2, fibroblast growth factor 2; FGF-R1, fibroblast growth factor receptor 1; HB-EGF, heparin-binding epidermal growth factor; HCC, hepatocellular carcinoma; HDMEC, human dermal microvascular endothelial cells; HEMVEC, human endometrial microvascular endothelial cells; HGF, hepatocyte growth factor; HMVEC, human microvascular endothelial cells; HPV16, human papillomavirus 16; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IDC, invasive ductal carcinoma; IGF, insulin-like growth factor; IGF-BP-3, insulin-like growth factor binding protein 3; JNK, jun N-terminal kinase; KDR, kinase-insert domain receptor also known as vascular endothelial growth factor receptor 2; KO, knockout; LEF/TCF, lymphoid enhancer factor/T cell factor (transcription factors); MCP-3, monocyte chemotactic protein 3; MDA, Monroe Dunaway Anderson Cancer Center; MEF-2, myocyte enhancer factor 2; MKP1, serine/threonine specific protein phosphatase; MMP, matrix metalloproteinases; MMP1, MMP inhibitor; MMTV, mouse mammary tumor virus; MOAP1, modulator of apoptosis 1; MVEC, microvascular endothelial cells; NF-κB, nuclear factor for IL-6; NFκB1, nuclear factor κB; NFκB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; NK cell, natural killer cell; Noxa, pro-apoptotic member of the Bcl-2 protein family; NSCLC, Non-Small Cell Lung Cancer; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PEAE3, polyomavirus enhancer activator 3; PI3K, phosphoinositide 3-kinase; PIA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; PTEN, phosphatase and tensin homolog; PVA, polyvinyl alcohol; RA, rheumatoid arthritis; Rh, retinoblastoma protein; Rac1b, ras-related C3 botulinum toxin substrate 1 isoform b; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; SH-PTP1, protein tyrosine phosphatase, non-receptor type 6; Sp1, selective promoter 1; STASIS, stress- or aberrant-signaling induced senescence; TACE, TNF alpha converting enzyme; TG, transgenic; TGFα, transforming growth factor alpha; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitors of metalloproteinases; TNFα, tumor necrosis factor alpha; TNF-R1, tumor necrosis factor receptor 1; TNF-R2, tumor necrosis factor receptor 2; TNFRSF21, tumor necrosis factor receptor superfamily, member 21; TRADD, tumor necrosis factor receptor-associated death domain; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R1, tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor-1; TYK, tyrosine kinase; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells; WAP, whey acidic protein; WAF1, wild-type p53-activated fragment 1; Wnt, wingless and Int signaling pathway.

I. INTRODUCTION

Tissue inhibitors of metalloproteinases (TIMPs) represent an ancient family of proteins, as the sequences homologous to mammalian TIMP are present in chicken, fish, fly, worm, and mollusk (Crassostrea gigas).1,2 Four distinct TIMP genes are present in the human genome. First identified based on its metalloproteinase inhibitory activity in 1986, TIMP1 was cloned and sequenced by Carmichael et al.3 and Docherty et al.4 Subsequently, cloning of TIMP2,5 TIMP3,6 and TIMP47 was achieved by the mid 1990s, with Leco et al. cloning these three murine timps.8–10 These proteins exert the final stage of regulation on the proteolytic activity of matrix metalloproteinases (MMP) following the activation of the latent enzyme, and they are also endogenous.
inhibitors of members of a disintegrin and metalloproteinase (ADAM) family. Collectively, MMPs are responsible for cleaving all of the major extracellular matrix (ECM) proteins and their balanced interaction with TIMP proteins regulates ECM homeostasis.

By virtue of controlling ECM turnover, TIMPs serve important roles in numerous physiological processes such as embryo implantation, reproductive tissue remodeling, and wound healing. On the other hand, an imbalance in MMP and TIMP expression and/or activity is found in various human diseases, including arthritis, cardiovascular disease, emphysema, retinopathies, and cancer. Much of our understanding of TIMPs has evolved from research in cancer biology. The process of tumorigenesis and the role of proteases within this process have been presented in a number of excellent papers. We begin this review with a brief account of the key events in tumorigenesis as well as in the field of metalloproteinases. We then focus on the role TIMPs play in tumorigenesis, with particular emphasis on genomic instability, cell proliferation, cell death, angiogenesis, invasion, and metastasis.

II. TUMOR DEVELOPMENT AND PROGRESSION

A. Tumor Development

Tumor development occurs through stepwise accumulation of phenotypic alterations that confer a growth advantage to the cancer cell. While the specific sequence in which these alterations occur is not the same for all tumor cells, they arise from genetic changes involving tumor suppressor genes and proto-oncogenes. The hallmarks of a cancer cell are self-sufficiency of growth signals, resistance to growth-inhibitory signals, resistance to apoptosis, unlimited replicative potential, sustained angiogenesis, and the acquisition of invasive and metastatic potential.

Self-sufficiency of growth signals is attained by alteration of intracellular pathways that allow constitutive signaling as well as by upregulation of growth-factor expression and its receptors. In fact, these molecules often represent the proto-oncogenes typically found mutated, amplified, or overexpressed in human cancers. These changes remove the dependence of the tumor cell on growth factors in the external environment. Because the tumor mass is heterogenous in nature, being composed of tumor cells and stromal cells such as fibroblasts, macrophage, neutrophils, and endothelial cells, self-sufficiency of growth signals also involves the ability to co-opt stromal cells to provide growth-stimulatory factors or to release sequestered factors within the tumor microenvironment. Cellular proliferation is normally kept in check by a number of growth-inhibiting mechanisms; this balance is tilted in favor of proliferation in tumor cells. This is in part achieved through insensitivity to inhibitory stimuli. For instance, Retinoblastoma protein Rb is a well-known tumor suppressor that functions to regulate cell-cycle checkpoints and whose
activity is typically lost through mutations,21 interactions with oncoproteins22, or overexpression of cyclins (D1 and E).20 In fact, many tumor-suppressor genes are found mutated in a number of familial human cancer syndromes. These include PTEN, which functions to inhibit AKT,23,24 and APC, which promotes rapid degradation of β-catenin and restricts WNT signaling.25

The molecular mechanisms that regulate programmed cell death were initially defined in Caenorhabditis elegans26 and are now well established as key regulators of cancer cell fate. Tumor cells acquire the ability to resist apoptosis. This is achieved by altering extracellular signals that enhance survival pathways (PI3K/AKT/PTEN), dysregulating death receptor signaling (FAS and TNF-R1)19, disabling apoptotic response following DNA damage (P53, ATM, BRCA)27,28, or developing defects in intrinsic mediators of apoptosis such as BCL-2. One particular protein, P53, plays a pivotal role in regulating apoptosis in response to a number of stimuli, including hypoxia and ionizing radiation.29–31 P53 can induce upregulation of pro-apoptotic genes such as BAX, NOXA and BID.32 In addition, upregulation of anti-apoptotic (BCL-2, Bcl-XL) or downregulation of pro-apoptotic genes (BIM, BID) also confers a survival advantage on the tumor cell.33 Other alterations include the upregulation of growth factors or their receptors, which affects pro-survival signaling (IGF axis).34

Normal cells have a finite replicative potential and undergo senescence, whereas tumor cells uncouple this constraint by acquiring an unlimited replicative potential. This limited replication is in part due to telomere attrition as a function of each cell division.35 The cumulative effect of telomere erosion is the inability to protect the ends of chromosomes, resulting in the formation of end-end chromosome fusions, karyotypic disarray, and death of the affected cell.35 Ninety percent of tumors avoid this fate through upregulation of the telomerase enzyme, which adds TTAGGG units to telomeres, while the others maintain telomere length through the mechanism of alternative lengthening of telomere (ALT).36 ALT occurs through homologous recombination between telomeres in different chromosomes.36 Both telomeric-dependent and -independent events (also known as stress- or aberrant-signaling induced senescence, STASIS) are mediated by pathways that converge on P53 and Rb. Abrogation of senescence can be achieved by inhibiting the activity of these tumor-suppressor proteins.37

The growth of the tumor mass is dependent on the formation of functional blood vessels to deliver both oxygen and nutrients. Normally, angiogenesis is transitory and is regulated through angiogenesis stimulators and inhibitors.38 It involves vasodilation and extravasation of plasma proteins, breakdown of basement membrane and ECM, proliferation and migration of endothelial cells into the perivascular space as a “migration column,” and re-building of a functional lumen and the underlying matrix.39 Activation of a pro-angiogenic switch in tumors can occur through increased
gene transcription of angiogenic stimulators (FGF-2, VEGF, PDGF, ANG1, and ANG2) or decreased expression of inhibitors (thrombospondin-1, and interferon-α). Pro-angiogenic gene expression occurs in response to hypoxia in the growing tumor mass and tumor-secreted growth factors, as well as through oncogene activation or inactivation of tumor suppressors. Beyond facilitating the growth of the primary tumor, angiogenesis is a central event in metastatic dissemination. Tumor angiogenesis is also affected by protease-mediated breakdown of the ECM, which facilitates the migration of activated endothelial cells. The breakdown of the ECM by proteases can also enhance the angiogenic process through the release of growth factors from the ECM. These proteases are expressed by the tumor cells itself and by stromal cells in response to the signals generated by the tumor.

B. Tumor Progression

Acquisition of invasive capacity is an essential step in tumor progression toward metastasis, and it involves alterations in proteins that regulate cell-cell and cell-ECM attachment as well as ECM-degrading proteases. Cell-adhesion molecules, such as cadherins, are often lost in epithelial tumors. A variety of mechanisms known to affect E-cadherin include mutation of the gene, transcriptional repression, or proteolysis of its ectodomain. Integrins are also important cell adhesion proteins that interact with receptor tyrosine kinases, disrupt cell-cell adhesion through phosphorylation of the E-cadherin-β-catenin complex, which results in its endocytosis, and activate the SNAIL/SLUG transcription factor that affects E-cadherin expression. Integrins also cooperate with these receptors to induce cell migration by phosphorylation of myosin light chain (MLC) kinase to induce contraction of actomyosin fibres. Tumor cells often switch the integrins present on their surface, replacing those that function to secure them to the ECM, and induce quiescence with other integrins that promote cell survival and a migratory phenotype. An additional important step in tumor cell invasion is the breakdown of connective tissue barriers, which is mediated by proteolytic activity. While some proteases are produced by tumor cells, many originate from stromal cells in response to growth factors, cytokines, and chemokines. Typically, these proteases, such as MMPs, become localized to the tumor cell surface at the invadopodia to mediate ECM breakdown and facilitate invasion.

Tumorigenesis culminates in metastatic dissemination, leading to the successful colonization of distant secondary sites by tumor cells. Similar to the multiple phenotypes that allow for growth advantage to the tumor cell during tumorigenesis, completion of the metastatic process requires changes in cell adhesion, migration, homing to target organs via chemokine/chemokine receptors, proteolysis, and ECM remodeling. In addition, the metastatic process is influenced by the stromal compartment. Thus, the outcome of
metastatic cell dissemination is determined not only by events in the tumor cell but also by successful interaction within the target organ.\textsuperscript{45}

III. METALLOPROTEINASES

Proteases play an important role in development, in numerous physiological processes, and in pathological conditions, such as cancer. On the basis of their mechanism of catalysis, proteases are divided into five classes: metallo, aspartic, cysteine, serine, and threonine.\textsuperscript{46} Both metallo and aspartic proteases are characterized by the use of an activated water molecule as the nucleophile to mediate the attack on the peptide bond of the substrate. In the other three classes of protease, the respective catalytic amino acid residue (cysteine, serine, or threonine) acts as a nucleophile.\textsuperscript{46} According to similarities in amino-acid sequences, the five classes of protease are subdivided into 63 families. These families are then further divided into clans that share similarities in 3D structure.\textsuperscript{46}

A. MMP Family

Among the proteases, the metalloprotease class has the largest number of members in humans.\textsuperscript{46} Within this class, the MMP family has been highlighted for its importance in ECM physiology.\textsuperscript{15,47} The MMP family is composed of 24 different zinc-dependent endopeptidases, which have been characterized in human, rodent, and amphibians.\textsuperscript{48} The general structure of an MMP is characterized by the presence of a pre-domain that acts as a signal peptide for secretion, a pro-domain important in maintaining its latency, a catalytic domain, and a hemopexin-like domain (with the exception of MMP-7 and MMP-26) that mediates interaction with the substrate or with TIMPs.\textsuperscript{1,16,49} The catalytic domain contains the active site that has a consensus motif of HEXXHXXGXXH, where the three histidines act as a zinc ligand, and the glutamic acid mediates hydrolysis of the peptide bond.\textsuperscript{16,50} The majority of MMPs are secreted as zymogens, in which the latency is maintained by interaction of the cysteine residue in the pro-domain sequence PRCGVPC with the catalytic zinc ion. This interaction prevents the zinc-dependent activation of water molecules required to mediate the nucleophilic attack on the peptide bonds of the substrate, and the presence of the pro-domain physically blocks access to the active site.\textsuperscript{50} Activation of the latent enzyme is dependent on the removal of the cysteine-zinc interaction. This process has been shown to occur \textit{in vitro} upon treatment with detergents, organomercurial, oxidants, and alkylating agents,\textsuperscript{51} or \textit{in vivo} through the activity of plasmin, cathepsin G, neutrophil elastase, or cellular oxidative changes.\textsuperscript{52–55} In addition, MMPs are capable of activating other MMPs. A well-studied mechanism is that of pro-mmp-2 activation at the cell surface, which involves the removal of its pro-domain in a two-step process through a tri-molecular complex
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composed of mt1-mmp (mmp14), pro-mmp-2, and timp2. Here, TIMP2 simultaneously binds to the hemopexin-like domain of MMP-2 and the catalytic domain of MT1-MMP, serving to sequester and localize pro-MMP2 to the cell membrane and facilitating the cleavage of the pro-domain by other TIMP2-free MT1-MMP molecules. The role of each TIMP in this process has been tested using mouse-embryo fibroblasts deficient in individual timps. Both timp2 and timp3 play a significant role in regulating this activation.

In addition to activation of the zymogen, MMP activity is also regulated at the level of transcription and through inhibition of the activated enzyme. The expression of MMPs is induced by a variety of external stimuli such as cytokines and growth factors, including interleukins, interferons, EGF, FGF, VEGF, TNF-α, TGF-β, and the extracellular matrix metalloproteinase inducer-EMMPRIN. Several cis-regulatory elements also influence MMP gene expression. For instance, the regulatory regions of inducible MMP genes contain AP-1 elements as well as PEA3 sites that bind the ETS family of transcription factors and cooperate with AP-1 for optimal expression of MMPs. Other cis-regulatory elements include the β-catenin-regulated LEF/TCF recognition site, TGF-β regulatory elements, AP-2, Sp1, Sp3, NF-κB, and CCAAT/enhancer-binding protein-β. The active MMP enzyme is inhibited by endogenous MMP inhibitors. The family of TIMPs has been identified as the primary inhibitors of MMPs in tissues. Other endogenous MMP inhibitors include α2-macroglobulin, which acts in tissue fluids and reversion-inducing cysteine-rich protein with Kazal motifs (RECK), which downregulates MMP-9 at an unidentified post-transcriptional level and can also inhibit MMP-2 activity following its MT1-MMP-mediated activation. In addition, thrombospondin-2 regulates MMP-2 by forming a complex that facilitates scavenger-receptor-mediated endocytosis, and thrombospondin-1 inhibits pro-MMP-2 and pro-MMP-9 activation and modulates MMP-2 production.

While the members of the MMP family collectively are capable of degrading all the major components of the ECM, specific MMPs show characteristic preferences for different ECM molecules as well as non-matrix molecules. These preferences for matrix substrates are often used to subdivide the MMP family into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, -11), and others. Collagenases primarily degrade fibrillar collagens, including collagen type I, II, III, and VII, whereas gelatinases preferentially degrade denatured collagen and collagen IV but can also degrade collagens I, V, and X, as well as laminin 5, as in the case of MMP-2. Stromelysins degrade a wider range of substrates, including collagen type IV, V, IX, and X as well as fibronectin, laminin, elastin, gelatin, and proteoglycan core proteins. In addition to these secreted MMPs, a number of MMPs (MMP-14, -15, -16, -17, -24, and -25) contain a transmembrane sequence downstream of the hemopexin-like domain that anchors the MMP to the cell membrane. These
membrane-type MMPs, also called MT1-MMP to MT6-MMP, are capable of degrading native-type I collagen, fibronectin, laminin, fibrin, gelatin, and cartilage proteoglycan core protein.\textsuperscript{61,62}

**B. Adamalysin Subfamily (ADAM and ADAMTS)**

In addition to proteases in the MMP family, members belonging to the ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motif) families also contain a zinc-dependent metalloproteinase domain and are thus grouped with the metzincin superfamily of metalloproteinases.\textsuperscript{64−66} Most of the interest in the ADAM molecules is associated with their ability to act as cell-surface sheddases.\textsuperscript{64,67,68} The proteolytic activity of ADAM17 results in the release of the membrane-anchored pro-TNF\textsubscript{α}, pro-TGF\textsubscript{α}, and pro-HB-EGF and is thus suggested to play a significant role in regulating cellular processes.\textsuperscript{65} ADAMTSs are secreted enzymes, and their activity has been implicated in the processing of the amino-propeptide of procollagens type I, II, and III as well as in the breakdown of proteoglycans (aggrecan, versican, and brevican).\textsuperscript{66,69}

**C. MMPs in Cancer**

While many of the key events during tumorigenesis are mediated by genetic and epigenetic changes, it is clear that alterations in the tumor microenvironment also act as inducers of tumor development.\textsuperscript{70} Both \textit{in vitro} and \textit{in vivo} studies support a predominantly stimulatory role of MMPs in tumorigenesis, angiogenesis, and metastatic dissemination.\textsuperscript{15,48,62} Clinical studies invariably show elevated expression of MMPs in human cancers, which increase with tumor progression.\textsuperscript{15,49} Unlike with classical oncogenes, upregulation of MMP expression does not generally occur through activating mutations. Examples of MMP gene alterations in cancers include the translocation of the chromosome region 1p36.2-p36.3, which contains \textit{MMP21-22}, in neuroblastoma; translocation or amplification of region 20q11, containing \textit{MMP24}, in multiple myeloma and breast cancer; and amplification of the 7.16−7.98 Mb region of chromosome 9 that contains 10 \textit{mmp} genes in tumors derived from \textit{p53\textsuperscript{−/−}} tetraploid mouse mammary epithelial cells.\textsuperscript{71−76} More typically, the increase in MMP levels in cancer cells results from changes in transcription produced by activation of oncogenes or loss of tumor suppressors. For instance, the \textit{src} oncogene induces MMP-7 promoter activity,\textsuperscript{77} whereas P53 downregulates the expression of MMP-1.\textsuperscript{78} As mentioned previously, tumor cells also upregulate the expression of MMPs in the host stroma through factors such as EMMPRIN.\textsuperscript{79}

The pro-tumorigenic function of MMPs was initially ascribed to their classical role in ECM breakdown. Subsequently, as novel non-matrix substrates have been identified for MMPs, it has become clear that their role in
The Role of Tissue Inhibitors of Metalloproteinase extend beyond this traditional view. MMP-mediated processing and release of bioactive molecules results in the activation of growth factors, suppression of apoptosis, release of angiogenic factors, and breakdown of cell-cell and cell-ECM constraints. These various activities generate a cellular microenvironment that facilitates growth and survival, angiogenesis, invasion, and metastatic dissemination. However, MMPs can in some cases act to limit the tumorigenic process by inhibiting angiogenesis through the generation of antiangiogenic factors (angiostatin, endostatin, and tumstatin), or to inhibit metastasis through the cleavage of chemokines such as CXCL12 that function to enhance metastasis.

D. ADAM and ADAMTS Enzymes in Cancer

Thus far, the evidence to support a role of ADAMs in tumorigenesis is not as extensive as for MMPs. Recent studies suggest that ADAM expression positively correlates with the progression of ovarian, lung, and breast cancers. The function of ADAMs as sheddases may underlie this effect. For instance, ADAM10, ADAM15, and ADAM17 have been shown to promote tumorigenesis, tumor cell migration, and invasion by inducing G-protein coupled receptor-EGFR transactivation. Information about the role of ADAMTS in cancer is similarly limited. The expression of ADAMTS4 and ADAMTS5 is upregulated in glioblastomas, and the ability of these enzymes to degrade proteoglycans may specifically contribute to the invasive potential of glioblastomas. ADAMTSs are also upregulated in breast and pancreatic cancers. Interestingly, ADAMTS1 has been shown to possess antiangiogenic activity, as the overexpression of its C-terminal region in CHO cells results in the reduction of tumor growth and lung metastasis.

IV. TIMPs

A. Characteristics of TIMPs

TIMP proteins are composed of two domains, an N-terminal domain responsible for the inhibition of MMP catalytic activity and a C terminus that mediates specific interactions with the hemopexin-like domain of pro-MMPs. Crystallography-based examination of the TIMP1/MMP-3 and TIMP2/MT1-MMP complexes has been conducted and reviewed in depth. These reports show that the wedge-like shape of the TIMP molecule binds with its edge into the active site cleft of the MMP. The majority of TIMP1/MMP-3 interaction is mediated through residues Cys-1 to Val-4 and Met-66 to Val-69 of TIMP1. Other regions involved in this TIMP/MMP interaction include the A-B loop, the E-F loop, (Thr-98 and Cys-99) and residues Leu-133 and Ser-134. In the case of TIMP2, similar regions mediate its interaction with MT1-MMP. The four TIMPs share a number of characteristics.
such as their overlapping MMP inhibitory activity, the presence of 12 cysteine residues that form six disulfide bonds that fold the protein into its two domains, the presence of 10 $\beta$-strands and four $\alpha$-helices, a conserved VIRAK region, and a total of 44 residues common to all TIMPs.\textsuperscript{92,96}

The evolution of the TIMP genes has been reviewed by Brew \textit{et al.}\textsuperscript{1} The two timp homologues in \textit{C. elegans} proteins have a single domain lacking the region corresponding to the C-terminal of TIMPs in all other species. Among mammalian TIMPs, TIMP1 and TIMP3 originated before TIMP2 and TIMP4, with TIMP1 undergoing the least evolutionary change. However, a more recent study shows that the N-terminal domain of human TIMP3 is more closely related to the Drosophila timp in terms of sequence, isoelectric point, and functional properties such as the inhibition of MT1-MMP and ADAM17 enzymes. It was suggested that TIMP3 may have preserved more of the functions of the ancestral protein.\textsuperscript{97} TIMP1 lacks the ability to inhibit these two enzymes but can inhibit ADAM10. Interestingly, when residue threonine 98 was altered to lysine on TIMP1, it acquired an ability to inhibit MT1-MMP. Additional mutations, that of valine 4 to serine, valine 69 to leucine, and the incorporation of the TIMP3-AB loop, conferred this chimeric TIMP1 with the ability to inhibit ADAM17, which surpassed that of the native TIMP3 protein.\textsuperscript{98,99} The addition of an N-terminal alanine extension or the substitution of threonine 2 for glycine residue in TIMP3 led to loss of its MMP-inhibitory activity, but an ability to inhibit TACE was retained.\textsuperscript{97}

The four TIMP genes are located on distinct chromosomes, with TIMP1 on the X-chromosome for both human and mouse, TIMP2 on chromosome 17 (human) and 11 (mouse), TIMP3 on chromosome 22 (human) and 10 (mouse), and TIMP4 on chromosome 3 (human) and 6 (mouse). Interestingly, genes for both human and mouse TIMP1, TIMP3, and TIMP4 reside within the introns of synapsin (SYN) I, III, and II genes, respectively, in the opposite transcriptional orientation.\textsuperscript{100–103} This indicates that prior to the divergence of the tetrapod lineage, the invertebrate SYN-TIMP locus replicated at least three times. Using the Fugu genome as a model, it is suggested that the lack of a syn-timp2 association may be due to duplication of this timp gene alone or to loss of its flanking syn region.\textsuperscript{1,103} Individual TIMPs have a number of distinct characteristics. TIMP1 poorly inhibits MT-MMPs,\textsuperscript{104} while TIMP2 is the only TIMP involved in the generation of the trimolecular complex necessary for activation of pro-MMP-2 at the cell surface.\textsuperscript{105} TIMP1 and \textsuperscript{3} are each capable of inhibiting ADAM10, but only TIMP3 inhibits ADAM12, ADAM17, and ADAM9. In addition, TIMP3 inhibits specific members of the ADAMTS family, namely ADAMTS4 and ADAMTS5.\textsuperscript{106,107} A key feature of TIMP3 is its localization to the ECM by its binding to heparin, heparan sulfate, and chondroitin sulfate moieties,\textsuperscript{108} which occurs through lysine and arginine groups in its N-terminus. TIMP3 is also the only TIMP gene in which mutations are linked to a human disease, Sorsby’s Fundus Dystrophy characterized by retinal degeneration.\textsuperscript{109} These mutations include Ser156Cys,
Gly166Cys, Gly167Cys, Tyr168Cys, S181C, and Tyr172Cys.\textsuperscript{110,111} TIMPs have specific organ expression patterns, with TIMP2 being the most widely expressed and TIMP4 being the most restricted, with high expression in heart, brain, skeletal muscle, ovaries, and testes.\textsuperscript{102,112} TIMP1 is highly expressed in the bone, and TIMP3 is abundant in kidney, heart, and lung. TIMP2, TIMP3, and TIMP4 are found to be silenced due to hypermethylation of their promoters in a number of human cancers.\textsuperscript{113–115}

The importance of TIMPs in normal physiology is underscored by a number of alterations (both spontaneous or in response to specific challenges) attributed to the ablation of specific \textit{timp} genes in mice. These include alterations to cardiovascular function, such as increased left ventricle end-diastolic volume in \textit{timp1}\textsuperscript{−/−} mice;\textsuperscript{116–118} LV dilation and dilated cardiomyopathy following aortic banding in \textit{timp3}\textsuperscript{−/−};\textsuperscript{119} and LV wall thickening in aged \textit{timp4}\textsuperscript{−/−} mice.\textsuperscript{120} Other alterations include increased tumor-cell metastasis in \textit{timp3}\textsuperscript{−/−} and \textit{timp4}\textsuperscript{−/−} mice.\textsuperscript{120,121} \textit{Timp4}\textsuperscript{−/−} mice have only been recently generated and are therefore less well studied.

### B. Regulation of TIMPs

The \textit{TIMP} genes respond differentially to a number of stimuli. TIMP1 and TIMP3 are upregulated in response to PMA and TGF-β, while these same agents downregulate TIMP2.\textsuperscript{122} All TIMP promoters have SP1 sites. \textit{TIMP1}, \textit{TIMP2}, and \textit{timp3} promoters have additional common elements, such as AP-1 and PEA3, that are also present in several \textit{mmp} promoters.\textsuperscript{122–124} Despite these similarities, there seems to be little organizational conservation of the common motifs in the \textit{Timp} promoters.\textsuperscript{124} Unlike human \textit{TIMP2} and murine \textit{timp3}, human \textit{TIMP1} and murine \textit{timp2} and 4 promoters lack the TATA box that is usually absent in housekeeping genes. TIMP2, which is typically constitutively expressed in most organs, contains MEF-2- and NF-IL6- binding sites in its promoter. The murine \textit{timp3} promoter uniquely has binding sites for the transcription factors NF-κB, c-MYC, and P53. NF-κB is an important signaling effector for the inflammatory- and death-receptor pathways, whereas c-MYC and P53 are critical regulators during apoptosis. This speaks to the specific roles of \textit{timp3} in regulating such processes.\textsuperscript{119,125} \textit{Timp4} contains a myogenin- binding site, likely important for its skeletal muscle-specific expression, as well as an AP-4 site. Biochemical studies show that TIMPs have overlapping MMP-inhibition profiles.\textsuperscript{126} A combination of organ-specific expression and differential regulation of both TIMPs and MMPs likely confers the specificity of TIMP:MMP interactions \textit{in vivo}.

### V. TIMPs IN CANCER

The role of TIMPs in cancer has been widely studied through clinical specimens and experimental systems. Clinical investigations have focused
on these proteins to determine their usefulness as prognostic and diagnostic markers, mostly relying on immunohistochemistry and in situ hybridization examination of surgical specimens. More recently, data are emerging on MMP- and TIMP-expression profiles through real-time and microarray expression analyses. Both MMPs and TIMPs are invariably altered within tumors, and MMPs as a whole are often found overexpressed in many human cancers. On the other hand, the family of TIMP proteins shows no clear trends. TIMPs, especially TIMP1 and TIMP2, are overexpressed in many human tumors, sometimes positively correlating with a poor prognosis.\textsuperscript{127–129} In contrast, downregulation, or silencing, has also been identified, especially for TIMP2, TIMP3, and TIMP4, in many human cancer lines and clinical specimens.\textsuperscript{113–115} TIMPs have also been extensively studied in experimental systems, often through the gain- or loss-of-function approaches using both cell lines and animal models. We will focus on these studies and summarize their effects on specific stages of tumorigenesis and progression and whether these effects are mediated through an MMP-dependent or MMP-independent mechanism.

A. TIMPs in Genomic Instability

The accumulation of somatic mutations forms the basis for tumorigenesis.\textsuperscript{130} Genetic alterations arise through a number of mechanisms with the end result of inactivating tumor-suppressor genes or activating oncogenes.\textsuperscript{131} To date, little evidence exists to support a direct role of MMPs in genomic instability, although the expression of certain MMPs correlates with changes in susceptibility to tumorigenesis. The ablation of \textit{mmp13} results in decreased number and size of DMBA-induced tumor lesions in the mammary gland and ovary.\textsuperscript{132} Similarly, ablation of \textit{mmp7} suppresses APC mutation-induced intestinal tumorigenesis in the Min/+ model.\textsuperscript{133} It has been suggested that the loss of cell adhesion can induce mutations by altering adhesion-dependent cell-cycle checkpoint controls and, in turn, that mutations in adhesion molecules that generate defective cadherins can affect cell-cell adhesion.\textsuperscript{134,135} MMPs have been implicated in the cleavage of the E-cadherin ectodomain responsible for the formation of adherens junctions,\textsuperscript{136} raising the possibility that MMP activity affects the adhesion-dependent accumulation of genetic instability. Mmp-3 overexpression driven by the whey acidic protein (WAP) promoter in the mammary gland results in increased mammary tumorigenesis associated with amplifications in chromosomes 4, 6, 7, and 15.\textsuperscript{137} More recently, mmp-3 overexpression was shown to induce upregulation of Rac1b and reactive oxygen species, allowing increased DNA damage and genomic instability.\textsuperscript{70}

Given that MMP activity may be linked to genetic stability, the question then follows whether TIMPs influence genomic instability. We provided the first evidence that downregulation of a timp imposed oncogenic
characteristics on otherwise non-tumorigenesis immortal cells. Specifically, antisense-RNA induced a 50% reduction of timp1 expression in Swiss 3T3 mouse fibroblasts that was sufficient to confer an ability to grow on soft-agar as well as to form tumors and metastases in nude mice.\textsuperscript{138} Further, such down-regulation of timp1 predisposed these cells to additional changes in gene expression.\textsuperscript{139} In the WAP-mmp-3 model noted above, the neoplastic changes associated with MMP-3 overexpression were quenched by transgenic TIMP1 overexpression. If the tumorigenic process in this model is dependent on the introduction of genomic instability involving MMP-3, then TIMP1 likely functions to prevent such genomic alteration. The effect of TIMP1 overexpression on chromosome amplification was not examined in this study.

Contrary to the idea that TIMPs may inhibit genomic instability, Rhee \textit{et al}.\textsuperscript{140} showed that TIMP1 overexpression enhances keratin-14-driven squamous cell carcinomas arising from the transgenic expression of the early region of human papilloma virus 16. Here, a TIMP1 increase was associated with greater keratinocyte proliferation and an earlier gain of chromosome 5 copy-number. To determine whether decreased TIMP1 affects the mutant frequency following an ethyl nitrosourea\textsuperscript{141} challenge, we used mice that had reduced timp1 expression due to an antisense timp1 transgene (Ta\textsuperscript{+}). Double transgenic mice were generated by crossing Ta\textsuperscript{+} mice with those harboring \textit{lacZ} as a reporter gene for somatic mutations. Ta\textsuperscript{+} \textit{lacZ}\textsuperscript{+} mice and the littermates control Ta\textsuperscript{−} \textit{lacZ}\textsuperscript{+} were administered ENU (150 mg/Kg of body weight, i.p.). Mutant frequency was measured in hepatic DNA at 15, 35, and 55 days post-injection and did not differ significantly between the two groups. This indicated that decreased timp1 levels did not affect the genomic instability introduced by the alkylating agent ENU.

If indeed TIMPs influence genomic stability, then it is necessary to determine the mechanisms by which they mediate this process. The effect of TIMP1 on genetic instability in MMP-3 overexpressing and keratin 14/HPV16 tumor models may arise indirectly by preventing the degradation of adhesion molecules or by affecting cellular proliferation.\textsuperscript{137,140} It is also possible that TIMPs may act directly to affect chromosomal integrity.\textsuperscript{140} Murine \textit{timp1} was first cloned as a cell cycle regulatable gene,\textsuperscript{142} and one study has suggested a cell-cycle-dependent accumulation of TIMP1 in the nucleus of human gingival fibroblasts.\textsuperscript{143} However, whether this accumulation can directly affect chromosomal stability remains undetermined. At present, the role of three other TIMPs in genomic instability remains unexplored.

\section*{B. TIMPs and Cell Proliferation: Differential and Cell-Type-Specific Effects}

As discussed in section II, an early event in tumorigenesis is the self-sufficiency of growth signals that contribute to the dysregulation of cell growth. An intriguing literature has developed over the years supporting
the role of TIMPs in regulating cell proliferation. When studied in the context of tumor growth, overexpression of TIMPs in transgenic tissues as well as in implanted cancer cells proves to be inhibitory. Hepatic timp1 overexpression suppresses T-antigen-induced liver hyperplasia in a transgenic model of hepatocellular carcinoma.\textsuperscript{144} Similarly, elevated levels of circulating timp1 decrease DMBA-induced mammary carcinogenesis.\textsuperscript{145} In a variety of tumor cell lines, overexpression of timp1 in melanoma, TIMP2 in haemangioma, timp3 in neuroblastoma, and TIMP4 in breast cancer also inhibit their growth as primary tumors when implanted subcutaneously into mice.\textsuperscript{146–149} In contrast to the general tumor-inhibitory theme of TIMP overexpression, evidence from clinical studies suggests a more complex role. In human cancers, high levels of TIMP expression sometimes correlate with a more favorable prognosis.\textsuperscript{150–152} However, it can also be associated with poor outcome in several tumor types.\textsuperscript{129,153–155} Thus, a direct relationship between TIMP expression and the outcome of human cancers has not emerged and underscores the need to define more directly the effects of altered TIMP expression on cell proliferation. Table 1\textsuperscript{a} summarizes studies in which the effects of individual TIMPs have been tested on cell proliferation using a variety of cell types and tissue-culture systems. For each study, we indicate the mode of TIMP alteration (recombinant or genetic), effect on cell growth (stimulatory or inhibitory), and the mode of action (MMP-dependent or MMP-independent). These studies illustrate both inhibitory and stimulatory effects on cell proliferation and are discussed below in that order.

1. TIMPs Inhibit Cell Proliferation

A few studies supported the inhibitory role of TIMP1 on cell proliferation \textit{in vitro}, specifically when TIMP1 was transduced in culture systems of vascular smooth-muscle cells and synovial fibroblasts by means of viral vectors (retrovirus and adenovirus).\textsuperscript{160,167} Comparatively strong data exist for a similar inhibitory function on cell proliferation \textit{in vivo}. Through genetic and biochemical manipulations, our laboratory assessed the effect of TIMP1 expression in transgenic models where cell proliferation \textit{in vivo} was the primary focus. Ductal mammary morphogenesis at puberty, which depends on epithelial-cell proliferation, is significantly enhanced upon mammary timp1 downregulation by antisense RNA. Conversely, it is decreased by locally-released recombinant TIMP1 implanted into the mammary gland.\textsuperscript{162}

Transgenic mice with altered hepatic timp1 expression have provided another system to examine timp1 effects on both preneoplastic hepatocyte proliferation as well as the normal hepatocyte cell cycle. During hepatocellular carcinoma (HCC) development, hepatic timp1 overexpression resulted in reduced hyperplasia while a TIMP1 reduction led to enhanced neoplastic proliferation.\textsuperscript{144} Further examination showed that hepatic timp1 reduced

\textsuperscript{a}Editor’s note: References 156 to 187 are cited sequentially for the first time in this table, and are subsequently in the text, where appropriate.
The Role of Tissue Inhibitors of Metalloproteinase

TABLE 1 Effects of TIMPs on Cell Proliferation *In Vitro*. Both stimulatory and inhibitory effects have been reported. Some of these effects arise through metalloproteinase (MP)-independent mechanisms. RA = rheumatoid arthritis; rb = recombinant bovine; TG = transgenic; KO = knockout; EC = endothelial cells; AoSMC = aortic smooth muscle cells; TYK = tyrosine kinase; PKA = protein kinase A; NA = not examined in the Study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Effect</th>
<th>MMP dependent</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP1</td>
<td>Increases</td>
<td>NA</td>
<td>Stimulates erythroid burst-forming units</td>
<td>[156]</td>
</tr>
<tr>
<td>Anti-TIMP1 antibody</td>
<td>Decreases</td>
<td>NA</td>
<td>Depletion of TIMP1 reduces growth of mouse erythroleukemia cell line ELM-I-1-3</td>
<td>[157]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of Gin1 fibroblast and Raji lymphoma cells</td>
<td>[158]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>No</td>
<td>Stimulates erythroid burst-forming units</td>
<td>[159]</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Decreases</td>
<td>NA</td>
<td>Decreases proliferation of SMC</td>
<td>[160]</td>
</tr>
<tr>
<td>rTimp1</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of epithelial corneal EC</td>
<td>[161]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Decrease</td>
<td>NA</td>
<td>Decreases proliferation of mammary ductal epithelial cells</td>
<td>[162]</td>
</tr>
<tr>
<td>TIMP1 TG</td>
<td>Decreases</td>
<td>Yes</td>
<td>Timp1 overexpression prevents cleavage of IGFBP-3, increases IGF-II sequestering, and decreases signaling through IGF-IR in hepatocytes</td>
<td>[164]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of UT7 leukemia cells, p38 activation (but not ERK1/2), and phosphorylation of JNK1/2</td>
<td>[165]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of MG-36 osteosarcoma cells, TYK dependent</td>
<td>[166]</td>
</tr>
<tr>
<td>Ad-TIMP1</td>
<td>Decrease</td>
<td>NA</td>
<td>Decreases proliferation of RA-synovial fibroblast muscle</td>
<td>[167]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of AoSMC, activation of ERK and PI3K</td>
<td>[168]</td>
</tr>
<tr>
<td>rTIMP1-GPI</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of HDMEC; suppresses proliferation of primary dermal fibroblasts</td>
<td>[169]</td>
</tr>
<tr>
<td>retrovirus</td>
<td>Increases</td>
<td>NA</td>
<td>Decreases in myeloid cell growth arrest</td>
<td>[170]</td>
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<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>Yes</td>
<td>Increases proliferation of MDA-MB-435 and activation of ERK and p38 pathways</td>
<td>[171]</td>
</tr>
<tr>
<td>AdTIMP1-KO, AS-timp1 TG</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of cardiac fibroblasts</td>
<td>[172]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Increases</td>
<td>Yes</td>
<td>Accelerates cell-cycle progression in hepatocyte (TG), increased HGF release</td>
<td>[173]</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Purified and rTIMP2</td>
<td>Decreases</td>
<td>No</td>
<td>Inhibits proliferation of HMVEC</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>No</td>
<td>Induces proliferation in Gin1 fibroblast and Raji lymphoma cells and other cell lines, growth of Raji cells inhibited by removal of TIMP2 from media</td>
<td>[175]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of foreskin Hs68 and fibrosarcoma HT-1080 fibroblasts cAMP upregulation and PKA activation</td>
<td>[176]</td>
</tr>
<tr>
<td>AdTIMP2</td>
<td>Decreases</td>
<td>No</td>
<td>Decreases proliferation of smooth-muscle cells</td>
<td>[177]</td>
</tr>
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</table>

(Continued on next page)
### Table 1 (Continued)

<table>
<thead>
<tr>
<th>Method</th>
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<th>MMP dependent</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbtimp2</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of rabbit epithelial cells</td>
<td>[161]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of dermal fibroblast</td>
<td>[178]</td>
</tr>
<tr>
<td>TIMP2 plasmid</td>
<td>Decreases</td>
<td>NA</td>
<td>Decreases growth rate of B16F10 melanoma cells</td>
<td>[179]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Decreases</td>
<td>No</td>
<td>Suppression of TYK growth factor-induced proliferation of A549, fibrosarcoma HT1080, fibroblast HS68, and breast carcinoma MCF7, disrupt EGFR phosphorylation and Grb-2 association, SH-PTP1 activity</td>
<td>[180]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of MG-36 osteosarcoma cells, cAMP/PKA-dependent</td>
<td>[166]</td>
</tr>
<tr>
<td>rTIMP2 c domain</td>
<td>Inhibits</td>
<td>No</td>
<td>Inhibits FGF-2- and VEGF-induced proliferation of EC, FGF-2 induced neovascularization and unstimulated angiogenesis in vivo in the CAM assay</td>
<td>[181]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Inhibits</td>
<td>No</td>
<td>Inhibits HMVEC proliferation in response to FGF/VEGF; decreases SHP1-integrin association; increases phosphatase activity against FGF-R1 and VEGF-R2</td>
<td>[182]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of A549 lung epithelial, upregulation of cyclin D1, activation of NF-6B, decreases in IkB</td>
<td>[183]</td>
</tr>
<tr>
<td>AdTIMP2</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of cardiac fibroblasts</td>
<td>[172]</td>
</tr>
<tr>
<td>timp2 plasmid</td>
<td>Decreases</td>
<td>No</td>
<td>TIMP2 inhibits PC12 proliferation via activation of integrin and cAMP/ERK pathways</td>
<td>[184]</td>
</tr>
<tr>
<td>TIMP3 purified</td>
<td>Increases</td>
<td>NA</td>
<td>Stimulates cell division of chicken embryo fibroblasts</td>
<td>[185]</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Increases</td>
<td>No</td>
<td>Increases number of smooth-muscle cells in S phase and BrdU uptake</td>
<td>[177]</td>
</tr>
<tr>
<td>TIMP3 vector</td>
<td>Increases</td>
<td>NA</td>
<td>Reduces growth rate of leiomyosarcoma cells</td>
<td>[186]</td>
</tr>
<tr>
<td>AdTIMP3</td>
<td>Decreases</td>
<td>NA</td>
<td>Decreases proliferation of RA-synovial fibroblast</td>
<td>[167]</td>
</tr>
<tr>
<td>TIMP4 rTIMP4</td>
<td>Decreases</td>
<td>No</td>
<td>Decreases proliferation of G401 Wilm’s tumor cells</td>
<td>[187]</td>
</tr>
<tr>
<td>rTIMP4</td>
<td>Increases</td>
<td>NA</td>
<td>Increases proliferation of MDA-MB-231 breast cancer cells</td>
<td>[187]</td>
</tr>
<tr>
<td>AdTIMP4</td>
<td>Increases</td>
<td>No</td>
<td>Increases proliferation of cardiac fibroblasts</td>
<td>[172]</td>
</tr>
</tbody>
</table>

The bioavailability of the mitogen IGF-II by inhibiting its proteolytic release from IGF-binding proteins and subsequent signaling through the type I IGF receptor. A direct effect on the hepatocyte cell-cycle was revealed in regenerating livers following partial hepatectomy. Here, hepatic timp1 levels directly influenced the timing of cell-cycle progression as reflected by the earlier peaks of cell-cycle markers (cyclin D1 for G1-S transition, PCNA for S phase, and phosphorylated histone H3 for M phase). In this case, excess timp1 inhibited the mmp-mediated processing of hepatocyte growth factor...
The Role of Tissue Inhibitors of Metalloproteinase

(hgf) to its active form. HGF is an important hepatic mitogen during liver regeneration.\textsuperscript{173,188}

TIMP2 overexpression also inhibits proliferation of normal and tumor cells.\textsuperscript{177,189} This effect can occur through MMP-dependent processes, such as the inhibition of collagenolysis, thus enhancing the inhibitory activity of fibrillar collagen on cellular proliferation.\textsuperscript{189,190} Alternatively, TIMP2 inhibits EGFR phosphorylation and Grb-2 association in an MMP-independent manner.\textsuperscript{180} In this case, TIMP2 was suggested to act through a putative TIMP-receptor on the plasma membrane, which increased SHP1-EGFR interaction, resulting in decreased phosphorylation of the receptor and subsequent decreased mitogenic signaling. This study also projected a similar inhibitory effect of TIMP2 on signaling of FGF-2 and PDGF.\textsuperscript{180}

A number of studies show that TIMP3 affects tumor cell viability and proliferation.\textsuperscript{191,192} For instance, TIMP3 overexpression in DLD-1 colon cancer cells results in a serum-dependent decrease in proliferation \textit{in vitro} as defined by a lower number of cells entering S-phase and complete loss of tumorigenicity \textit{in vivo}.\textsuperscript{191} This latter group further showed that TIMP3 overexpression delays cell-cycle progression in G1 phase. Similarly, in the human leiomyosarcoma cell line SK-LMS-1,\textsuperscript{193} TIMP3 overexpression retarded cellular growth, and TIMP3 antisense expression led to enhanced proliferation.\textsuperscript{186} TIMP3 overexpression inhibited smooth-muscle cells proliferation \textit{in vitro} with an upregulation of cyclin-dependent kinase inhibitor p21 (waf1), but, paradoxically, this was associated with an increased number of cells in S-phase.\textsuperscript{177} However, additional studies have failed to show a direct effect of timp3 overexpression on tumor cell proliferation of NXS2 neuroblastoma or B16F10 melanoma cell lines\textsuperscript{149} thus suggesting that the effect of TIMP3 on proliferation may be cell-type specific or is determined by the dependence of the cell line on growth factor modulated by MMPs.

Celiker \textit{et al.}\textsuperscript{187} showed that rhTIMP4 inhibits the growth of the Wilm’s tumor-derived cell line G401 \textit{in vitro}, and that upregulation of TIMP4 through naked TIMP4 DNA constructs, delivered intramuscularly, results in slower tumor growth \textit{in vivo}. More interestingly, this study suggests that this effect mediated by rhTIMP4 is likely independent of its MMP inhibitory activity, since it could not be achieved with synthetic MMP inhibitors and was exerted at concentrations lower than that needed for MMP inhibition.\textsuperscript{187} Furthermore, treatment with rhTIMP4 led to nuclear localization of TIMP4 in G401 cells, suggesting a direct role in regulating cellular proliferation through interactions with intracellular growth regulatory proteins.\textsuperscript{187}

2. TIMPs Promote Cell Proliferation

TIMPs can also function to enhance cell proliferation, in many instances as a result of MMP-independent mechanisms. TIMP1 was initially characterized as erythroid-potentiating activity (EPA) due to its ability to promote
the burst-forming units of erythroid precursor cells. TIMP2 has similarly displayed EPA. These two TIMPs have also been shown to enhance the growth of a number of transformed and tumor cell lines. While this role of TIMP1 has been linked to inhibition of MMP activity, it has also been shown that TIMP1 and TIMP2 growth-promoting effects may arise from mechanisms that are MMP-independent. For instance, the use of alkylated TIMPs, which are unable to inhibit MMP activity, induced DNA replication in human gingival fibroblast Gin-1 and Burkitt’s lymphoma Raji cell lines. In the osteosarcoma cell line MG-63, TIMP1 and TIMP2 mediated increased tyrosine phosphorylation and activation of ERKs P42/P44. The use of a PKA inhibitor (H89) or a tyrosine-kinase inhibitor (Herbimycin) showed that TIMP2 mediated Ras activation through PKA, while TIMP1 activated Ras via tyrosine-kinase pathways. Here, TIMP1 activation of RAS led to activation of RAF and the MAPK cascade, but TIMP2-mediated Ras activation instead resulted in increased RAS interaction with PI3K. TIMP2 also stimulated fibroblast (HS68) and fibrosarcoma (HT1080) proliferation through stimulation of cAMP production and PKA activation. Lizarraga et al. have shown that the addition of exogenous rhTIMP2 induces cell proliferation of A549 lung epithelial cells, and that this is associated with downregulation of IκBα and concomitant NF-κB activation. These pro-mitogenic effects may occur through interaction of TIMPs with putative TIMP-receptors on the plasma membrane, which allows activation of the above-mentioned signal transduction pathways. However, the identity of these receptors remains unknown at present. Such pro-mitogenic TIMP activity may perhaps be important in several human cancers where high TIMP1 and TIMP2 expression is associated with a poorer prognosis (for example, see Refs. 129–200).

Despite the fact that TIMP3 and TIMP4 expression are reported in tumor cells, the evidence for their pro-mitogenic role is not as extensive as for other TIMPs. Chicken timp3 (ChIMP) added as a purified protein stimulated the proliferation of growth-retarded, non-transformed chicken embryo fibroblasts. In contrast to the growth-inhibitory effect shown with G401 cells, rhTIMP4 also has been shown to stimulate the growth of MDA-MB-231 breast cancer cells.

The above studies highlight the complex role of TIMPs in cell proliferation and tumor growth. Experiments in Burkitt’s lymphoma cells have indicated a bimodal effect of TIMP1 overexpression, where initial enhancement of tumor growth was followed by a subsequent enhancement of tumor necrosis leading to tumor regression. Not only do the effects differ among the four TIMPs, but also each TIMP can exert inhibitory or stimulatory activity depending on the specific cell type and the system involved. Further, their direct in vivo effects on cell proliferation are made more difficult to discern since they simultaneously modulate related processes such as angiogenesis and apoptosis. Clearly, the mechanisms involved are diverse and in part depend on regulating the bioavailability of growth factors, through
C. The Effect on Apoptosis Differs Among TIMPs

Normally, anchoring of cells to the ECM provides cues for cell polarity, function, and survival, ultimately influencing cell fate. The lack of cell-ECM interaction is known to trigger cell death through anoikis, whereas tumor cells utilize a number of means to evade apoptosis including anchorage-independent growth and activation of anti-apoptotic factors. Compared to TIMPs, few studies have directly examined the effects of metalloproteinases on apoptosis. Table 2 shows that TIMP1 and TIMP3 have been more extensively studied for their impact on apoptosis in a variety of cell types, both via their genetic manipulation and through recombinant proteins. Again, the TIMPs either suppress or promote apoptosis, and these effects appear to be cell-type specific and sometimes independent of their MMP-inhibitory activity. The mechanisms underlying apoptosis extend beyond the physical integrity of the ECM and implicate signaling pathways that regulate apoptosis.

TIMP1 overexpression or the recombinant protein inhibits apoptosis in a wide variety of normal and tumor cell lines. This anti-apoptotic role is commonly mediated by AKT phosphorylation and activation of PI3Kinase signaling, which can lead to phosphorylation of Bad, preventing its interaction with BCL-2 and BCL-XL. Liu et al. recently showed that TIMP1 also inhibits TRAIL-mediated apoptosis in MCF10A breast cancer cells in an MMP-independent manner. Jung et al. highlighted CD63 as a cell surface-binding partner for TIMP1, which facilitates TIMP1:β1 integrin interaction and the subsequent activation of β1integrin/FAK survival pathways. However, other studies suggest that the anti-apoptotic function of TIMP1 occurs in an MMP-dependent manner.

In contrast to TIMP1, TIMP3 overexpression has repeatedly been shown to increase apoptosis in several normal and tumor cell lines. The TIMP3 pro-death domain was found to be located within the three N-terminal loops and involved MMP-inhibition. In melanoma and colon adenocarcinoma cell lines, it stabilized the death receptors TNF-RI, FAS and TRAIL-RI, resulting in activation of caspase 8 and caspase 3. Using human embryonic kidney 293 cells, Bond et al. showed that caspase 8 activation occurred downstream of mitochondrial activation through a type-II apoptotic pathway. Recently, microarray analysis of TIMP3-overexpressing glioma cells (Gli36) showed an upregulation of 13% of all apoptosis-associated genes present on the HG-U133A gene chip. These genes included members of

Editor’s note: References 20 to 23 are cited sequentially for the first time in this table, and subsequently in the text, if appropriate.
TABLE 2 Effects of TIMPs on Apoptosis In Vitro and In Vivo. Both MP-dependent and MP-independent effects have been reported. VSMC = vascular smooth muscle cells; AS = antisense; TG = transgenic; KO = knockout; NA = not examined in the study cited.

<table>
<thead>
<tr>
<th>Method</th>
<th>Effect</th>
<th>MMP dependent</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits stromelysin-1 induced apoptosis of alveolar epithelial cells</td>
<td>[206]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Inhibits</td>
<td>No</td>
<td>Inhibits apoptosis in BL cells in response to cold shock, serum starvation and fas activation</td>
<td>[207]</td>
</tr>
<tr>
<td>rTIMP1, TIMP1 vector</td>
<td>Inhibits</td>
<td>No</td>
<td>Inhibits apoptosis of breast epithelial cells</td>
<td>[208]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Increases</td>
<td>NA</td>
<td>Increases tumor apoptosis (pancreatic cancer cell line PANC-1)</td>
<td>[209]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>No</td>
<td>Protects rat mesangial cells from apoptosis, decreases expression of bax</td>
<td>[210]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Reduces caspase 3 activity; enhances BCL-2 expression in hepatic stellate cells (HSC)</td>
<td>[211]</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases apoptosis in Hodgkin/Reed-Sternberg cells</td>
<td>[212]</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits caspase 3 activity and apoptosis in HSC</td>
<td>[213]</td>
</tr>
<tr>
<td>rTIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Enhances survival of UT-7 erythroid cells, increases activity of PI-3K/AKT, JAK2</td>
<td>[91]</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits apoptosis in breast carcinoma cells T-47D, upregulation of BcL-2 tyrosine and BAD phosphorylation, maintained BCL-XL expression</td>
<td>[214]</td>
</tr>
<tr>
<td>rTIMP1, AS &amp; TIMP1 vector</td>
<td>Inhibits</td>
<td>No</td>
<td>Increase caspase activity and apoptosis in MCF10A cells with AS-TIMP1 increases ERK/PI-3K activity with TIMP1 overexpression</td>
<td>[215]</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits TNF-induced apoptosis, activation of PI-3K/AKT in endothelial cells and Bcl-XL; increases activation of AKT/ERK through G protein, PTK, and PI-3K</td>
<td>[216]</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Protects granulocytes from spontaneous apoptosis</td>
<td>[217]</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Reduces cyclohexamide-induced apoptosis in HSC; reduces cleavage of N-cadherin</td>
<td>[218]</td>
</tr>
<tr>
<td>Bovine timp1</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Reduces radiation-induced apoptosis in endothelial cells</td>
<td>[219]</td>
</tr>
<tr>
<td>rTIMP1, TIMP1 vector</td>
<td>Inhibits</td>
<td>No</td>
<td>Protects MCF10 cells from TRAIL-induced cell death and caspase (3,8,9) activity, involvement of FAK and PI-3K activation</td>
<td>[220]</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits apoptosis of B16F10 melanoma cells</td>
<td>[178]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Increases</td>
<td>Yes</td>
<td>Increases apoptosis in activated T lymphocytes and Tsup or Jurkat lymphoma cell lines</td>
<td>[221]</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Inhibits apoptosis in HCC tumor</td>
<td>[192]</td>
</tr>
<tr>
<td>TIMP3 vector</td>
<td>Increases</td>
<td>yes</td>
<td>Increases apoptosis in HCC tumor</td>
<td>[193]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Sensitizes colon cells DLD to cytotoxic signals, prevents cleavage of TNFRI</td>
<td>[222]</td>
</tr>
<tr>
<td>rTIMP3, Ad-TIMP3</td>
<td>Increases</td>
<td>yes</td>
<td>Induces apoptosis in melanoma cells SK-Mel-5 and A2058</td>
<td>[177]</td>
</tr>
<tr>
<td>rTIMP3, Ad-TIMP3</td>
<td>Increases</td>
<td>yes</td>
<td>Induces apoptosis in VSMC</td>
<td>[223]</td>
</tr>
</tbody>
</table>

(Continued on next page)
TABLE 2 (Continued)

<table>
<thead>
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<th>Method</th>
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<th>Observations</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>rTIMP3, Ad-TIMP3</td>
<td>Increases</td>
<td>yes</td>
<td>N-terminus induces apoptosis in Hela and SMC</td>
<td>[224]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Increases apoptosis in neointima and media</td>
<td>[225]</td>
</tr>
<tr>
<td>KO</td>
<td>Inhibits</td>
<td>yes</td>
<td>Accelerates apoptosis mammory gland in the absence of timp3</td>
<td>[226]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Induces of caspase 8/9 activation and cleavage of PPAR and</td>
<td>[227]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Induces apoptosis in RPE and MCF-7cells</td>
<td>[228]</td>
</tr>
<tr>
<td>rTIMP3, Ad-TIMP3</td>
<td>Increases</td>
<td>yes</td>
<td>Stabilizes of death receptors (TNF-R, FAS, TRAIL-R1) in A2058 melanoma cells; activates caspase 8</td>
<td>[229]</td>
</tr>
<tr>
<td>TIMP3 vector</td>
<td>Increases</td>
<td>NA</td>
<td>Increases apoptosis in HCC tumor</td>
<td>[192]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>no</td>
<td>Sensitzes fibroblast to Fas/CD95 apoptosis in synovial fibroblasts; reverses anti-apoptotic effect of TNF-α on Fas-induced apoptosis; inhibits NF-6B activation, nuclear localization, and synthesis of Fas/CD95</td>
<td>[230]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Increases</td>
<td>NA</td>
<td>Induces apoptosis in cardiac fibroblast</td>
<td>[172]</td>
</tr>
<tr>
<td>TIMP4</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases apoptosis in MDA-MB-435 derived tumors; increases expression of BCL2 and BCL-XL</td>
<td>[231]</td>
</tr>
<tr>
<td>Purified TIMP4</td>
<td>Increases</td>
<td>NA</td>
<td>Induces apoptosis in transformed cardiac fibroblast but not in normal fibroblast</td>
<td>[232]</td>
</tr>
<tr>
<td>Ad-TIMP4</td>
<td>Increases</td>
<td>NA</td>
<td>Induces apoptosis in VSMC and balloon-injured carotid arteries</td>
<td>[43]</td>
</tr>
</tbody>
</table>

the TNF receptor superfamily (e.g. TNFR5, CD95/FAS2, and TNFRSF21), caspase family (e.g. CASP1, 4, 7, and 8), BCL-2 family (e.g. BNIP1, BNIP3, and MOAP1), and Death Domain family (e.g. NFKB2 and TRADD). Paradoxically, timp3−/− mice have accelerated epithelial apoptosis during mammary gland involution, at least in part due to the compromised cell-ECM adhesion.

Overexpression of TIMP2 or TIMP3 exerts a direct cytotoxic effect on hepatocellular carcinoma and colon cancer cells. Exogenous TIMP2 also inhibits shedding of TNF-RI and TNF-RII in Colo25 and SWS626 cells in vitro, and as is the case with TIMP3, the stabilization of these receptors could result in increased TNF signaling and apoptosis. Recombinant TIMP2 induces apoptosis in activated peripheral blood T cells as well as in Tsup and Jurkat T lymphoblastic lymphoma cell lines. In this study, the pro-apoptotic effect could not be achieved by using TIMP2 that lacked the MMP-inhibitory N-terminal domain, suggesting that this effect is MMP dependent. Analysis of cell surface expression of Fas ligand suggests that this effect may be due to the ability of TIMP2 to inhibit its shedding. This link between TIMPs and the shedding of FAS ligand may be cell specific since neither TIMP1 nor TIMP2 affects the levels of FAS ligand shedding in Ewing’s sarcoma family of tumors.
Valente et al.\textsuperscript{178} showed that TIMP2 overexpression in B16F10 cells resulted in protection against mitomycin-induced apoptosis.

TIMP4 acted as an anti-apoptotic factor in MDA-MB-435 cells both \textit{in vitro} and \textit{in vivo},\textsuperscript{231} whereas in smooth-muscle cells and transformed cardiac fibroblasts it had a pro-apoptotic effect.\textsuperscript{232,239} In MDA-MB-435 cells, Jiang \textit{et al.} reported that TIMP4 expression correlated with increased expression of anti-apoptotic molecules BCL-2 and BCL-X\textsubscript{L} \textsuperscript{231,239} a finding that contradicted the previously reported anti-tumor effect associated with transfection of a TIMP4 vector into the same MDA-MB-435 cells.\textsuperscript{147} This discrepancy was rationalized by the authors on the basis of the different levels of elevation achieved in the two studies. As the newest member of the family, the effects of TIMP4 on apoptosis remain to be further studied.

Thus, as with cellular proliferation, it is difficult to generalize the overall effect of the TIMP family on apoptosis. TIMP1 predominantly mediated an anti-apoptotic effect, whereas either supraphysiological levels or the loss of TIMP3 promoted apoptosis, and TIMP2 and TIMP4 exerted either pro- or anti-apoptotic effects, depending on the study.

D. TIMPs Use Different Mechanisms to Inhibit Angiogenesis

Angiogenesis is essential for the growth of the primary tumor and its metastases as it provides nutrients and oxygen. Within the multifaceted angiogenic process,\textsuperscript{240} ECM turnover is essential for capillary growth,\textsuperscript{149} and is mediated by the activity of MMPs and serine proteases.\textsuperscript{241} As inhibitors of MMPs, TIMPs have been proposed to play a regulatory role during angiogenesis. Table 3\textsuperscript{a} summarizes the effects of individual TIMPs on angiogenesis, again indicating whether the mode of action is MMP dependent or independent. With the exception of a very few studies of TIMP1, an inhibitory function has been observed for all TIMPs. Overexpression of TIMP1 in tumor cells (Burkitt’s lymphoma, Ehrlich cells, and Kaposi’s Sarcoma-derived KS-IMM cells) results in significantly decreased tumor angiogenesis and growth.\textsuperscript{202,242,243} Similarly in bovine aortic endothelial cells, TIMP1 significantly decreased their migration and invasion\textsuperscript{242,244,245} in an MMP-dependent manner. TIMP1 can also inhibit angiogenesis independent of its MMP inhibitory role. Reed \textit{et al.}\textsuperscript{246} showed that PVA sponges treated with anti-TIMP1 antibody resulted in enhanced angiogenesis that was not associated with changes in collagenase or gelatinase activity. This effect was ascribed to changes in cell morphology whereby a more elongated shape, greater alignment, and extension of the actin cytoskeleton and increased expression of $\alpha_2\beta_1$ integrin were observed.\textsuperscript{246}

TIMP2 also inhibits various processes of angiogenesis, and the MMP-independent effect of this TIMP plays a prominent role. Fernandez \textit{et al.}\textsuperscript{181}

\textsuperscript{a}Editor’s note: References 244 to 265 are cited for the first time in this table, and subsequently in the text, if appropriate.
### TABLE 3 Effects of TIMPs on Angiogenesis In Vitro and In Vivo

In general, increased TIMP levels inhibit angiogenesis. Both MP-dependent and MP-independent effects have been reported. EC = endothelial cells; ad = adenovirus; KO = knockout; rTIMP = recombinant TIMP; NA = not examined in study cited. HUVEC = Human umbilical vein endothelial cells, HDMEC = Human dermal microvascular endothelial cells, HEMVEC = Human endometrial microvascular endothelial cells, HMVEC = human microvascular endothelial cells.

<table>
<thead>
<tr>
<th>Method</th>
<th>Effect</th>
<th>MMP dependent</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP1</td>
<td>rTIMP1</td>
<td>Inhibits</td>
<td>NA Inhibits FGF-2-induced neovascularization; decreases HMVEC migration</td>
<td>[244]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases bovine aortic EC migration/invasion</td>
<td>[245]</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases HCC angiogenesis</td>
<td>[248]</td>
</tr>
<tr>
<td>Retrovirus-TIMP1</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases EC migration and angiogenesis in Burkitt’s lymphoma</td>
<td>[202]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases angiogenesis in PANC-1 pancreatic cancer cells</td>
<td>[209]</td>
</tr>
<tr>
<td>TIMP1, transgenic</td>
<td>Ab</td>
<td>Inhibits</td>
<td>No Increases EC migration</td>
<td>[242]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Increases levels of VE cadherin and suppression of HDMEC migration</td>
<td>[249]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>No</td>
<td>Decreases HMVEC migration, dephosphorylation of focal contact molecules FAK and paxillin; increases expression of PTEN</td>
<td>[249]</td>
</tr>
<tr>
<td>TIMP1 vector</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Suppresses HMVEC spontaneous or VEGF-induced migration</td>
<td>[250]</td>
</tr>
<tr>
<td>TIMP2</td>
<td>rTIMP2</td>
<td>Inhibits</td>
<td>NA Inhibits EC tubule formation</td>
<td>[252]</td>
</tr>
<tr>
<td>TIMP2 vector</td>
<td>Inhibits</td>
<td>Yes</td>
<td>NA Inhibits MT1-MMP-mediated fibronolysis during EC invasion</td>
<td>[253]</td>
</tr>
<tr>
<td>TIMP2 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases invasiveness/migration of EC and tumor angiogenesis</td>
<td>[178]</td>
</tr>
<tr>
<td>retrovirus-TIMP2</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases invasiveness/migration of EC and tumor angiogenesis</td>
<td>[178]</td>
</tr>
<tr>
<td>retrovirus-TIMP2</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases HUVEC and HDMEC tubulogenesis in fibrin</td>
<td>[256]</td>
</tr>
<tr>
<td>retrovirus-TIMP2</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Decreases HUVEC and HDMEC tubulogenesis in fibrin</td>
<td>[256]</td>
</tr>
<tr>
<td>TIMP2 domain</td>
<td>Inhibits</td>
<td>Yes</td>
<td>NA Inhibits embryonic EC proliferation</td>
<td>[181]</td>
</tr>
<tr>
<td>TIMP2 C domain</td>
<td>Inhibits</td>
<td>No</td>
<td>NA Inhibits embryonic &amp; FGF-2-driven EC proliferation</td>
<td>[181]</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Inhibits</td>
<td>No</td>
<td>Decreases SHP1-integrin association and increase phosphatase activity against FGF-R1 and VEGF-R2 in HMVEC</td>
<td>[182]</td>
</tr>
</tbody>
</table>

(Continued on next page)
TABLE 3 (Continued)

<table>
<thead>
<tr>
<th>Method</th>
<th>Effect</th>
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<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP2 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases vessel formation in HCC tumor</td>
<td>[192]</td>
</tr>
<tr>
<td>retrovirus-TIMP2</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits angiogenesis in MC38 colon cancer cells, upregulation of mpk1 phosphatase, and inactivation of mapk pathways</td>
<td>[258]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Inhibits</td>
<td>No</td>
<td>Suppresses HMVEC spontaneous or VEGF-induced migration, upregulation of RECK</td>
<td>[250]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits MT1-MMP-mediated upregulation of VEGF in MCF7 cells</td>
<td>[259]</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits tumor angiogenesis in MMTV-Wnt1 mouse</td>
<td>[260]</td>
</tr>
<tr>
<td>rTIMP2</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits EC invasion and tubule morphogenesis</td>
<td>[261]</td>
</tr>
<tr>
<td>TIMP3 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases tubulogenesis, pericyte recruitment; decreases VE cadherin</td>
<td>[149]</td>
</tr>
<tr>
<td>rTIMP3</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits SP1-induced EC invasiveness in fibrin and collagen</td>
<td>[257]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Decreases HMVEC tubulogenesis in fibrin/collagen</td>
<td>[262]</td>
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<tr>
<td>rTIMP3</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases EC migration and tubulogenesis</td>
<td>[263]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Inhibits</td>
<td>No</td>
<td>Competes for binding to VEGF2</td>
<td>[203]</td>
</tr>
<tr>
<td>TIMP3 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases vessel formation in HCC tumor</td>
<td>[192]</td>
</tr>
<tr>
<td>Ad-TIMP3</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits VEGF-induced tubulogenesis of HEMVEC</td>
<td>[251]</td>
</tr>
<tr>
<td>KO</td>
<td>Enhances</td>
<td>NA</td>
<td>Enhances angiogenesis in tumor and in response to FGF-2</td>
<td>[264]</td>
</tr>
<tr>
<td>rTIMP3</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits EC invasion and tubule morphogenesis</td>
<td>[261]</td>
</tr>
<tr>
<td>TIMP4 vector</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases angiogenesis in MDA-MB-435 cells</td>
<td>[147]</td>
</tr>
<tr>
<td>rTIMP4</td>
<td>Inhibits</td>
<td>NA</td>
<td>Decreases HUVEC and HDMEC tubulogenesis in fibrin</td>
<td>[256]</td>
</tr>
<tr>
<td>rTIMP4</td>
<td>Inhibits</td>
<td>Yes</td>
<td>Inhibits formation of lumen by EC in fibrin and collagen</td>
<td>[257]</td>
</tr>
<tr>
<td>rTIMP4</td>
<td>Inhibits</td>
<td>NA</td>
<td>Inhibits migration of EC</td>
<td>[265]</td>
</tr>
</tbody>
</table>

reported that the N-terminal domain of TIMP2 exerts an MMP-dependent suppression of embryonic neovascularization in the chicken chorioallantoic membrane (CAM) assay, whereas the C-terminal domain (specifically the C-terminal loop 6 in the intact TIMP2 molecule) inhibits both capillary endothelial cell proliferation and embryonic- and FGF-2-driven angiogenesis in an MMP-independent manner. TIMP2 inhibits endothelial cell-mediated fibrinolysis and neovascularization by inhibiting MT1-MMP fibrinolytic activity.253 Using a TIMP2 mutant with a lower inhibition constant for MT1-MMP, it was later shown that the anti-angiogenic activity of TIMP2 depended on its ability to inhibit this membrane-anchored MMP.256 TIMP2 activity against MT1-MMP inhibits both the upregulation of VEGF-A levels...
associated with induced overexpression of this membrane-type MMP in MCF-7 cells,\textsuperscript{259} as well as the angiogenic response by endothelial cells growing in a collagen-rich matrix.\textsuperscript{266}

TIMP2 also inhibits the FGF-2- and VEGF-induced proliferation of endothelial cells by increasing phosphatase activity against FGFR1 and KDR.\textsuperscript{175,182} This activity depends on TIMP2 interacting directly with $\alpha_3\beta_1$ integrin, which results in a decrease in the integrin-SHP-1 phosphatase association.\textsuperscript{182} Similarly, TIMP2 overexpression increased mpk1 phosphatase activity, resulting in decreased growth and angiogenesis of tumors generated from MC38 murine tumor cells.\textsuperscript{258} Upregulation of phosphatase Ptpn16 was also found in endothelial cells that inactivated MAPK pathways and led to the inhibition of angiogenesis.\textsuperscript{258} TIMP2 interaction with $\alpha_3\beta_1$ integrin induces upregulation of RECK expression,\textsuperscript{250} resulting in inhibition of endothelial cell migration. In parallel, TIMP2 downregulates the expression of VEGF, reducing the ability of tumor cells to recruit endothelial cells.\textsuperscript{148,254}

TIMP3 overexpression in several \textit{in vitro} and \textit{in vivo} systems effectively inhibits angiogenesis and tumor growth.\textsuperscript{234,267} This inhibitory effect stems from a decrease in endothelial cell migration and tubule formation \textit{in vitro}.\textsuperscript{149,252} Recently, Saunders \textit{et al.}\textsuperscript{261} have shown that TIMP3 and TIMP2 is primarily expressed in pericytes, whereas TIMP2 is expressed in endothelial cells; moreover, pericyte-derived TIMP3 inhibits EC tube formation and vascular assembly \textit{in vitro} and \textit{in vivo}, and in conjunction with EC-derived TIMP2, it stabilizes pre-existing EC tubular networks. Within the developing tumor, TIMP3 overexpression results in the formation of fewer functional blood vessels, decreased pericyte recruitment, and decreased VE cadherin expression.\textsuperscript{149} Similarly, co-injection of tumor cells with TIMP3 retroviral vector-producing cells successfully decreased pericyte recruitment, drastically reducing the vascular maturity index.\textsuperscript{267} TIMP3 inhibits capillary tubule formation by endothelial cells in both fibrin/collagen and fibrin/matrigel matrices, an effect related to the inhibition of MT1-MMP activity.\textsuperscript{262} A mutation in TIMP3 associated with Sorsby’s Fundus Dystrophy (SFD)\textsuperscript{109} induces increased choroidal neovascularization due to its reduced MMP inhibitory activity.\textsuperscript{268} However, Yeow \textit{et al.}\textsuperscript{111} found no significant decrease in MMP inhibitory activity in TIMP3 mutants and suggested that SFD progression may instead be due to changes in TIMP3 interaction with other proteins and altered cell adhesion.

A complete absence of TIMP3 in the host leads to a higher density of functional blood vessels in FGF-2-supplemented Gelfoam and matrigel plugs and also promoted tumor angiogenesis in \textit{timp3} deficient mice.\textsuperscript{264} In this case the anti-angiogenic role of \textit{timp3} appears to be mediated primarily through its MMP-inhibitory activity. In contrast, however, Qi \textit{et al.}\textsuperscript{203} proposed that TIMP3 inhibits VEGF-induced angiogenesis in an MMP-independent manner by competing with the binding of VEGF to its receptor KDR.

Because TIMP4 inhibits the fibrinolytic/collagenolytic activities of MT1-MMP and, potentially, the activation of MMP-2, it has been proposed that TIMP4 acts as a suppressor of angiogenesis.\textsuperscript{253,266,269} However, recent data
from our laboratory challenges the direct role of TIMP4 in regulating MT1-MMP-mediated activation of pro-MMP-2 at the cell surface, since \textit{timp4}^{-/-} mouse embryo fibroblasts do not show a reduction in mmp-2 activation following cytochalasin D or concanavalin A treatments.\textsuperscript{57} Concrete evidence to support an anti-angiogenic role for TIMP4 has been recently presented showing that TIMP4 inhibits capillary endothelial cell migration but does not affect endothelial cell proliferation or angiogenesis \textit{in vivo}.\textsuperscript{265} Additional circumstantial evidence shows that TIMP4 inhibits PMA-induced activation of MMP-2 in HUVECs and in glioma (U87) and fibrosarcoma (HT1080) cell lines;\textsuperscript{256} moreover, reduced TIMP4 in high-grade gliomas may allow for increased MT-MMP activity and angiogenesis.\textsuperscript{270}

TIMP4 expression in developing blood vessels during experimental corneal neovascularization is indicative of its function in corneal wound healing and angiogenesis.\textsuperscript{263} Recombinant TIMP4 inhibited tubulogenesis by endothelial cells in a fibrin matrix at similar concentrations necessary for TIMP2 inhibition,\textsuperscript{256} and TIMP4 overexpressing MDA-MB-435 breast cancer cells generated tumors with decreased microvasculature.\textsuperscript{147} However, recent testing in \textit{timp4}^{-/-} mice has revealed only a transient decrease in re-epithelialization of dermal wounds and no alterations in their angiogenic response to FGF-supplemented Gelfoam plugs and in intramuscular melanoma tumors.\textsuperscript{120}

Similar to the pro- and anti-proliferative role of TIMPs on tumor cell growth, there are a few instances where TIMP activity leads to enhanced angiogenesis. TIMP1 overexpression in an NMU-induced rat mammary carcinoma cell line or MCF-7 cells resulted in VEGF upregulation and enhanced angiogenesis in the growing tumors.\textsuperscript{247} Also, transgenic mice that co-overexpress VEGF and TIMP1 in the retina showed increased retinal neovascularization compared to single VEGF transgenics.\textsuperscript{271} Overall, most of the experimental evidence shows a significant anti-angiogenic capacity for TIMPs.

E. TIMPs Affect Key Steps in the Metastatic Process

Metastasis is a multi-step process responsible for tumor cell dissemination and growth at secondary sites. Tumor cells that invade surrounding tissues and metastasize through the blood stream or lymphatic system must penetrate several structural barriers, including basement membranes and the interstitial connective tissue.\textsuperscript{972} As such, breaching the ECM is one of the defining steps of tumor progression and is reflected in tumor grading.\textsuperscript{44,50} For example, human breast cancers are graded as ductal carcimona \textit{in situ} (DCIS) or invasive ductal carcinoma (IDC) on the basis of ECM disruption. The poorer prognosis of IDC gives weight to the importance of this step in the metastatic process. The physical aspects of cell invasion and ECM degradation are mediated primarily by the MMP family [reviewed in Ref. \textsuperscript{54}]. Table 4 illustrates the numerous reports focused on TIMPs, which are
The Role of Tissue Inhibitors of Metalloproteinase

TABLE 4 Effects of TIMPs on cell invasion and metastasis. In general, all TIMPs inhibit invasive and metastatic capacity of tumor cells.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Knockout</strong></td>
<td></td>
</tr>
<tr>
<td><em>Timp1</em></td>
<td>Increased invasiveness of <em>timp1</em>&lt;sup&gt;−/−&lt;/sup&gt; ES cells [296]</td>
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<td></td>
<td>No change in lung metastasis in <em>Timp1</em>&lt;sup&gt;−/−&lt;/sup&gt; mouse</td>
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<td></td>
<td>Increase/decrease in metastatic potential of <em>timp1</em>&lt;sup&gt;−/−&lt;/sup&gt; ES cells [297]</td>
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<tr>
<td><em>Timp2</em></td>
<td>N/A</td>
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<tr>
<td><em>Timp3</em></td>
<td>Increased metastasis to multiple organs by B16F10 and EL-4 cells [121]</td>
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<tr>
<td><em>Timp4</em></td>
<td>Increased lung colonization by B16F10 cells [120]</td>
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<td><strong>In vivo models</strong></td>
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<tr>
<td>TIMP1</td>
<td>Antisense <em>timp1</em>&lt;sup&gt;−/−&lt;/sup&gt; confers metastatic potential to Swiss 3T3 cells [138]</td>
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<td></td>
<td>Decreased metastatic ability of KKLS gastric cells in chick embryo assay [298]</td>
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<td></td>
<td>Decreased metastatic dissemination of B16F10 to lung [146]</td>
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<tr>
<td></td>
<td>Decreased metastatic ability of B16F10 but does not affect extravasation [299]</td>
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<tr>
<td></td>
<td>Antisense <em>timp1</em>&lt;sup&gt;−/−&lt;/sup&gt; antisense increases metastasis to liver by EsbL lymphoma cells</td>
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<td></td>
<td>Decreased metastasis to brain in transgenic mouse [301]</td>
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<td></td>
<td>Decreased liver metastasis by KM12SM colon cancer [302]</td>
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<td>Decreased metastasis to liver and abdominal wall by pancreatic cell line PANC-1 [299]</td>
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<td></td>
<td>Decreased metastatic invasion of lung by B16 melanoma [303]</td>
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<td></td>
<td>Ad-TIMP1 inhibits liver metastasis by L-CL.5s lymphoma and CT26 colorectal cells [304]</td>
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<tr>
<td>TIMP2</td>
<td>Inhibited local tissue invasion and reduced lung metastasis by 4R ras-transfected rat embryo cells [305]</td>
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<td>No effect on metastatic potential of M24.net melanoma to lungs and lymph nodes [189]</td>
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<td></td>
<td>Decreased invasiveness of tumor cell coinjected with retroviral TIMP2 vector [306]</td>
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<td>Decreased experimental metastasis to lungs by B16F10 cells [178]</td>
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<td></td>
<td>AdTIMP2 expression in liver reduces metastasis by colorectal LS174T cells [236]</td>
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<td></td>
<td>Decreased metastatic potential of LLC, C51, and MDA-MB-231 cells [255]</td>
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<td></td>
<td>Inhibited lung metastasis in MMTV-neu mice [307]</td>
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<td></td>
<td>Prevents distant pulmonary metastasis by MDA-MB-435 breast carcinoma [308]</td>
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<tr>
<td>TIMP3</td>
<td>N/A</td>
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<tr>
<td>TIMP4</td>
<td>Reduced metastatic potential of MDA-MB-435 [147]</td>
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<tr>
<td><strong>In vitro models</strong></td>
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<tr>
<td>TIMP1</td>
<td>Decreased invasive potential in B16F10 melanoma [309]</td>
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<td></td>
<td>Reduced SK-Mel5 and A2058 melanoma invasiveness [222]</td>
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<td></td>
<td>Decreased invasiveness of smooth-muscle cells [177]</td>
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<tr>
<td></td>
<td>Decreased migration and increased adhesion in hepatoma cells HEPG2 [276]</td>
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<td></td>
<td>Decreased invasiveness of clearCa28 renal carcinoma [310]</td>
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<td></td>
<td>Increased adhesion/invasiveness of Caki-1 [275]</td>
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<tr>
<td></td>
<td>AdTIMP1 expression decreases invasion of PANC-1 [311]</td>
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<td></td>
<td>AdTIMP3 reduces invasion of rheumatoid arthritis synovial fibroblasts [167]</td>
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<td></td>
<td>Decreased invasiveness of fibromatosis cells [312]</td>
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<tr>
<td>TIMP2</td>
<td>Reduced invasion and attachment by SK-Mel5 and A2058 melanoma [222]</td>
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<td></td>
<td>Decreased invasiveness of smooth-muscle cells [177]</td>
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<td></td>
<td>Decreased invasiveness of clearCa28 renal carcinoma [310]</td>
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grouped as either in vitro or in vivo studies. Notably, the majority of these studies demonstrate an inhibitory effect of TIMPs on tumor cell invasion and metastasis, indicating that the MMP/TIMP axis plays an influential role in tumor progression. While the presence of TIMPs often associates with decreased metastatic dissemination in experimental models, their specific effects on individual steps of metastasis and the relevant molecular mechanisms are poorly identified. Below, we discuss how the TIMPs influence the successive stages of metastasis from tumor cell migration, to survival in the circulation, to invasion.

1. TIMPs in Tumor Cell Migration

The breakdown of both cell-cell and cell-ECM adhesion is a first step in tumor cell migration. TIMP1-underexpressing fibroblasts have compromised cell-cell and cell-ECM contact, which can be rescued by treatment with synthetic metalloproteinase inhibitors. Conversely, TIMP1 overexpression results in increased adhesion of Caki cells to laminin, and TIMP1 treatment enhanced cell-cell adhesion and decreased migration of HepG2 hepatoma cells. Underexpression of TIMP2 or increase in the MMP-2/TIMP2 ratio also results in decreased cell-ECM attachment and an increase in tumor cell motility. Additionally, TIMP2 inhibits cell-cell aggregation mediated by MMP-7 in vitro, both TIMP1 and TIMP2 inhibit the pro-migratory activity arising from MMP-9-mediated activation of αvβ3 integrin. Recent evidence also suggests that, at least during myogenesis, TIMP2 regulates the levels of β1 integrin expression and that, through upregulation of RAP1, TIMP2 enhances hMVEC spreading and hinders cell migration. The loss of the cadherin-mediated adherens junction is an essential element of tumor cell invasion. Indeed, E-cadherin expression is often lost in epithelial tumors. In addition to changes in E-cadherin expression, proteolysis of the extracellular cadherin domain is considered an important mechanism in tumor cell invasion. MMP-3 and MMP-7 can cleave the E-cadherin

<table>
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<tr>
<th>Observation</th>
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<tr>
<td>AdTIMP2 expression decreases invasion of PANC-1</td>
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<tr>
<td>Inhibited migration and invasion of MCF10A</td>
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<tr>
<td>Reduced invasion by MDA-MB-435 and MDA-MB-231 breast carcinoma cells</td>
<td>[308]</td>
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<tr>
<td>Increased invasive potential by U87MG glioblastoma</td>
<td>[314]</td>
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<tr>
<td>TIMP3</td>
<td>No effect on invasiveness of JB6 tumor cells</td>
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<tr>
<td>Reduced invasion SK-Mel5 and A2058 melanoma</td>
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<tr>
<td>Decreased invasiveness of smooth-muscle cells</td>
<td>[177]</td>
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<tr>
<td>Decreased invasiveness of leiomyosarcoma cells</td>
<td>[186]</td>
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<tr>
<td>AdTIMP3 reduced invasion by Hela and HT1080</td>
<td>[223]</td>
</tr>
<tr>
<td>Shark TIMP3 reduced invasive potential of HT1080</td>
<td>[316]</td>
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<tr>
<td>AdTIMP3 reduced invasion of rheumatoid arthritis synovial fibroblasts</td>
<td>[167]</td>
</tr>
<tr>
<td>TIMP4</td>
<td>Reduced invasive potential of MDA-MB-435</td>
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ectodomain, generating a soluble 80-kDa fragment,\textsuperscript{136} while MT1-MMP activity results in the release of a 40-kDa E-cadherin fragment.\textsuperscript{284} In cultures of normal rat kidney (NRK) cells, recombinant TIMP2 and TIMP3 were shown to inhibit the cleavage of E-cadherin, whereas TIMP1 did not prevent this cleavage.\textsuperscript{285} Interestingly, TIMP3 also prevented the decrease of N-cadherin expression in these cells. Since TIMP3 is a robust inhibitor of several ADAMs, it is possible that members of the ADAM protease family play a role in E-cadherin cleavage.\textsuperscript{285} Thus, while TIMP inhibition of E-cadherin cleavage has the potential to restrain cell migration, to date no studies have directly tested this during tumor cell migration or invasion.

\subsection*{2. TIMPs May Affect Immune Response to Tumor}

Metalloprotease activity is increasingly being recognized as affecting fundamental steps of the immune system.\textsuperscript{68} Once the tumor cell has intravasated, its survival depends on successfully evading immune system responses.\textsuperscript{48} Within the circulation, intravasated tumor cells interact with platelets, enhancing not only their survival but also facilitating tumor cell attachment to the endothelial cells within a target organ.\textsuperscript{286} MMPs have been implicated in this process.\textsuperscript{287,288} Addition of recombinant TIMPs significantly reduces platelet recruitment and self-aggregation.\textsuperscript{289,290} Given these results, it is possible that TIMPs may inhibit the interaction of tumor cells with platelets, lowering their metastatic potential.

MMPs can affect additional aspects of the immune response against tumor cells. These include cleavage and activation of latent TGF-\(\beta\), which may promote its immunosuppressive activity;\textsuperscript{48,291} cell surface shedding of ICAM-1, which reduces NK cell cytotoxicity;\textsuperscript{292} MMP-2 cleavage of monocyte chemoattractant protein 3 (MCP-3), which attracts macrophages to the site of inflammation;\textsuperscript{293} and MMP-mediated cleavage of IL-2\(\alpha\), which downregulates T-cell proliferation.\textsuperscript{294} As these processes are dependent on MMP proteolytic activity, it is reasonable to assume that TIMPs will enhance the immune response against the tumor cell. Accordingly, the addition of recombinant TIMP2 to cultured fibroblasts decreases the MMP-mediated cleavage of MCP-3.\textsuperscript{293} Similarly, exogenous TIMP2 inhibits IL-2R\(\alpha\) cleavage and can thus enhance T-cell proliferation.\textsuperscript{294} Additionally, TIMP1 enhances activation of granulocytes and reduces their apoptosis.\textsuperscript{217}

Other studies, however, suggest that TIMPs are immuno-suppressors. TIMP1 and TIMP2 inhibit T-cell-mediated cytotoxicity against tumor-associated antigen-presenting cells \textit{in vitro}.\textsuperscript{212} In addition, TIMP2 induces apoptosis in activated T cells.\textsuperscript{221} Mohammed \textit{et al}.\textsuperscript{295} showed that timp3 ablation results in increased shedding of hepatic tnf, leading to significant liver inflammation in aged mice as characterized by lymphocytic infiltration and necrosis. TIMP3 was shown to be an innate negative regulator of inflammation.\textsuperscript{125,295} Whether these TIMP-mediated pro- or
anti-inflammatory effects play a significant role in the survival of intravasated tumor cells remains unaddressed.

3. TIMPs Inhibit Tumor Cell Invasion and Metastasis

Table 4 summarizes studies that utilized the modulation of TIMP gene expression to assess their effects on tumor cell invasion and metastasis. Intravasation and extravasation occur at the tumor-parenchymal interface and involve the stromal compartment. An important limiting step in metastasis is the ability of the tumor cell to degrade the ECM. MMP overexpression significantly enhances the invasive and metastatic potential of tumor cell lines in a number of in vitro and in vivo studies. Conversely, the reduction of MMP levels compromises the extent of invasion and metastasis of tumor cells. The lack of membrane-anchored MT1-MMP is especially restrictive to cell traffic through the ECM when tested in three-dimensional systems. In addition, mmp-2- and mmp-9-deficient mice display decreased levels of experimental metastasis.

With few exceptions, overexpression of individual TIMPs resulted in a significant decrease in invasion as well as metastasis. Transgenic mice that overexpressed timp1 in the brain showed resistance to metastatic colonization by the fibrosarcoma cell line FSL/10, and TIMP1 adenovirus injection into the liver inhibited metastasis by T-cell lymphoma (L-Cl.5s) and colorectal carcinoma (CT-26) cell lines. Consistent with this, antisense timp1 expression, which lowered hepatic timp1, resulted in increased liver metastasis by ESbL T-Lymphoma cells. Directed mutagenesis of timp1 residues involving its MMP-inhibitory activity also resulted in increased migration and invasion of B16F10 melanoma cells. Thus, TIMP1 acts as a blocker of metastasis against a number of tumor types when tested in experimental metastasis assays.

Unfortunately, a transgenic approach has not been utilized to test whether TIMP2, -3, or -4 overexpression exerts a similar resistance to metastasis. When TIMP2 was overexpressed in cultured tumor cells, it reduced their ability to invade in the transwell assay as well as their metastatic potential in vivo. Overexpression of TIMP3 decreased the invasion by melanoma cell lines SK-Mel-5 and A2058, and overexpression of TIMP4 hindered invasion and metastasis of breast cancer MDA-MB-435 cells. Thus far the effect of host timp ablation on metastasis has only been examined with respect to timp1 and timp3 (Table 4). When timp1−/− cell lines were tested for metastatic potential in timp1-deficient or wild type hosts, an inconsistent metastatic outcome was observed. However, timp1−/− embryonic stem (ES) cells showed enhanced invasion in vitro. It will be important to subject timp1−/− host to the classical tumor cell lines such as B16F10 melanoma and MDA series of breast cancer cells that have been extensively tested.

Editor’s note: Reference 296 to 316 are cited sequentially for the first time in this table, and subsequently in the text, if appropriate.
recently tested the *timp3*-deficient mice in metastasis assays and found that metastatic colonization of lung and bone by B16F10 melanoma and of kidney and liver by EL-4 lymphoma cell lines is significantly enhanced.\(^{121}\)

Contrary to the above literature, exogenous TIMP1 increased the migration of the melanoma cell line PM-WK.\(^{326}\) Similarly, high levels of TIMP1 expression in the stroma surrounding the tumor have also been associated with increased invasiveness,\(^{327}\) as well as metastatic potential and decreased survival in human cancers.\(^{328}\)

### VI. CONCLUSIONS

Overall, the literature reviewed here shows that TIMPs share conserved evolutionary structural characteristics and ability to inhibit MMPs. Despite these similarities, TIMPs often differ in the effect and the manner (MMP-dependent or MMP-independent) in which they impact specific processes during tumorigenesis. The arrows in Figure 1 indicate the stages at which MMPs and TIMPs have been implicated during cancer development and progression. It also summarizes the general trend associated with each of the TIMPs at specific stages. Since TIMPs have displayed paradoxical effects

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** MMPs and TIMPs Affect Multiple Stages of the process of cancer development and progression. Currently, there is no direct evidence to support the effect of TIMPs on genetic instability, intravasation, arrest in the secondary site and extravasation. While TIMPs generally inhibit angiogenesis and local invasion, their effects on tumor cell proliferation and apoptosis are more complex. TIMP1, -2, and -4 enhance or inhibit tumor growth, whereas TIMP3 generally inhibits tumor growth. + indicates a stimulatory role; – indicates an inhibitory role.
on cell proliferation and cell death, it is difficult to generalize their effect on tumor growth. The nature of this effect varies according to the cell type and models used. In the case of tumor cell invasion and metastasis, almost without exception, all studies document an inhibitory function for TIMPs. By the same token, angiogenesis is almost always inhibited by TIMP overexpression. An understanding of the molecular mechanisms by which TIMPs operate is limited and remains a challenge for this field. Given the broad impact of TIMP and MMP activity on tumorigenesis and metastasis, a deeper understanding of their roles will enhance our ability to develop better therapeutic approaches in cancer treatment.

Based on the initial findings that MMPs are frequently overexpressed in malignant tumors, a number of synthetic MMP inhibitors were developed as potential therapeutic agents against cancer. However, given the complexity of the pro- and anti-tumorigenic roles described for MMPs and TIMPs, it is not surprising that such a simplistic approach proved ineffective. Indeed, the majority of synthetic MMPIs used in clinical trials of late-stage malignancies showed only a borderline beneficial effect, and in some instances their activity was associated with a negative outcome for the patient. The more recent studies also document a lack of effect, for prinomastat in Non-Small Cell Lung Cancer (NSCLC) and for BMS-275291 (in combination with paclitaxel and carboplatin) in advanced NSCLC. Future approaches using MMPI in cancer therapy must build on a better understanding of the complex roles of MMPs in tumorigenesis.

This review highlights the complexity of TIMP effects on processes underlying cancer biology, and this emerging knowledge will help guide the development of the next generation of MMPIs. Phase II clinical trials using neovastat, which inhibits VEGF-signaling pathways to induce endothelial cell apoptosis along with inhibiting MMP activity, have shown promise, and phase III clinical trials of neovastat in combination with chemotherapy or radiation are currently underway for stage III NSCLC. Such compounds hold the potential to serve as cancer therapeutic and chemopreventative agents.

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The Role of Tissue Inhibitors of Metalloproteinase


W. Cruz-Munoz and R. Khokha


The Role of Tissue Inhibitors of Metalloproteinase


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