

REVIEW

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# Molecular mechanisms of tissue inhibitor of metalloproteinase 2 in the tumor microenvironment

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## Abstract

There has been a recent paradigm shift in the way we target cancer, drawing a greater focus on the role of the tumor microenvironment (TME) in cancer development, progression and metastasis. Within the TME, there is a crosstalk in signaling and communication between the malignant cells and the surrounding extracellular matrix. Matrix metalloproteinases (MMPs) are zinc-dependent endoproteases that have the ability to degrade the matrix surrounding a tumor and mediate tumor growth, angiogenesis and metastatic disease. Their endogenous inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs), primarily function to prevent degradation of the ECM via inhibition of MMPs. However, recent studies demonstrate that TIMP family members also possess MMP-independent functions. One TIMP member in particular, TIMP-2, has many distinct properties and functions, that occur independent of MMP inhibition, including the inhibition of tumor growth and reduction of angiogenesis through decreased endothelial cell proliferation and migration. The MMP-independent molecular mechanisms and signaling pathways elicited by TIMP-2 in the TME are described in this review.

**Keywords:** Tissue inhibitor of metalloproteinase-2 (TIMP-2), Matrix metalloproteinase (MMPs), Tumor microenvironment (TME), Angiogenesis

## Review

### Introduction

Historically, much of cancer research has focused on the proliferation of malignant cells and how oncogenes become activated while tumor suppressor genes become inactivated [1]. More recently, there has been a change in the ways we investigate tumors and neoplastic growth that has led to an expansion of research interests to include a broader focus, not only on the tumor cells alone, but also on how the tumor microenvironment (TME) affects cancer initiation and progression. The TME provides great promise for the identification of new therapeutic targets and novel cancer therapies that can only be fully exploited by understanding the molecular

mechanisms involved in TME regulation of tumorigenesis and the development of metastatic disease.

### MMPs and ECM degradation

The TME is composed of cellular and non-cellular components. The extracellular matrix (ECM) is the non-cellular, structural element of the TME that plays a central role in tumor cell communication. There are two types of ECM: a continuous layer of matrix that constitutes the basement membrane (BM) and the stroma or interstitial matrix. BM, a key structure of the ECM, is a subepithelial/endothelial layer of fibrous, cross-linked proteins (such as Type IV collagen), glycoproteins and associated proteoglycans. The BM provides not only physical support but also metabolic support to the cellular components (i.e. oxygenation and waste removal). The BM separates the epithelial compartment from the surrounding stromal tissue. The BM does not contain pores large enough to allow cellular transmigration. Thus, extensive proteolytic disruption and/or remodeling of the basement membrane

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remain the hallmark of the metastatic potential in malignant cancers. The ECM in the connective tissue (the interstitial matrix) consists of fibers (Type I collagen, reticular, and elastic fibers), ground substances (unstructured amorphous material that surrounds cells and fibers) primarily composed of glycosaminoglycans, glycoproteins and proteoglycans, as well as interstitial fluid. Within the interstitial matrix lay stromal cells including fibroblasts, pericytes and immune cells that constitute part of the cellular components of the TME. Important signaling events and communication between tumor cells and a variety of cellular components of the TME strongly influence tumor progression [2,3].

The ECM also contains secreted proteins, produced by the tumor cells and/or activated stromal cells, which elicit signaling pathways and promote tumor development [2,3]. Upon ECM breakdown, ECM-bound peptide growth factors, such as Insulin-like Growth Factor (IGF), vascular endothelial growth factor (VEGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) are released [4-6]. Collagens type IV and XVIII derived fragments in the ECM such as arrestin, canstatin, tumstatin, restin, and vastatin can either have pro- or anti-angiogenic functions [7]. It is, therefore, apparent that the ECM plays major, determining role on whether cancer will develop and progress or become dormant. With respect to tumor growth, the ECM provides a supporting role, and communicates with the tumor through signaling between growth factor, cytokine and cellular adhesion receptors, making the understanding of its influence on the tumor behavior not only complex but also of great importance.

The Matrix Metalloproteinase (MMP) family members are a group of metzincin metalloproteases, along with a disintegrin and metalloproteinases (ADAMs) and ADAM proteases with thrombospondin motifs (ADAMTSs), that function primarily extracellularly, and are critical for the maintenance and remodeling of tissues, largely by degrading the ECM. MMPs are zinc-dependent endopeptidases that cleave peptide bonds on the nonterminal ends of their numerous target proteins [8,9]. The MMPs are categorized based on whether they are secreted or remain bound to the cell membrane. Additionally, they can be organized by their structure and substrate specificity: collagenases, membrane type, stromelysins and matrilysins (Table 1). MMPs are produced and secreted without biological activity as zymogens. MMPs contain a cysteine-sulfhydryl group in their propeptide domain that associates with a bound zinc ion in the proteins catalytic domain. This association maintains the MMPs' zymogen form and keeps them inactive. The disruption of the interaction between the zinc ion and the cysteine residue, known as the 'cysteine switch', which is usually initiated by a serine protease, leads to the cleavage of a pro-peptide and the activation of the MMPs [10].

MMPs have long been the focus of investigation for potential use as disease biomarkers [11]. Levels of secreted and active forms of MMPs are usually increased during cancer progression, and have been associated with the development of tumor metastasis [2]. Thus, synthetic MMP inhibitors have been evaluated as therapeutics in clinical trials for cancer treatment. Surprisingly, they proved to be largely ineffective [12]. This outcome may be mainly due

**Table 1 Matrix metalloproteinases (MMPs) categories**

Secreted MMPs		Membrane bound MMPs		
		Type I transmembrane	GPI- anchored	Type II transmembrane
MMP-1	MMP-2	MMP-14	MMP-17	MMP-23A
MMP-3	MMP-7	MMP-15	MMP-25	MMP-23B
MMP-8	MMP-9	MMP-16		
MMP-10	MMP-11	MMP-24		
MMP-12	MMP-13			
MMP-19	MMP-20			
MMP-21	MMP-27			
MMP-28				
Structure and substrate specificity				
Collagenases	Gelatinases	Stromelysins	Matrilysins	Membrane-Type
MMP-1	MMP-2	MMP-3	MMP-7	MMP-14
MMP-8	MMP-9	MMP-10	MMP-26	MMP-15
MMP-13		MMP-11		MMP-17
MMP-18				MMP-24
				MMP-25

to the large number of MMP family members, where the benefits of inhibiting a few are compensated by the activity of other MMPs. Furthermore, recent studies have reported that MMPs can both promote and inhibit tumor growth and progression [13].

#### **TIMPs and their interaction with the TME**

Tissue inhibitors of matrix metalloproteinases (TIMPs) are a protein family that functions as natural MMP inhibitors, along with inhibiting the activity of ADAMs and ADAMTs families of metalloproteinases, albeit with distinct affinities [14]. Whereas three of the four TIMPs (TIMP-1, -3, and -4) are nested within the *synapsin* gene family [15], TIMP-2 contains a nested gene, *differential display clone 8* (DDC8), in its first intron [16]. There are some similarities among the TIMP family members. They each share 40% of their basic structure that their amino and carboxyl ends each contain six cysteine residues, which form 3 conserved disulfide bonds on both the N-terminal and C-terminal ends of the protein [17,18]. The various functions of TIMPs have been described [19-21]. Recent evidence has shown that TIMPs control important cellular processes including proliferation, apoptosis and angiogenesis by mechanisms independent of their MMP inhibitory activity [14].

As its name implies, TIMPs inhibit MMP activation by direct interaction. TIMP inhibition of MMP occurs primarily through their N-domains [22]. However, a unique feature of TIMP-2 is its ability to activate MMP-2. The activation of MMP-2 occurs via a distinctive mechanism involving MT1-MMP (MMP-14), a membrane bound MMP, and TIMP-2 [23]. The N-terminus of TIMP-2 binds and inhibits MT1-MMP, whereas the C-terminus can bind to secreted pro-MMP-2 (zymogen, inactive form). Upon binding of TIMP-2 to MT1-MMP, a neighboring MMP-14 that is not inhibited (free of TIMP-2) can cleave and activate a bound pro-MMP-2. It has also been shown that this activation is accelerated if the C-terminus of MMP-2 is bound to alpha<sub>v</sub>beta<sub>3</sub> ( $\alpha_v\beta_3$ ) integrin receptor on the cell surface that is proximally located to the MT1-MMP [24]. TIMPs (and MMPs) play an active role in tumor cell adhesion [25]. Members of the TIMP family have been shown to engage with the cell surface by binding to cellular receptors; TIMP-1 interacts with CD63, a member of the tetraspanin family that interact with cell adhesion molecules, such as  $\beta_1$  integrin. This interaction results in inhibition of caspase-mediated apoptosis in mammary epithelial cells. Moreover, TIMP-2 inhibits angiogenesis by interacting with alpha<sub>3</sub>beta<sub>1</sub> ( $\alpha_3\beta_1$ ) integrin on endothelial cells; this occurs in an MMP independent fashion. TIMP-2 has also been shown to interact, via the C-terminal loop 6 domain, with the insulin-like growth factor-I receptor (IGF-I-R). TIMP-3 also binds to VEGF receptor 2 (VEGFR2), acting as a Vascular Endothelial Growth Factor A (VEGF-A)

antagonist and blocking endothelial cell proliferation. In conclusion, TIMPs role in tumor cell growth involves a combination of mechanisms including inhibition of MMP-mediated ECM degradation and interactions with the cellular part of the TME [25].

#### **TIMP-2 MMP-independent functions**

There are many TIMP-2 functions that take place independent of its MMP inhibitory activity [26]. In general, MMP-independent TIMP functions, including those of TIMP-2, have only recently been established and there is contradictory evidence depending on the tumor or repair mechanisms under study. This only reinforces the need for more studies on the mechanisms of TIMP MMP-independent functions in order to understand the complexities of these MMP-independent activities. There is a collection of research studies focusing on the TIMP-2 effects either on tumor cells, and how they might influence the TME, or on endothelial cells and tumor endothelium. See Table 2 for a summary of the TIMP-2 MMP-independent functions.

#### **Effects on endothelial cell function and angiogenesis**

TIMP-2 can inhibit endothelial cell proliferation, independent of MMP inhibition, through binding to endothelial cell receptors, eliciting a series of signaling events [20]. We have shown that TIMP-2 inhibits endothelial cell growth mediated by angiogenic factors, including VEGF-A and Fibroblast Growth Factor 2 (FGF-2), by binding to the alpha<sub>3</sub>beta<sub>1</sub> integrin receptor ( $\alpha_3\beta_1$ ) on endothelial cells [27]. This leads to a decrease in the amount of protein tyrosine phosphatase (PTP), SHP-1, that associates with the  $\beta_1$  integrin subunit coupled with the increase in the dissociation of HSP60 from this same subunit. This result leads to a signaling cascade of events mediated by the inactivation of receptor tyrosine kinases via SHP-1. TIMP-2 is able to reduce this  $\alpha_3\beta_1$  activation by promoting the dissociation between the integrin and HSP60 [27]. TIMP-2 increases SHP-1 activity associated with FGFR1 (Fibroblast Growth Factor Receptor 1) and VEGFR2 (Vascular Endothelial Growth Factor Receptor 2), upon growth factor stimulation, leading to the belief that TIMP-2 induces a switch of SHP-1 binding from  $\alpha_3\beta_1$  to FGFR1 or VEGFR2, as well as an increase in SHP-1 activity. Additional studies showed that the TIMP-2 N-terminus is involved in the antiangiogenic effects and inhibited VEGF-A mediated endothelial cell growth stimulation. This N-terminus domain is solvent exposed, flexible and is unique to TIMP-2, compared to the other TIMP family members [27,28]. More recently, the N-terminus TIMP-2 derived 18-mer peptide, peptide 9, was found to inhibit human umbilical vein endothelial cell migration, proliferation, and tubular formation via inhibition of VEGF-A-stimulated cell proliferation, leading to the induction of

**Table 2 MMP-independent functions of TIMP-2**

Affected protein	Effect on function	Mechanism	Reference
VEGF-A	Reduced human microvascular endothelial cell (hMVECs) growth. Reduced human umbilical vein endothelial cell migration, proliferation, and tubular formation	TIMP-2 N-terminus binds to $\alpha_3\beta_1$ integrin receptor. TIMP-2 peptide 9 inhibits VEGF-A, resulting in increased cAMP/PKA levels and the induction of p27 <sup>Kip1</sup>	[27-29]
VEGFR2 and FGFR1	Decreased endothelial cell proliferation and angiogenesis	TIMP-2 binds to the $\alpha_3\beta_1$ integrin receptor; SHP-1 inactivates/dephosphorylates VEGFR2 and FGFR1	[20,27,30]
VEGF-A	Decreased vascular permeability	Inhibition of VEGF-A leads to increased cAMP levels. Increasing VE-cadherin association with the actin cytoskeleton, increasing cell-cell contacts	[31]
Decreased ERK and AKT phosphorylation	Inhibition of endothelial cell proliferation and angiogenesis	TIMP-2 binding and inhibition of IGF-R1 via Loop 6, C-terminus	[32]
RECK and p27 <sup>Kip1</sup>	Suppression of endothelial cell migration	Transcriptional regulation	[33,34]
MDSCs	Reduction of angiogenesis and A549 tumor growth	TIMP-2 inhibition of the recruitment of MDSCs	[35]
EGFR	Decreased EGFR activation and growth suppression on A549 cells	Binding of TIMP-2 to increase cytosolic cAMP levels, preventing SHP-1 from dissociating EGFR, leading to hypophosphorylation and inactivation of EGFR	[36]
FAK and AKT, Decreased FAK and AKT phosphorylation	A549 cell migration and invasion. In A549 xenograft tissues by immunohistochemistry	Not determined	[37]
EFEMP1, Fibronectin, E-cadherin, IGF-R1, SEMA-3A, ANGPT1	A549 lung cancer xenografts Inhibition of tumor growth <i>in vivo</i>	Transcriptional regulation	[38]
ABCB1, ABCG2, AKR1C1	Decreased expression in side population in A549 cells, increased chemosensitivity	Transcriptional regulation	[39,30]

p27<sup>Kip1</sup> from increased cAMP/protein kinase A (PKA) levels [29]. In another study, it was also shown that, in addition to its antiangiogenic effects, TIMP-2 mediated inhibition of vascular permeability via a  $\alpha_3\beta_1$ -SHP-1-cAMP/PKA dependent mechanism involves vascular E-cadherin (VE-cadherin) [29,31]. More specifically, TIMP-2 treatment increased VE-cadherin distribution in cell-cell contacts through increased association with the actin cytoskeleton.

TIMP-2 was also confirmed to inhibit endothelial cell proliferation and angiogenesis through its C-terminus, Loop 6 domain [32]. TIMP-2 Loop 6 inhibits downstream signaling of Insulin-like Growth Factor Receptor 1 (IGF-R1), specifically AKT and Erk phosphorylation, yet does not alter the MT1-MMP expression levels [32]. It was also shown that Loop 6 binds to IGF-R1 and initiates the downstream inhibitory signaling in endothelial cells.

Furthermore, TIMP-2 suppresses endothelial cell migration by inducing the reversion-inducing-cysteine-rich protein with Kazal motif (RECK) expression, an MMP inhibitor, which acts as an endothelial cell suppressor of angiogenesis [33]. TIMP-2 treatment is also able to inhibit endothelial cell growth, by mediating the G1 growth arrest through the activation of the cyclin-dependent kinase

inhibitor p27<sup>Kip1</sup> synthesis, resulting in angiogenesis inhibition [34].

Tumor-bearing TIMP-2 deficient (-/-) mice were found to have a significantly increased number of inflammatory cells in their tumors, as well as increased myeloid-derived suppressor cells (MDSC) coupled with increased MMPs activation and VEGFR1 expression [35]. This same study found that forced TIMP-2 expression in A549 cells significantly reduced recruited MDSCs, which are believed to induce angiogenesis and cancer immunosuppression, in tumors and suppressed angiogenesis and tumor growth. Collectively, the above studies have provided extended mechanistic insights on how TIMP-2 regulates endothelial proliferation and angiogenesis within the TME.

#### **Effects on the TME mediated through changes on tumor cell function**

For over 20 years now, numerous studies have shown that ectopic expression of TIMP-2 in tumor xenografts or syngeneic models inhibits tumor growth and angiogenesis [27,40,41]. There is also growing evidence from clinical-pathological analyses to support that loss of TIMP-2 expression occurs in many types of human cancer, in particular at the late stages of tumor progression [42-44]. However, there are contradicting findings from

clinical studies addressing the association of TIMP-2 expression in the development and progression of several cancers [45,46]. TIMP-2 not only inhibits but also activates MMP-2. Therefore, changes in TIMP-2 and MMP-2 levels (or MMP/TIMP-2 ratio) and activities would also determine whether TIMP-2 role is tumor promoting or inhibitory in a particular type of cancer [47,48]. More clinical research and mechanistic studies are needed to address this paradox.

It is, however, well documented how TIMP-2 inhibits tumor growth and progression. In a similar mechanism to endothelial cells, TIMP-2 was shown to prevent the activation tyrosine kinase-type receptors (TKR) on human A549 (lung carcinoma), MCF7 (mammary adenocarcinoma), HT1080 fibrosarcoma and Hs68 dermal fibroblast cells [49]. MMPs cleave TGF- $\alpha$  (Transforming Growth Factor alpha), a membrane bound protein that serves as an activating ligand for EGFR (Epidermal Growth Factor Receptor). EGFR is overexpressed in many human cancers and its activation leads to increased cell growth and proliferation through numerous signaling pathways [36]. It was shown that TIMP-2 binding to the cell surface results in increased intracellular cAMP levels. This prevents SHP-1 from dissociating from the EGFR, leading to reduced EGFR phosphorylation, resulting in decreased EGFR activation and growth suppression.

The Focal Adhesion Kinase (FAK) is a signaling protein involved in cellular adhesion and is associated with cellular migration [50]. Elevated FAK phosphorylation correlates with tumor invasiveness and ability to metastasize, where increased FAK activation usually leads to increased cellular migration and invasion. Furthermore, AKT is a key protein kinase involved in important signaling pathways leading to increased cellular proliferation and survival in many cancers [51]. However, as we have previously shown, both FAK and AKT activation (phosphorylation) is inhibited by TIMP-2 through mechanisms other than MMP-2 inhibition [37].

In a recent study, we performed gene expression microarray analysis in lung cancer A549 cells overexpressing TIMP-2 or the mutant Ala + TIMP-2 (devoid of MMP inhibitory activity), to address TIMP-2 mediated transcriptional regulation [38]. The use of Ingenuity Pathway Analysis (IPA), a functional analysis program, enabled us to identify novel biological mechanisms and functions associated with TIMP-2 overexpression in A549 cells. EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) is a secreted protein that was determined to inhibit cancerous glioma growth through the reduction of EGFR levels and intracellular AKT phosphorylation [52]. In our system, *EFEMP1* expression was up-regulated in TIMP-2 overexpressing cells, rendering a 6.507 decrease fold-change in cancer development and metastasis-associated functions [38]. In the same study, fibronectin-1,

another secreted ECM protein involved in tumor cell growth promotion, was also down regulated upon TIMP-2 forced expression. Finally, forced TIMP-2 expression in A549 cells led to increased *SEMA3A* (Semaphorin-3A) and decreased *ANGPT1* (Angiopoietin 1) transcriptional levels, acting to inhibit angiogenesis pathways [38]. These data suggest that TIMP-2 may regulate the TME by affecting transcription, expression and, therefore, protein secretion important in tumor development and progression.

E-cadherin, a protein involved in cell-cell communication and adhesion, is expressed in all epithelial tissues. However, in many solid malignancies, the expression of E-cadherin is lost, which leads to tumor invasiveness and increased metastatic potential [53]. Furthermore, E-cadherin loss often leads to an epithelial to mesenchymal transition (EMT) phenotype [53]. We have previously demonstrated that TIMP-2 overexpression in lung A549 tumor cells results in up-regulated E-cadherin transcription and protein levels [38]. Additionally, beta-catenin ( $\beta$ -catenin), a protein that directly binds to the C-terminus of the E-cadherin mediating cellular adhesion, was also found to be up-regulated with overexpression of TIMP-2 [38]. EGF has been shown to induce a cell morphology change, EMT, in A549 cells, by internalizing E-cadherin, and down regulating its transcription [54]. However, with TIMP-2 overexpression, both E-cadherin and  $\beta$ -catenin remained strongly localized to the plasma membrane after cell treatment with EGF. These same observations were confirmed upon exogenous treatment with recombinant Ala + TIMP-2 mutant [38]. Taken together, these studies suggest that TIMP-2 promotes an anti-tumor transcriptional profile in cancer cells.

Additionally, the A549 TIMP-2 transcriptome analysis revealed another novel TIMP-2 function [38]. We uncovered that both TIMP-2 and the mutant Ala + TIMP-2 affected the A549 side population (SP) phenotype and functional properties. More specifically, TIMP-2 was determined to decrease the SP in A549 lung cancer cells through decreased transcription of ABCB1 (ATP-binding cassette sub-family G member 1), ABCG2 (ATP-binding cassette sub-family G member 2), and AKR1C1 (Aldo-keto reductase family 1 member C1) [39]. As a result, TIMP-2 overexpression increased A549 tumor cell chemosensitivity to cytotoxic drugs (including doxorubicin and topotecan), suggesting the importance of utilizing TIMP-2 as an inhibitor of tumor growth in combination with well-known chemotherapeutics.

## Conclusions

TIMP-2 provides a novel therapy for cancer treatment. A further understanding of the underlying molecular mechanisms and signaling pathways involving TIMP-2 and the regulation of tumor growth and progression will allow for its full exploitation as a candidate for cancer therapy. As

more and more research is being conducted on TIMP-2 molecular mechanisms, its growth inhibitory effects on the tumor cells, and regulation of the tumor microenvironment, this endogenous antitumor and angiogenesis inhibitor may prove to be a powerful new remedy in the ongoing battle against cancer.

#### Abbreviations

TME: Tumor microenvironment; ECM: Extracellular matrix; BM: Basement membrane; IGF: Insulin-like growth factor; VEGF: Vascular endothelial growth factor; TGF- $\beta$ : Transforming growth factor- $\beta$ ; MMP: Matrix metalloproteinase; ADAM: A disintegrin and metalloproteinases; ADAMT: ADAM proteases with thrombospondin motifs; TIMP: Tissue inhibitor of metalloproteinase; VEGFR2: VEGF receptor 2; VEGF-A: Vascular endothelial growth factor A; FGF-2: Fibroblast growth factor 2;  $\alpha_3\beta_1$ : Alpha 3 beta 1 integrin receptor;  $\alpha_v\beta_3$ : Alpha v beta 3 integrin receptor; PTP: Protein tyrosine phosphatase; IGF-R1: Insulin-like growth factor receptor 1; FGFR1: Fibroblast growth factor receptor 1; VEGFR2: Vascular endothelial growth factor receptor 2; PKA: Protein Kinase A; VE-cadherin: Vascular Endothelial cadherin; RECK: Reversion-inducing-cysteine-rich protein with Kazal motif; MDSC: Myeloid-derived suppressor cells; TKR: Tyrosine kinase-type receptors; TGF- $\alpha$ : Transforming growth factor alpha; EGFR: Epidermal growth factor receptor; FAK: Focal adhesion kinase; IPA: Ingenuity pathway analysis; EFEMP1: EGF-containing fibulin-like extracellular matrix protein 1; SEMA3A: Semaphorin-3A; ANGPT1: Angiopoietin 1; EMT: Epithelial to mesenchymal transition; SP: Side Population; ABCB1: ATP-binding cassette sub-family G member 1; ABCG2: ATP-binding cassette sub-family G member 2; AKR1C1: Aldo-keto reductase family 1 member C1; hMVECs: Human microvascular endothelial cells.

#### Competing interest

The authors declare that they have no competing financial and non-financial interests to declare.

#### Authors' contributions

TR, GB, SJT, WGSS and DB have been involved in drafting the manuscript and revising it. WGSS and DB have also given final approval of the version to be published. All authors read and approved the final manuscript.

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