ARTICLE

Analysis of the effects of Tissue Inhibitor of Metalloproteinases-1, -2 and -3 N- and C-terminal domains on signaling markers during *X. laevis* development

M. A. Nieuwesteeg, J. A. Willson, M. Cepeda, M. A. Fox, and *S. Damjanovski

All Res. J. Biol., **2014**, 5, 30-36

All your results are good results!

THE ALL RESULTS JOURNALS

Issue 4, Vol 5, 2014, 30-36

Analysis of the effects of Tissue Inhibitor of Metalloproteinases-1, -2 and -3 N- and C-terminal domains on signaling markers during *X. laevis* development

M. A. Nieuwesteeg, J. A. Willson, M. Cepeda, M. A. Fox, and *S. Damjanovski

*Corresponding Author: Dr. Sashko Damjanovski, sdamjano@uwo.ca
Department of Biology, University of Western Ontario, 1151 Richmond Street, London Ontario, Canada, N6A5B7

Abstract: Extracellular matrix (ECM) remodeling is accomplished largely by matrix metalloproteinases (MMPs), which cleave individual components of the ECM to affect many cellular functions. Tissue inhibitors of metalloproteinases (TIMPs) are secreted MMP inhibitors, which, along with the cell surface MMP inhibitor reversion-inducing cysteine-rich protein with Kazal motifs (RECK), bind to MMPs and inhibit their activity. Although TIMPs were originally characterized based on their MMP-inhibitory activities, TIMPs are now known to be multifunctional proteins, with structurally and functionally distinct N- and C-terminal domains. TIMP N-terminal domains bind to and inhibit MMPs, while their C-terminal domains have demonstrated *in vitro* cell signaling activity in apoptosis, cell proliferation, and cell migration pathways. This study utilized TIMP N- and C-terminal domain constructs to examine individual domain functions related to cell proliferation, apoptosis and RECK expression in *Xenopus laevis* embryos. Western blot analysis revealed that none of the TIMP constructs altered cell proliferation as measured by phospho-histone-3 levels. Conversely, TIMP-1 full-length and C-terminal domain constructs both elevated both RECK and apoptosis levels, the latter as measured by caspase-3. Conversely TIMP-2 C-terminal and TIMP-3 N-terminal domain constructs both decreased RECK levels. **Keywords**: TIMP, *Xenopus laevis*, RECK, apoptosis

Introduction

Extracellular matrix (ECM) remodeling is accomplished largely by matrix metalloproteinases (MMPs), which cleave individual components of the ECM to facilitate cell migration. Although tissue inhibitors of metalloproteinase (TIMP) family members were originally identified and characterized based on their abilities to bind to and inhibit MMPs, it is now acknowledged that TIMPs are multifunctional proteins, which can have pleiotropic effects on ECM remodeling and cell behavior[1]. *In vivo*, TIMP knockout mouse models display developmental deficiencies, demonstrating the importance of TIMPs in regulating normal development. In particular, TIMP-1 and -2 knockouts have been associated with defects in brain and neural development. *TIMP-1* null mice had diminished neuronal development and impaired learning and memory[2], while *TIMP-2* null mice showed decreased neurite outgrowth, delayed neuronal differentiation and considerable motor dysfunction[3]. In contrast, *TIMP-3* null mice predominantly showed increased apoptosis in cells of the mammary glands, and defective alveolar and lung development[4,5], both of which have been attributed to enhanced MMP activity[6]. In *X. laevis*, ectopic expression of TIMP-3 has been associated with perturbation of head and neural structures[7], a finding that we have corroborated using full-length and N-terminal domain TIMP-3 constructs (but not C-terminal TIMP-3), also indicating that the role of TIMP-3 in development is dependent on its MMP-inhibitory activities[8].

While the TIMP N-terminal domains have been shown to impede MMP activity in many cell types and model organisms, their C-terminal domains have demonstrated cell signaling activity in specific cell types *in vitro*. Characterization of TIMP C-terminal domain function has been difficult as their roles in cell signaling vary, not only between the different TIMPs, but also depending on the cell line under study[1]. The seemingly contradictory roles of TIMPs in up-regulating or down-regulating apoptosis and cell proliferation pathways indicate that TIMPs may have cell or tissue-specific roles, where TIMP function may depend on both the availability of cell surface receptors and the stoichiometry of free TIMPs and MMPs within the ECM.

TIMP-1 inhibits apoptosis in Burkitt's lymphoma cell lines, and this inhibition was demonstrated to occur through the TIMP-1 C-terminal domain binding to the CD63-β1-integrin

complex in MCF10A cells[10]. TIMP-1 has also been shown to have both cell growth-promoting and cell growth-inhibiting activities in various cell lines, which are independent from the N-terminal domain-mediated inhibition of MMPs[11,12].

Likewise, there are several conflicting reports in the literature regarding the role of TIMP-2 in mediating cell growth and apoptosis pathways. In the MCF-7 breast cancer cell line TIMP-2 demonstrated cell growth promoting activity, which was mediated by its C-terminal domain interacting with MT1-MMP on the cell surface to activate the MAPK pathway[13]. In contrast, in cultured human endothelial cells TIMP-2 inhibited cell growth by binding to α3β1 integrin receptors, leading to upregulation of the cyclin dependent kinase inhibitor p27kip1[14]. In addition to both promoting and inhibiting cell growth, TIMP-2 has been shown to both suppress apoptosis (in melanoma cells), and promote apoptosis (in human T-lymphocytes)[15,16].

In contrast to TIMP-1 and -2, which are peri-cellular, TIMP-3 is sequestered in the ECM away from the cell surface and consequently may have limited direct ability to participate in cell surface signaling events[1]. However, TIMP-3 is a good inhibitor of ADAM family proteases, particularly ADAM-17 (also known as TACE [TNF-α converting enzyme]). ADAM-17 facilitates the shedding of transforming growth factor-α (TNF-α) as well as various cell surface receptors including FAS and TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1)[17]. Thus, some TIMP-3 effects are thought to occur indirectly as a result of ADAM inhibition, rather than as a result of direct signaling activity[1].

In addition to linking TIMPs with both apoptotic and cell growth pathways, *in vitro* studies have also connected TIMP functions to that of RECK (a cell surface MMP inhibitor)[18]. TIMP-1 overexpression can down regulate RECK in both *Xenopus* embryos and mouse cells[19]. TIMP-2 has been shown to inhibit endothelial cell migration by elevating RECK levels[20], while in melanomas TIMP-2 down regulated RECK[21]. Furthermore, mouse TIMP-3 and RECK share a common regulatory mechanism involving microRNA (miRNA-712)[22]. Taken together TIMPs 1, 2 and 3 can have differing roles in cell proliferation, apoptosis and regulation of RECK.

We have previously demonstrated that ectopically injected full length and individual N- or C-terminal domains of TIMP-1, -2 and -3 had varying effects on *Xenopus* embryo morphology and survival[8,23]. In general, injection of individual N- or C-terminal domains had a more deleterious effect than injection of the full length protein, with TIMP-1 C, TIMP-2 C and N, and TIMP-3 N having the most damaging effects. While all TIMP N-terminal domains bind to and inhibit MMPs, different TIMPs have varying effects on apoptosis[16]. Although specific C-terminal interactions have been associated with cell proliferation[10,13] and cell migration[20], the purpose of this investigation was to examine whether TIMP C-terminal domains have unique abilities to alter these signaling pathways *in vivo* in frog embryos. Specifically, changes in apoptosis were assayed using an active caspase-3 antibody, proliferation via a phosho-histone-3 (PH3) antibody, and RECK protein levels using a RECK antibody. We show that no TIMP construct altered phospho-histone-3 levels. Only TIMP-1 could regulate caspase-3 levels, while different TIMP-1, TIMP-2 and TIMP-3 domains could regulate RECK levels.

Results

The presence of equal levels of ectopic proteins for all constructs has been previously confirmed in embryonic protein extracts using an HA-antibody[8,23] . As a control for RNA injection artifacts, injection of equivalent levels of GFP mRNA and production of high levels of GFP protein did not have any developmental effects, while the phenotypic consequences of TIMP-1 and -2 and -3 mRNA overexpression has been previously reported[8,23].

PH3 levels were not altered by the overexpression of any TIMP construct in X. laevis embryo protein lysates

In order to characterize the roles of the TIMP-1, -2 and -3 N- and C-terminal domains in regulating cell proliferation levels, phospho-histone-3 was used as an indicator. Western blot analysis of stage 30 whole embryo protein lysates showed that the overexpression of full-length, N-terminal or C-terminal domain TIMP constructs did not significantly alter PH3 levels when compared to whole embryo lysates from control embryos (TIMP-1, Fig 1A; TIMP-2, Fig 1B; TIMP-3, Fig 1C). This suggests that TIMPs do not significantly impact cell proliferation in early *X. laevis* embryos.

Only overexpression of full-length and C-terminal TIMP-1 constructs increased active caspase-3 levels in X. laevis embryo protein lysates.

Active caspase-3 was used as an indicator of apoptosis to measure differences in the abilities of TIMP constructs to alter this pathway during development. Western blot analysis from stage 30 whole embryo protein lysates demonstrated increased caspase-3 levels only following overexpression of full-length (T1FL) and C-terminal (T1C) TIMP-1 constructs (asterisks Fig 2A, $p < 0.05$). The N-terminal TIMP-1 construct (T1N) did not significantly alter caspase-3 (Fig 2A). Similarly, Western blot analysis demonstrated that no TIMP-2 or TIMP-3 construct significantly altered caspase-3 levels relative to control embryos (Figs 2B, 2C).

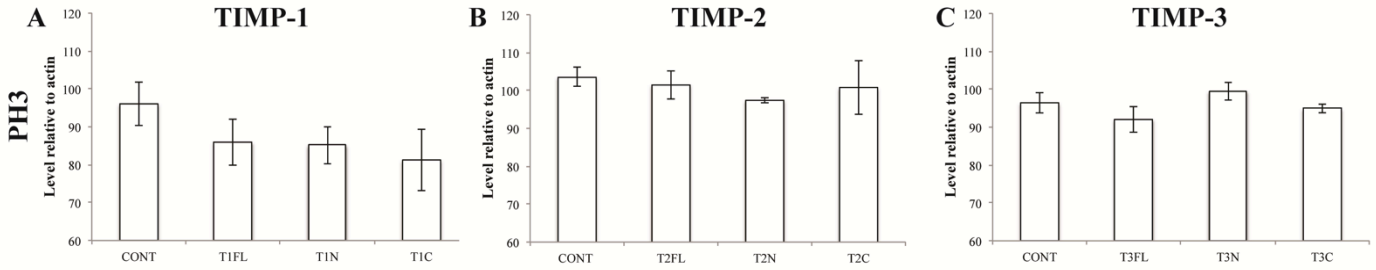

Figure 1. Injection of TIMP constructs did not alter PH3 levels in *X. laevis* embryo protein extracts. Embryos were injected at the 1 cell stage with 4 ng of each TIMP full-length, N-terminal domain, or C-terminal domain mRNA construct. Western blot analysis was performed to measure changes in phospho-histone-3 (PH3) levels from stage 30 whole embryo protein lysates. PH3 levels were measured relative to β-actin. Results are displayed as mean ± SE using protein isolated from 10 injected embryos from 3 independent experiments. No significant changes in PH3 levels relative to control uninjected (CONT) embryos were observed following overexpression of any TIMP construct, as analyzed by one-way ANOVA and Dunnett's multiple comparisons test ($p > 0.05$). Constructs are; full length, N-terminal domain and C-terminal domain of A; TIMP-1 (T1FL, T1N, T1C), B; TIMP-2 (T2FL, T2N, T2C) and C; TIMP-3 (T3FL, T3N, T3C), respectively.

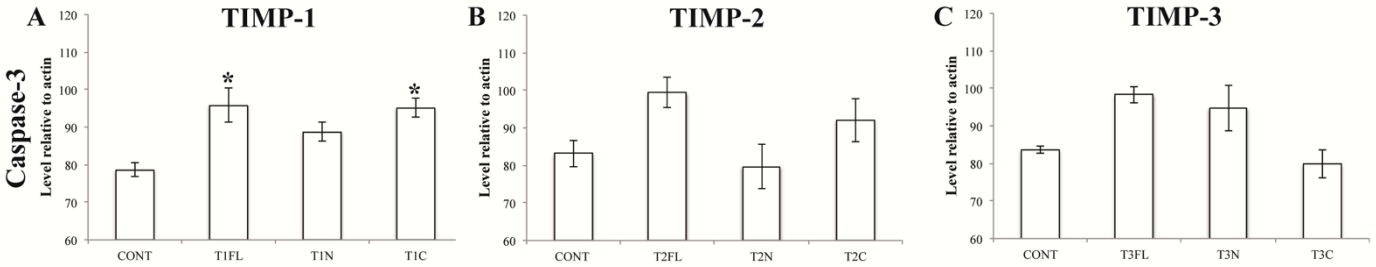

Figure 2. Only T1FL and T1C constructs increased active caspase-3 levels in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of each TIMP full-length, N-terminal domain, or C-terminal domain mRNA construct. Western blot analysis was performed to measure changes in caspase-3 levels from stage 30 whole embryo protein lysates. Caspase-3 levels were measured relative to β-actin. Results are displayed as mean ± SE using protein isolated from 10 injected embryos from 3 independent experiments. Only embryos injected with TIFL and T1C constructs significantly increased caspase-3 levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett's multiple comparisons test *($p < 0.05$). Constructs are; full length, N-terminal domain and C-terminal domain of A; TIMP-1 (T1FL,T1N, T1C), B; TIMP-2 (T2FL, T2N, T2C) and C; TIMP-3 (T3FL, T3N, T3C) respectively.

Overexpression of several TIMP constructs altered RECK protein levels in X. laevis embryo protein lysates.

Western blot analyses from stage 30 embryos showed that RECK expression significantly increased following overexpression of TIFL and T1C constructs (asterisks Fig 3A, $p < 0.05$). In contrast, while the full-length (T2FL) and N-terminal domain TIMP-2 (T2N) constructs did not significantly alter RECK levels, the C-terminal domain TIMP-2 (T2C) construct resulted in significantly decreased levels of RECK relative to control embryos (asterisk Fig 3B, $p < 0.05$). Interestingly, with TIMP-3, the full-length (T3FL) and C-terminal domain (T3C) constructs did not result in altered RECK levels compared to controls, whereas the N-terminal domain (T3N) construct significantly decreased RECK levels compared to controls (asterisks Fig 3B and C, $p < 0.05$).

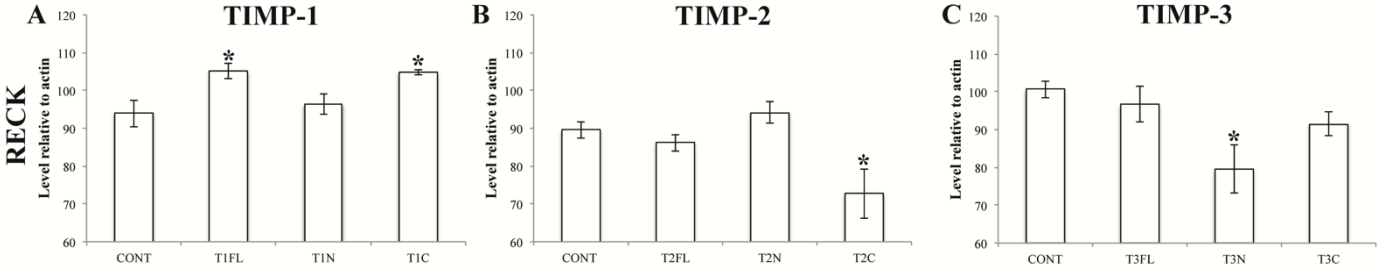

Figure 3. T1FL and T1C increased RECK, while T2C and T3N decreased RECK in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of each TIMP full-length, N-terminal domain, or C-terminal domain mRNA construct. Western blot analysis was performed to

measure changes in RECK levels from stage 30 whole embryo protein lysates. RECK levels were measured relative to β-actin. Results are displayed as mean ± SE using protein isolated from 10 injected embryos from 3 independent experiments. Embryos injected with TIFL and T1C, constructs significantly increased RECK levels relative to control uninjected (CONT) embryos, whereas embryos injected with T2C and T3N constructs decreased RECK levels relative to control (CONT) uninjected embryos. All data were analyzed by one-way ANOVA and Dunnett's multiple comparisons test *($p < 0.05$). Constructs are; full length, N-terminal domain and C-terminal domain of A; TIMP-1 (T1FL, T1N, T1C), B; TIMP-2 (T2FL, T2N, T2C) and C; TIMP-3 (T3FL, T3N, T3C), respectively.

Discussion

We have previously shown that overexpression of both TIMP-1 C- and TIMP-2 C-terminal domains (but not the TIMP-3 C-terminal domain) resulted in severe developmental defects. Furthermore, we demonstrated that the TIMP-2 C-terminal domain altered MMP levels independent of MMP inhibition[8]. In order to better understand the unique contributions the TIMP C-terminal domains may have on the regulation of signaling pathways during development, we overexpressed TIMP-1, -2 or -3 full-length, N-terminal and C-terminal domain constructs in *X. laevis* embryos, and examined changes in levels of PH3 as an indicator of cell proliferation and active caspase-3 as an indicator of apoptosis. Additionally, we examined changes in RECK levels, as the TIMP-2 C-terminal domain has previously been shown to increase RECK protein levels in cultured human endothelial cells[20].

Ectopic expression of TIMP constructs did not alter PH3 levels in X. laevis embryos

Overexpression of TIMP-1, -2 or -3 full-length, N-terminal or C-terminal domain constructs did not result in significantly altered cell proliferation in *X. laevis* embryos, as indicated by changes in the levels of PH3 (Fig 1A, B and C), suggesting that there may be no direct or indirect role for TIMPs in regulating this pathway during *X. laevis* development, during a time were very rapid early embryonic cleavage cycles are autonomously controlled. Interestingly, even the C-terminal domain constructs of TIMP-1 and -2 (which have been demonstrated in various cancer and fibroblast cell lines to either promote or inhibit cell proliferation as reviewed in Stetler-Stevenson 2008[1]), did not alter the rapid proliferation in injected relative to control embryos. As past *in vitro* proliferation studies with TIMPs were performed using homogenous populations of differentiated cells, this mechanism or pathway may not be maintained in the undifferentiated rapidly dividing cells of an embryo. Many pluripotent and multipotent cells in the embryo divide autonomous such that TIMPs may not have the ability to alter the proliferation of significant cell numbers in the whole embryo, at least as demonstrated by the PH3 assays.

The TIMP-1 C-terminal domain increased active caspase-3 levels in X. laevis embryos

To investigate whether any of the *X. laevis* TIMPs had the ability to alter cell death during development (particularly through their C-terminal domains), we examined changes in active caspase-3 as an indicator of apoptosis following overexpression of each TIMP construct. The T1FL and T1C constructs increased caspase-3 levels relative to control embryos (Fig 2A). As the C-terminal domain construct has no direct MMP-inhibitory activity, these data suggest that these subtle changes in apoptosis are mediated by another mechanism. While the TIMP-1 C-terminal domain has been linked to changes in apoptosis in both human lymphoma and mammary epithelia cell lines[10,24], these *in vitro* studies are associated with suppression of apoptosis, rather than an increase in apoptosis observed here. Although the importance of the TIMP-1 C-terminal domain in regulating caspase-3 levels seems to be maintained in *X. laevis*, the effects of this protein may be different during development. Interestingly, a recent study using hematopoietic cells in culture has reported that TIMP-1 can indeed lead to increased activation of caspase-3; however, this activation was associated with cell differentiation through activation of MEK1, MEK6, and p38α[25]. Nevertheless, the role of TIMP-1 in regulating the amount of active caspase-3 during *X. laevis* development seems to be specific to the TIMP-1 C-terminal domain, as both the T1C and T1FL constructs (which contain a functional C-terminus) increased apoptosis (but not the T1N construct). Furthermore, none of the TIMP-2 or -3 constructs resulted in significantly altered levels of caspase-3 relative to control embryos, demonstrating that this effect is unique to TIMP-1.

The TIMP-1 and -2 C-terminal domains had opposing effects on RECK protein levels in X. laevis embryos

RECK is a potent cell surface inhibitor of MMPs, thus, alterations in RECK expression may have considerable effects on cell migration during embryogenesis. As TIMP-2 has previously been demonstrated to increase RECK expression *in vitro*, here we investigated whether the *X. laevis* TIMPs or their individual domains may contribute to the regulation of RECK expression *in vivo*, during development. Intriguingly, the TIMP-1 full-length and C-terminal domain constructs resulted in significantly increased levels of RECK protein when quantified using Western blot analysis (Fig 3A). The T1N construct, which only functions in inhibition of MMP activity, did not result in changes in RECK levels relative to control embryos. Thus, these data suggest that the observed increases in RECK following overexpression of T1FL and T1C constructs may be due to a C-terminal domain specific mechanism. While no previous

reports have linked the individual TIMP-1 C-terminal domain to the regulation of RECK levels, RECK levels have been linked to the expression of TIMP-1[22] thus suggesting that they share a regulatory mechanism.

Following overexpression of TIMP-2 full-length, N-, and C-terminal domain constructs, we found that only the T2C construct significantly decreased RECK expression relative to controls (Fig 3B). This finding is in contrast to the report by Oh et al.[20], which showed that the TIMP-2 C-terminal domain increased RECK expression through binding to α3β1 integrin receptors on the surface of human microvascular endothelial cells. As the effect of TIMP-2 on the regulation of RECK expression has currently only been examined using cultured human endothelial cells, it is possible that TIMP-2 may have varying effects on RECK levels, or may alter RECK expression through different pathways, depending on the requirements of different tissues and organisms. Indeed, recently published work suggests that TIMP-2 levels drop as RECK levels decrease[21]. We demonstrated here that TIMP-2 has the ability to alter RECK through its C-terminal domain, independent of MMP inhibition, during *X. laevis* development.

Overexpression of full-length and C-terminal domain TIMP-3 did not alter RECK levels relative to control embryos, suggesting that the TIMP-3 C-terminal domain does not participate in signaling events that regulate RECK expression in *X. laevis* embryos. The T3N construct, however, did result in significantly decreased RECK levels relative to control embryos when analyzed by Western blot (Fig 3C). As the TIMP N-terminal domains are involved in MMP inhibition and have not been associated with direct signaling activity, it is unlikely that the T3N construct decreased RECK levels through a typically associated cell-surface signaling mechanism. Under normal conditions, full-length TIMP-3 is sequestered in the ECM away from the cell surface, a function that is mediated by its C-terminal domain[26]. Thus, whereas the T3FL and T3C constructs may remain bound away from the cell, this ability would be impaired with the T3N construct. The T3N construct is free to act pericellularly, and therefore may result in inappropriate feedback to the cell to downregulate expression of other MMP inhibitors, like RECK.

Conclusions

We have shown for the first time that the TIMP-1 and -2 C-terminal domains have the ability to influence caspase-3 and RECK levels in *X. laevis* embryos. This research indicates that TIMPs may be important not only for directly mediating MMP activity during development, but independent of their MMP inhibitory role, TIMPs may regulate cell signaling pathways that influence apoptosis and/or cell differentiation through caspase-3, and cell migration through RECK.

Materials and Methods

Animals

Adult *X. laevis* were purchased from Xenopus I Inc (Dexter, MI), fertilized and reared in accordance with standard protocols, and staged according to Nieuwkoop and Faber[27]. Animals were housed and treated in accordance with UWO and CCAC guidelines.

Generation of TIMP constructs for microinjection

Briefly, PCR was used to produce HA-tagged full-length, N-terminal and C-terminal domain constructs for TIMP-1, -2 and -3. Details regarding TIMP-2 and-3 construct generation can be found in [8]. TIMP-1 was cloned from total cDNA from stage 35 embryos based on unannotated *X. laevis* clone AAI41767.1. Forward and reverse primers (5'GACAGAAGGACTGCCCAGCC, 5'CAAAACACTTCTCCTTCGAG) were used, and the full-length *X. laevis* TIMP-1 sequence was confirmed at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario, and submitted as GenBank ID: 1628144. Using the full-length TIMP-1 clone as a template, PCR was used to generate full-length, N-terminal or C-terminal TIMP-1 (hemagglutinin) HA-tagged constructs as described previously[8]. This unobtrusive HA-tag allows the use of sensitive and specific HA antibodies to be used for the detection of ectopic protein production. Respective PCR forward and reverse primers were as follows:

T1FL 5'AGATCTATGTTGTACCTTGTGGTTGTG, 5'CAGTCTGCTGCCACAACACAATACCCATACGATGTTCCAGATTACGCTACTAGT;

T1N 5'AGATCTATGTTGTACCTTGTGGTTGTG, 5'GTGTATCGCAAAGCCTGTTCCTACCCATACGATGTTCCAGATTACGCTACTAGT

T1C signal sequence and reverse link 5'AGATCTATGTTGTACCTTGTGGTTGTG, 5'CTCAGCCAGGAGGTGTTGGGGTGCAACATCGTCCCCTGCTAT,

T1C-terminal link 5'TGCAACATCGTCCCCTGCTAT, 5'CAGTCTGCTGCCACAACACAATACCCATACGATGTTCCAGATTACGCTACTAGT.

Microinjection of TIMP-1, -2 and -3 mRNA constructs in X. laevis embryos

Fertilized *X. laevis* embryos were microinjected with TIMP-1, -2 or -3 full-length, N-terminal or C-terminal domain mRNA constructs. Embryos were injected at the one cell stage with 4 ng of mRNA in a volume of 2.3 nl, using 10 μm diameter glass. Embryos were maintained in 1X (Marc's modified ringers) MMR containing 4% Ficoll for 5 hours

following injection, then transferred to 0.1 X MMR solution for rearing.

Protein preparations and Western blotting

Protein was extracted from stage 30 embryos injected with the various TIMP constructs, or uninjected control embryos. Protein extraction, quantification and Western blotting were performed from 10 pooled embryos from 3 independent experiments, as previously described[8]. Primary antibodies used were RECK (H-300) (rabbit polyclonal, 1:200 dilution; Santa Cruz), p-Histone H3 (ser-10) (rabbit polyclonal, 1:200 dilution; Santa Cruz), anti-active caspase-3 (rabbit polyclonal, 1:500 dilution; Abcam), and anti-β-actin (C4) (mouse monoclonal, 1:1000 dilution; Santa Cruz). Secondary antibodies used were goat anti-rabbit HRP (1:5000 dilution; Life Technologies) or goat anti-mouse HRP (1:5000 dilution; Bio-Rad). Western blots were visualized and photographed using Bio-Rad Quantity One 4.4.0 software. Densitometry was performed using ImageJ software, where levels of PH3, RECK or caspase-3 were standardized to β-actin, and plotted as mean ± SE based on 3 independent experiments.

Statistical Analysis

Western blot densitometry data were log transformed (Log10) to achieve a normal distribution. All statistical analysis was performed using the IBM SPSS Statistic 19 program. Results were presented as mean ± SE. Statistical significance was determined using One Way analyses of variance followed by Dunnett's multiple comparisons test. Differences were considered statistically significant when $p < 0.05$.

Acknowledgements

Supported by a Discovery Grant to S.D. from the Natural Sciences and Engineering Research Council of Canada.

References

1. Stetler-Stevenson, W.G., (2008) Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Science signaling 1, re6.
2. Chaillan, F.A., Rivera, S., Marchetti, E., Jourquin, J., Werb, Z., Soloway, P.D., Khrestchatisky, M., Roman, F,S, (2006) Involvement of tissue inhibition of metalloproteinases-1 in learning and memory in mice. Behavioural brain research 173, 191-198.
3. Jaworski, D.M., Soloway, P., Caterina, J., Falls, W.A., (2006) Tissue inhibitor of metalloproteinase-2 (TIMP-2)-deficient mice display motor deficits. Journal of neurobiology 66, 82-94.
4. Leco, K.J., Waterhouse, P., Sanchez, O.H., Gowing, K.L., Poole, A.R., Wakeham, A., Mak, T.W., Khokha, R., (2001) Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). J Clin Invest. 108, 817-829.
5. Fata, J.E., Leco, K.J., Voura, E.B., Yu, H.Y., Waterhouse, P., Murphy, G., Moorehead, R.A., Khokha, R. (2001) Accelerated apoptosis in the Timp-3-deficient mammary gland. J Clin Invest. 108, 831-841.
6. Brew, K., Nagase, H. (2010) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochimica et biophysica acta. 1803, 55-71.
7. Pickard, B., Damjanovski, S. (2004) Overexpression of the tissue inhibitor of metalloproteinase-3 during *Xenopus* embryogenesis affects head and axial tissue formation. Cell Res. 14, 389-399.
8. Nieuwesteeg, M.A., Walsh, L.A., Fox, M.A., Damjanovski, S., (2012) Domain specific overexpression of TIMP-2 and TIMP-3 reveals MMP-independent functions of TIMPs during *Xenopus laevis* development. Biochemistry and cell biology 90, 585-595.
9. Guedez, L., Stetler-Stevenson, W.G., Wolf,f L., Wang, J., Fukushima, P., Mansoor, A., Stetler-Stevenson, M., (1998) *In vitro* suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. J Clin Invest. 102, 2002-2010.
10. Jung, K.K., Liu, X.W., Chirco, R., Fridman, R., Kim, H.R., (2006) Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. The EMBO journal 25, 3934-3942.
11. Hayakawa, T., Yamashita, K., Tanzawa, K., Uchijima, E., Iwata, K., (1992) Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. FEBS letters 298, 29-32.
12. Taube, M.E., Liu, X.W., Fridman, R., Kim, H.R., (2006) TIMP-1 regulation of cell cycle in human breast epithelial cells via stabilization of p27(KIP1) protein. Oncogene 25, 3041-3048.
13. D'Alessio, S., Ferrari, G., Cinnante, K., Scheerer, W., Galloway, A.C., Roses, D.F., Rozanov, D.V., Remacle, A.G., Oh, E.S., Shiryaev, S.A., et al. (2008) Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism. The Journal of biological chemistry 283, 87-99.
14. Seo, D.W., Li, H.M., Qu, C.K., Oh, J., Kim, Y.S., Diaz, T., Wei, B.Y., Han, J.W., Stetler-Stevenson, W.G., (2006) Shp-1 mediates the antiproliferative activity of tissue inhibitor of metalloproteinase-2 in human microvascular endothelial cells. Journal of Biological Chemistry 281, 3711-3721.
15. Lim, M.S., Guedez, L., Stetler-Stevenson, W.G., Stetler-Stevenson, M., (1999) Tissue inhibitor of metalloproteinase-2 induces apoptosis in human T lymphocytes. Annals of the New York Academy of Sciences 878, 522-523.
16. Valente, P., Fassina, G., Melchiori, A., Masiello, L., Cilli, M., Vacca, A., Onisto, M., Santi, L., Stetler-Stevenson, W.G., Albini, A., (1998) TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. Int J Cancer 75, 246-253.
17. Black, R.A., (2002) Tumor necrosis factor-alpha converting enzyme. The international journal of biochemistry and cell biology 34, 1-5.
18. Takahashi, C., Sheng, Z., Horan, T.P., Kitayama, H., Maki, M., Hitomi, K., Kitaura, Y., Takai, S., Sasahara, R.M., Horimoto, A., Ikawa, Y., Ratzkin, B.J., Arakawa, T., Noda, M., (1998) Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Natl Acad Sci USA 95, 13221–13226.
19. Walsh, L.A., Roy, D.M., Reyngold, M., Giri, D., Snyder, A., Turcan, S., Badwe, C.R., Lyman, J., Bromberg, J., King, T.A., Chan, T.A., (2014) RECK controls breast cancer metastasis by modulating a convergent, STAT3-dependent neoangiogenic switch. Oncogene 2014 Jun 16. doi: 10.1038/onc.2014.175.
20. Oh, J., Seo, D.W., Diaz, T., Wei, B., Ward, Y., Ray, J.M., Morioka, Y., Shi, S., Kitayama, H., Takahashi, C., et al. (2004) Tissue inhibitors of metalloproteinase 2 inhibits endothelial cell migration through increased expression of RECK. Cancer research 64, 9062-9069.
21. Jacomasso, T., Trombetta-Lima, M., Sogayar, M.C., Winnischofer, S.M., (2014) Downregulation of reversion-inducing cysteine-rich protein with Kazal motifs in malignant melanoma: inverse correlation with membrane-type 1-matrix metalloproteinase

and tissue inhibitor of metalloproteinase 2. Melanoma Res. 24(1), 32-9. doi: 10.1097/CMR.0000000000000039.
22. Kim, C.W., Kumar, S., Son, D.J., Jang, I-H., Griendling, K.K., Jo, H., (2014) Prevention of Abdominal Aortic Aneurysm by Anti–MicroRNA-712 or Anti–MicroRNA-205 in Angiotensin II–Infused Mice. Arteriosclerosis, Thrombosis, and Vascular Biology 2014 34, 1412-1421 doi: 10.1161/ATVBAHA.113.303134
23. Nieuwesteeg, M.A., Willson, J.A., Cepeda, M., Fox, M.A., Damjanovski, S., (2014) Functional characterization of tissue inhibitor of metalloproteinase-1 (TIMP-1) N- and C-terminal domains during *Xenopus laevis* development. ScientificWorldJournal 2014 Jan 30, 2014, 467907. doi: 10.1155/2014/467907. eCollection 2014.
24. Li, G., Fridman, R., Kim H.R., (1999) Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. Cancer research 59, 6267-6275.
25. Dasse, E., Bridoux, L., Baranek, T., Lambert, E., Salesse, S., Sowa, M.L., Martiny, L., Trentesaux, C., Petitfrere, E., (2007) Tissue inhibitor of metalloproteinase-1 promotes hematopoietic differentiation via caspase-3 upstream the MEKK1/MEK6/p38alpha pathway. Leukemia 21, 595-603.
26. Yu, W.H., Yu, S., Meng, Q., Brew, K., Woessner, J.F. Jr., (2000) TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. The Journal of biological chemistry 275, 31226-31232.
27. Nieuwkoop, P.D., Faber, J., (1956) Normal table of *Xenopus laevis*: A systematic chronological survey of the development of the fertilized egg until the end of metamorphosis. (North Holland Publication, Amsterdam).