Membrane Type-1 Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases-2 RNA Levels Mimic Each Other during *Xenopus laevis* Metamorphosis

Logan A. Walsh, Deanna A. Carere, Colin A. Cooper, Sashko Damjanovski*  
Department of Biology, University of Western Ontario, London, Ontario, Canada

Matrix metalloproteinases (MMPs) and their endogenous inhibitors TIMPs (tissue inhibitors of MMPs), are two protein families that work together to remodel the extracellular matrix (ECM). TIMPs serve not only to inhibit MMP activity, but also aid in the activation of MMPs that are secreted as inactivezymogens. *Xenopus laevis* metamorphosis is an ideal model for studying MMP and TIMP expression levels because all tissues are remodeled under the control of one molecule, thyroid hormone. Here, using RT-PCR analysis, we examine the metamorphic RNA levels of two membrane-type MMPs (MT1-MMP, MT3-MMP), two TIMPs (TIMP-2, TIMP-3) and a potent gelatinase (Gel-A) that can be activated by the combinatorial activity of a MT-MMP and a TIMP. In the metamorphic tail and intestine the RNA levels of TIMP-2 and MT1-MMP mirror each other, and closely resemble that of Gel-A as all three are elevated during periods of cell death and proliferation. Conversely, MT3-MMP and TIMP-3 do not have similar RNA level patterns nor do they mimic the RNA levels of the other genes examined. Intriguingly, TIMP-3, which has been shown to have anti-apoptotic activity, is found at low levels in tissues during periods of apoptosis.

**INTRODUCTION**

Amphibian metamorphosis is a late developmental event that has been used to examine numerous processes including; cell signaling, receptor function, gene regulation, morphogenesis, and effects of environmental toxins [1–4]. While intricate, the entire metamorphic process is controlled by one molecule, thyroid hormone (T3). During *Xenopus laevis* metamorphosis all tissues are altered in some way, where structures are either created *de novo* (such as the limbs), removed completely (such as the tail), or remodeled (skin, the head and gills, and internal organs such as the intestine amongst others). This T3 dependent process is exemplified in the intestine (Figure 1) where embryonic epithelial cell death and adult epithelial cell proliferation facilitate the metamorphoses of an herbivorous tadpole into an omnivorous frog [5]. Removal of the tail, on the other hand, is achieved largely through apoptotic events late in the metamorphic process (Figure 1). While the ECM remodeling in both of these organs is facilitated by a similar array of molecules [6–9], the different cellular responses (proliferation vs. death) to this remodeling allow for the investigation of the possible functions of the molecules that remodel the ECM.

Amongst the important molecules that are modulated by T3 during metamorphosis are matrix metalloproteinases (MMPs) [10]. Indeed, the first MMP identified, collagenase, was isolated from the resorbing frog tail [11]. MMPs are a family of 25 proteins that function to cleave and remodel the extracellular matrix (ECM) and other cell surface proteins. Accordingly, MMPs influence most cellular functions during development and in a number of pathologies [12–15].

MMPs are tightly regulated at transcriptional and posttranslational levels. Most MMPs are secreted as inactivezymogens (pro-MMPs) that must to be activated extracellularly, often by other already active MMPs. Interestingly membrane-type MMPs (MT-MMPs), a family of six molecules, are activated intracellularly in the Golgi by furin [16]. Active MT-MMPs have been shown to play an important role at the cell surface in not only cleaving ECM molecules, but also in activating other pro-MMPs, a process which counter-intuitively involves tissue inhibitor of metalloproteinases (TIMPs).

TIMPs are a family of four secreted molecules that together can inhibit the activity of all MMPs [17], but also work with MT-MMPs in distinct stoichiometric ratios to activate other pro-MMPs near the cell surface. For example, two MT1-MMP molecules act with one TIMP-2 molecule to activate a pro-Gelatinase-A (Gel-A) molecule [18]. Gel-A (also known as MMP-2) is a powerful enzyme that cleaves a broad range of ECM and cell surface substrates and whose mis-regulation has been associated with a number of developmental anomalies and pathological conditions [19].

We have previously described the expression patterns of *X. laevis* MT3-MMP and TIMP-3 during early developmental stages. MT3-MMP is localized in anterior and neural structures, while TIMP-3 is more ubiquitously expressed. Ectopic expression of either gene by injection of mRNA into fertilized embryos resulted in death [15,20,21]. In this study we investigated the RNA levels of three MMPs (MT1-MMP, MT3-MMP, Gel-A) and two TIMPs (TIMP-2 and TIMP-3) during *Xenopus* metamorphosis. Though the embryonic expression of some of these molecules had previously been examined, frog metamorphosis allowed us the opportunity to see in particular how MT-MMPs and TIMPs might be regulated together in a developmental process that is controlled by one molecule–T3.

**Academic Editor:** Sebastian Fugmann, National Institute on Aging, United States of America

**Received** August 24, 2007; **Accepted** September 16, 2007; **Published** October 3, 2007

**Copyright:** © 2007 Walsh et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This research was supported by an NSERC Discovery Grant to SD, and Ontario Graduate Scholarship funding to LW and CC. The research funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* To whom correspondence should be addressed. E-mail: sdamjano@uwo.ca
RESULTS AND DISCUSSION

Natural metamorphosis of the intestine

All five genes examined displayed increases in RNA levels as intestine metamorphosis commenced (Figure 2, stage 56 vs. 58). During intestine metamorphosis MT1-MMP, TIMP-2 and TIMP-3 RNA levels followed a general ‘M’ pattern, starting low, increasing, decreasing, increasing and then decreasing again as metamorphosis progressed (Figure 2). The first peak of RNA at about stage 58 corresponded to approximately when larval epithelial cells were beginning to undergo apoptosis (Figure 1).

Several other MMPs (stomelysin-3, collagenase-3, collagenase-4 and MT1-MMP) have already been described to be expressed during intestine metamorphosis [3,9], where MMP activation at this stage is involved in remodeling of the basement membrane resulting in the death of overlying cells [5]. Increased TIMP-2 RNA, in conjunction with MT1-MMP may also increase ECM remodeling by possibly activating Gel-A (whose RNA is also increased at this time). Indeed, when representative RT-PCR RNA levels of MT1-MMP and TIMP-2 are compared, they mirror each other in both the metamorphic intestine and tail (Figure 3).

The second peak of MMP and TIMP RNA levels in the intestine at about stage 63 correlates with the remodeling and cell proliferation associated with the morphogenesis of the multiple folds eventually seen in the post-metamorphic intestine (Figure 1). The continued high levels of RNA of MT3-MMP and Gel-A after this stage suggest that these two molecules, but not the other three genes examined here, play a role in later metamorphic remodeling events in the intestine.

Natural metamorphosis of the tail

The RNA levels of the five genes were distinct during tail metamorphosis versus the metamorphic intestine (compare Figure 2 and Figure 4). MT3-MMP RNA displayed one peak at stage 61, while TIMP-3 RNA levels dipped at stage 61. MT1-MMP, TIMP-2 and Gel-A RNA levels increased as metamorphosis progressed and were maintained until the end of metamorphosis. Metamorphosis of the tail is a late event and one of the last to occur as all tail tissues are remodeled and removed in an efficient and safe manner. MMPs (Stromelysin-3, as well as collagenase-3 and –4) have already been shown to be expressed in distinct tail domains [3] and here high levels of MT1-MMP, MT3-
MMP and Gel-A also support the role for these MMPs in tail resorption. The high levels of TIMP-2 supports two possible roles for this molecule. It can either work with MT1-MMP in activating other pro-MMPs, or conversely, if protein levels are high enough, TIMP-2 can inhibit and slow ECM remodeling to allow tail resorption to take place. TIMP-3 may then be playing an important role in modulating the speed of ECM degradation at late stages of metamorphosis (after stage 63), presumably a time when many MMPs are active and would otherwise degrade the ECM and induce apoptosis.

Precocious T3 induction of metamorphosis

In an effort to better understand the role of these five genes during metamorphosis, we examined if they were directly responsive to thyroid hormone induced metamorphosis. MMPs can contain thyroid hormone response elements in their DNA regulatory regions and can be transcribed directly by T3 and its receptor [22]. Genes that respond directly to T3 increase their transcription within hours. Our data suggest that while these genes are modulated during natural metamorphosis, we find that none of the genes we examined appear to be direct T3 responsive genes, as there was no increase in their RNA levels after 1 day of treatment (Figure 5). These studies also correlate well with other studies that observed an increase of transcription of four of these genes (MT1-MMP, MT3-MMP, TIMP-2 and Gel-A) by day three of T3 treatment [9,23]. This suggests that there are numerous intervening players that are regulating their expression after the initial induction of metamorphosis by T3.

While four of our genes were eventually upregulated in the tail by the addition of T3, TIMP-3 RNA levels remained low (Figure 5). As the tail is one of the last tissues to undergo metamorphosis, it responds to T3 days after other tissues do.
during natural metamorphosis. It is likely that 3 days of T3 treatment is comparable to only the very early stages of tail metamorphosis. TIMP-3 levels do rise late in natural metamorphosis only after stage 61 (Figure 2 and Figure 4). However, this often takes many days after natural metamorphosis has started, and to expose tadpoles to high levels of exogenous T3 for long periods of time to induce complete tail regression is impractical, as it is most often lethal. Parenthetically, TIMP-3 levels may remain low during early stages as TIMP-3 has been shown to have anti-apoptotic properties [24], characteristics that would be detrimental to the regression of the tail.

Conclusions
The RNA levels of MT1-MMP and TIMP-2 mirror each other during natural and induced metamorphosis of both the intestine and the tail. Their RNA patterns are closely matched by Gel-A, except during late intestine metamorphosis where Gel-A levels stay elevated. It is known that MT1-MMP and TIMP-2 can work together to activate Gel-A and our RNA data support this activation scenario. By comparison, MT3-MMP and TIMP-3 do not share similar RNA patterns during intestine metamorphosis, tail metamorphosis, nor in response to T3. This suggests that MMP and TIMP expression patterns during development should be considered collectively as certain pairings of these molecules appear to play significant roles together. While there appears to be a clear elevation of genes in response to apoptotic and cell proliferation events both in the intestine and tail, the level of active proteins and the exact cellular distribution of these proteins remains to be elucidated.

MATERIALS AND METHODS
Animals and Treatments
Free-swimming *Xenopus laevis* tadpoles (Boreal Northwest) were raised in dechlorinated tap water. For precocious induction of metamorphosis, 10nM thyroid hormone (3,3',5' triiodothyronine: T3) was added to the rearing water of stage 55 and 56 embryos. All tadpoles were staged according to Nieuwkoop and Faber [25]. Animal care met the principles and guidelines of the Canadian Council on Animal Care, and the University of Western Ontario Animal Use Sub-Committee.

RNA Extraction and Semi-quantitative RT-PCR
Analysis
Total RNA from *Xenopus* intestine and tail was isolated at stages 56, 58, 61, 63 and 65, using TRIZol (Invitrogen) as per the manufacturer's instructions. Stage 65 tail is very small and largely a-cellular and thus was not used. Total intestine and tail RNA was also isolated by the same means from T3-treated tadpoles prior to treatment (Day 0), 1 day, or 3 days after T3 treatment. RNA was treated with RNase-free DNase I (Fermentas) to remove any DNA contamination. RNA integrity was checked by visualizing the 18S and 28S ribosomal RNAs with gel electrophoresis. First-strand cDNA synthesis was performed on 5 µg of total RNA using SuperScript™ II Reverse Transcriptase (Invitrogen). PCRs were performed according to the manufacturer's instruction using Platinum® Taq DNA Polymerase (Invitrogen).

The *Xenopus* MMP and TIMP specific primers used were as follows: MT1-MMP, 5'-ATGATGACCGGAGGAAATC-3' and 5'-TGCGACAGTTCAGGTCATA-3'; MT3-MMP, 5'-CCATGGTCCTGGCTCCCCTCA-3' and 5'-TGGAACCATAAATTCTTCT-3'; Gel-A, 5'-CGAGGGAACCTGCAAACACAGA-3' and 5'-GATGGAGGAGGGGAAACAAAT-3'; TIMP-2, 5'-TCCCTGTTGCGATAGCTTCTGCTTG-3' and 5'-GACATCTTCCATTTGTCACA-3'; TIMP-3, 5'-GCAATAAGCTGCTGGGAATC-3' and 5'-TCATGTTCTCACCCTTCCTG-3'. The EF1-α control primers, 5'-CTCTAACCACCCAGGCGGAAT-3' and 5'-GGGAGTGTCAGAGAGGAAGC-3', were used as standards, and as they flanked an intron in genomic DNA, they were also controlled for genomic DNA contamination. EF1-α PCR reactions were always run in parallel with all genes of interest.

Aliquots were taken at different cycles to ensure that the PCR products analyzed were from the log growth phase of the reaction. RT-PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and visualized using the Bio-Rad Gel Doc 1000 system. The intensities of the unsaturated product bands were quantified using Bio-Rad Quantity One c. 4.40 software and compared to EF1-α. All experiments were repeated at least three times with consistent results, and the identity of the resulting amplicons was confirmed by sequencing at the Robarts Research Institute (London, ON). The ratio for each gene interest to EF1-α, at different stages and conditions, were compared and recorded as an increase or decrease compared to the previous developmental stage or treatment condition. The resultant graphs illustrated general trends of RNA level increase or decrease relative to EF1-α.

The scale of the Y-axis is not standardized between genes and should not be used to quantify or compare RNA gene against each other.

ACKNOWLEDGMENTS
We would like to thank Dr. Kim Roberts for her critical evaluation of this manuscript.

Author Contributions
Conceived and designed the experiments: SD. Performed the experiments: LW. Analyzed the data: LW. Contributed reagents/materials/analysis tools: DC CC. Wrote the paper: LW.

REFERENCES
1. Dodd MHI, Dodd JM (1976) The biology of metamorphosis; London B, ed. New York Academic Press, pp 467–599.
2. Shi yb (1999) Amphibian Metamorphosis: From Morphology to Molecular Biology. New York: John Wiley and Sons.
3. Damjanovski S, Ishizuya-Oka A, Shi YB (1999) Spatial and temporal regulation of collagenases-3, -4, and stromelysin -3 implicates distinct functions in apoptosis and tissue remodeling in Xenopus laevis. J Cell Biol 150: 1177–1188.
4. Crump D, Werry K, Veldhoen N, Van Aggelen G, Helbing CC (2002) Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and tissue remodeling during frog metamorphosis. Cell Res 9: 91–105.
5. Ishizuya-Oka A, Li Q, Amano T, Damjanovski S, Ueda S, et al. (2000) Requirement for matrix metalloproteinase stimuylin-3 in cell migration and apoptosis during tissue remodeling in Xenopus laevis. J Cell Biol 150: 1177–1180.
6. Shi YB, Ishizuya-Oka A (2001) Thyroid hormone regulation of apoptotic tissue remodeling: implications from molecular analysis of amphibian metamorphosis. Prog Nucleic Acid Res Mol Biol 65: 53–100.
7. Damjanovski S, Puzianowska-Kuznicka M, Ishuzuya-Oka A, Shi YB (2000) Differential regulation of three thyroid hormone-responsive matrix metalloproteinase genes implicates distinct functions during frog embryogenesis. Faseb J 14: 503–510.
8. Hasebe T, Hartman R, Fu L, Amano T, Shi YB (2007) Evidence for a cooperative role of gelatinase A and membrane type-1 matrix metalloproteinase during Xenopus laevis development. Mech Dev 124: 11–22.
9. Hasebe T, Hartman R, Matsuda H, Shi YB (2006) Spatial and temporal expression profiles suggest the involvement of gelatinase A and membrane type 1 matrix metalloproteinase in amphibian metamorphosis. Cell Tissue Res 324: 105–116.
10. Das B, Cai L, Carter MG, Piao YL, Sharrow AA, et al. (2006) Gene expression changes at metamorphosis induced by thyroid hormone in Xenopus laevis tadpoles. Dev Biol 291: 342–355.
11. Gross J, Nagai Y (1965) Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc Natl Acad Sci U S A 54: 1197–1204.
12. Damjanovski S, Amano T, Li Q, Pei D, Shi YB (2001) Overexpression of matrix metalloproteinases leads to lethality in transgenic Xenopus laevis: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dyn 221: 37–47.
13. Arvelo F, Coté C (2006) [Metalloproteinases in tumor progression. Review]. Invest Clin 47: 185–205.
14. Ishizuya-Oka A, Ueda S, Inokuchi T, Amano T, Damjanovski S, et al. (2001) Thyroid hormone-induced expression of sonic hedgehog correlates with adult epithelial development during remodeling of the Xenopus stomach and intestine. Differentiation 69: 27–37.
15. 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.
16. Kang T, Nagase H, Pei D (2002) Activation of membrane-type matrix metalloproteinase-3 zymogen by the proprotein convertase furin in the trans-Golgi network. Cancer Res 62: 673–681.
17. Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69: 562–573.
18. Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17: 463–516.
19. Somiari SB, Shriver CD, Heckman C, Olsen C, Hu H, et al. (2006) Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer. Cancer Lett 233: 98–107.
20. Hammoud L, Walsh LA, Damjanovski S (2006) Cloning and developmental characterization of Xenopus laevis membrane type-3 matrix metalloproteinase (MT3-MMP). Biochem Cell Biol 84: 167–177.
21. Walsh LA, Cooper CA, Damjanovski S (2007) Soluble membrane-type 3 matrix metalloproteinase causes changes in gene expression and increased gelatinase activity during Xenopus laevis development. Int J Dev Biol 51: 389–396.
22. Fu L, Tomita A, Wang H, Buchholz DR, Shi YB (2006) Transcriptional regulation of the xenopus laevis stromelysin-3 gene by thyroid hormone is mediated by a DNA element in the first intron. J Biol Chem.
23. Jung JC, Lee CJ, Edwards DR, Fiss ME (2002) Matrix metalloproteinases mediate the dismantling of mesenchymal structures in the tadpole tail during thyroid hormone-induced tail resorption. Dev Dyn 223: 402–413.
24. Lambert E, Dasse E, Haye B, Peintre F (2004) TIMPs as multifacial proteins. Crit Rev Oncol Hematol 49: 187–198.
25. Nieuwkoop PD, Faber J (1956) Normal table of Xenopus laevis (Daudin); a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Amsterdam: North-Holland Pub. Co. pp 243.
26. Shi YB, Su Y, Li Q, Damjanovski S (1998) Auto-regulation of thyroid hormone receptor genes during metamorphosis: roles in apoptosis and cell proliferation. Int J Dev Biol 42: 107–116.