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Overexpression of the tissue inhibitor of metalloproteinase-3 during *Xenopus* embryogenesis affects head and axial tissue formation

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ABSTRACT

Tissue inhibitors of metalloproteinases (TIMPs) modulate extracellular matrix remodeling during embryonic development and disease. TIMP-3 expression was examined during *Xenopus laevis* embryogenesis: TIMP-3 transcripts detected in the maternal pool of RNA increased at the mid-blastula transition, decreased dramatically during gastrulation and increased again during neurulation and axis elongation. Interestingly, the decrease during gastrulation was not seen in LiCl treated (dorsalized) embryos. Whole mount *in situ* hybridization of TIMP-3 using DIG-labeled RNA probes demonstrated that the transcripts were present in all dorsal tissues during embryogenesis, but were prominent only in head structures starting at stage 35. Overexpression of TIMP-3 through transgenesis and RNA injections led to developmental abnormalities and death. Both overexpression strategies resulted in post-gastrulation perturbation including those to neural and head structures, as well as truncated axes. However, RNA injections resulted in more severe early defects such as failure of neural tube closure, and transgenesis caused truncated axes and head abnormalities. No transgenic embryo expressing TIMP-3 survived past stage 40.

Keywords: *Xenopus,* TIMP-3, transgenesis, neurulation

INTRODUCTION

The remodeling of the extracellular matrix (ECM) is crucial for the proper development and maintenance of multicellular organisms. Controlled degradation of proteinaceous compounds in the ECM is mediated largely through matrix metalloproteinases (MMPs) and their inhibitors -- tissue inhibitors of metalloproteinases (TIMPs). Given the fact that TIMPs inhibit all known MMPs and that a proper balance of MMPs and TIMPs is essential for normal cellular function, it is not surprising that TIMPs are associated with numerous human disease processes. TIMP-3 plays multiple roles in angiogenesis, cancers, and diseases including Sorsby Fundus Dystrophy, an inherited eye disease [1].

MMPs are extracellular zinc proteases that display a wide range of substrate specificity and are tightly regulated, since uncontrolled proteolysis contributes to abnormal development and the generation of many pathological conditions [2-5]. Based on structural relationships and substrate specificities, MMPs have been classified into a number of groups including collagenases (whose primary substrates are collagens), gelatinases (which digest gelatin, and component molecules of the basement membrane), stromelysins (which can degrade almost any ECM component), and membrane type MMPs (MT-MMPs; - which can digest a number of collagens, glycoproteins and proteoglycans). Though individual MMP mouse knockout studies have provided insight into function, these studies are often complicated by MMP functional redundancies producing viable offspring [6, 7]. Overexpression studies, on the other hand, have demonstrated that proper control of MMP activity is required during cellular differentiation and development [8, 9]. Interestingly, TIMPs together with MT-MMPs also facilitate the activation of some MMPs [10]. Therefore, TIMPs can perform a variety of biological functions during development including inhibition of MMPs and facilitation of MMP activation [11]. To date, however, there are few studies that directly examine the expression, distribution and roles played by TIMPs during embryogenesis [12, 13].

MMP mediated remodeling of the ECM plays important developmental functions, and amphibians are used routinely to describe how disruption of the ECM can have detrimental effects on vertebrate development [14]. Since the

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discovery of the first MMP (*Xenopus* collagenase was isolated from resorbing tail during metamorphosis [15]), collagenases are now known to be involved in a variety of processes during *Xenopus* development [16], and their overexpression leads to abnormal gastrulation and death [9]. Transgenic overexpression of several MMPs (ST3, col4, MT5-MMP) is also known to affect gastrulation and axial development in *Xenopus* [9]. Since proper ECM structure is a requisite for vertebrate gastrulation, and despite the fact that the roles played by many ECM molecules and MMP during early embryogenesis have been examined, there remains a void regarding the crucial roles that TIMPs play during these processes.

TIMPs are conserved in a wide range of organisms including *Xenopus*, chicken, mouse, human, cow, rat and Drosophila [3, 17-22]. Of the four vertebrate TIMPs, TIMP-3 is the only one capable of binding directly to the ECM, and is the only TIMP capable of inhibiting several types of ADAM proteases [23, 24]. Previous studies by Yang and Kurkinen [3] have shown that *Xenopus* TIMP-3 mRNA is expressed maternally in the egg, is present during development, and is expressed in specific tissues of the adult. Details of the spatial and temporal examination of TIMP-3 during *Xenopus* development has not yet been undertaken, nor have there been any reports about the developmental consequences of overexpressing this important regulator of the ECM.

To address these issues, we here examine the spatial and temporal expression of TIMP-3 during *Xenopus* embryogenesis. Having catalogued the expression pattern, we disrupted TIMP-3 levels using transgenics and RNA microinjection to elucidate TIMP-3 functions during development. We report here that TIMP-3 RNA is present throughout *Xenopus* development, with relative levels highest prior to gastrulation, falling during gastrulation, but still present during developmental stages requiring extensive tissue remodeling. More importantly, TIMP-3 is spatially regulated during early embryogenesis and is expressed in dorso-anterior structures. As development progresses, however, TIMP-3 levels decrease in these dorso-anterior structures but persist in the head. Coincidently, overexpression of TIMP-3 during embryogenesis leads to defects in the head and to the dorsal axis. These findings, in agreement with extensive data already collected regarding the roles of ECM remodelling during embryogenesis, indicate that TIMP-3 plays a crucial role in the normal embryonic events associated with *Xenopus* development.

MATERIALS AND METHODS

Embryo rearing

Xenopus laevis embryos were fertilized and grown using standard protocols [25] and all were staged according to Nieuwkoop and Faber [26].

LiCl and UV light treatment

Three hours after fertilization, embryos were transferred for 10 min to a dish containing 0.1M LiCl [27]. The embryos were then rinsed three times with 0.1X MMR, and then returned to 0.1 × MMR for rearing. For UV light treatment, fertilized and dejellied embryos were transferred to Pyrex® quartz dishes and exposed for 60 sec to short wave UV light (254 nm) from a Model UVGL-25 Mineralight® lamp (UVP Inc). The embryos were returned to 0.1 × MMR and reared at 20ºC.

RNA isolation

Total RNA was isolated from *Xenopus* embryos using TRIzol Reagent according to the manufacturer's instructions (Invitrogen). RNA was quantified by spectrophotometry and RNA integrity was assessed by 1.2% agarose formaldehyde gel electrophoresis as described in Sambrook *et al* [28].

Cloning of *Xenopus* TIMP-3

Yang and Kurkinen [3] cloned *Xenopus laevis* TIMP-3 from oocyte RNA (Accession AF042493). We cloned *Xenopus* TIMP-3 from total oocyte cDNA using an Invitrogen SuperScript™ One-Step RT-PCR kit with Platinum® Taq using conditions supplied by the manufacturer. The PCR primers for TIMP-3 amplification were 5'-*TGGTCGACGGTAC*ATGTCTGTGTGTGCTCTGAC-3' and 5'-*TAACCGGTGGATCCAT*GGGATCCGTGGTGTTTATTG-3'. The italicized bases denote restriction enzyme cleavage sites. The primers amplify the ATG start site and the full length coding sequence [3], excluding the termination codon. The full-length 700 bp TIMP-3 amplicon was cloned into the pCR®II-TOPO vector as per manufacturer's instructions (Invitrogen). Recombinants were restriction mapped and those containing the expected insert size were sequenced at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario.

Sub-cloning of TIMP-3 and transgenesis

Full-length *Xenopus* cDNA TIMP-3 was cloned into the Clontech plasmid pEGFP-N1, which contains a CMV promoter and an enhanced GFP reporter. The full length coding region of TIMP-3 was inserted into the *Age* I/*Kpn* I restriction sites downstream of the CMV promoter to allow the CMV driven expression of an in-frame fusion protein of TIMP-3 with GFP at its carboxyl end. Both pEGFP-N1 with TIMP-3, and empty pEGFP-N1 (control) plasmid were linearized with *Not* I for transgenesis. The transgenic procedure was performed as in Kroll and Amaya [27], except no protease inhibitors were used in the preparation of the egg extract or the sperm nuclei. The sperm nuclei preparation was modified as in Huang *et al* [30]. Embryos were sorted at the two-cell stage for normal cleavage patterns and assessed for phenotype and GFP expression during subsequent development. Only embryos that developed normally pass gastrulation (as judged by external criteria) to Nieuwkoop and Faber [26] stage 14 were used and scored in subsequent assays. Embryos were fixed in PBS containing 3.7% formaldehyde for future analysis.

Sub-cloning of TIMP-3 and RNA injection

Full length TIMP-3 cDNA was ligated into the *EcoR* V/*Spe* I

restriction sites of the T7TS plasmid. This plasmid adds *Xenopus* β-globin 5' and 3' UTRs to mRNAs aiding in their *in situ* stability and translation of *in vitro* transcribed cRNA. In addition, a construct lacking the ATG start and the N-terminal most 100 amino acids was created using the restriction enzyme *Hinc* II. This construct can be bound to by ribosomes, but cannot be translated. All constructs were linearized with *Xba* I such that T7 RNA polymerase to produce sense cRNA. Capped poly(A)-tailed cRNAs were synthesized using the RiboMAX™ Large Scale RNA Production System-T7 as per manufacturer's instructions (Promega). The RNA was quantitated using optical density measurements and its integrity assessed by 1.2% formaldehyde gel electrophoresis.

Just prior to injection stage 2 *Xenopus* embryos were incubated in 1X MMR with 4% Ficoll. Glass needles with 10 μm diameters were used, and one cell of each two-cell embryo was injected with 4.6 nl of water containing between 10 and 900 pg of cRNA. Embryos were allowed to heal for several hours in the 1× MMR/4% Ficoll solution then transferred to 0.1×MMR solution containing 50 μg/ml gentamycin. Embryos were monitored throughout development for phenotypic abnormalities, and then fixed in PBS containing 3.7% formaldehyde for future analysis and photography.

RNA probe synthesis and whole mount *in situ* hybridization

The full length 700 bp TIMP-3 cDNA in the TOPO plasmid was used to generate RNA probes. Digoxigenin-labeled sense and antisense transcripts were generated using T7 and SP6 viral promoter, respectively. RNA probes were visualized and the amounts quantitated compared to a standard using gel electrophoresis.

Albino *Xenopus* embryos were used for whole mount *in situ* hybridization as described in Harland [31]. Following the colorimetric reaction, embryos were fixed in Bouin's overnight. Embryos were then washed in methanol and cleared in a 2:1 mix of benzyl benzoate and benzl alcohol for photography (see below).

Cryosectioning

In situ stained embryos were re-hydrated with a methanol/PBS series, transferred to a solution containing 0.5 M sucrose in PBS overnight, embedded in O.C.T. Compound (Electron Microscopy Sciences) and then frozen in liquid nitrogen cooled isopentane. Transverse 20 μm sections of embryos were cut at -20º C, then transferred to slides and allowed to dry overnight at room temperature. Slides were briefly washed in PBS to dissolve excess O.C.T, then mounted with Cytoseal™ 60 and photographed (see below).

RT-PCR analysis of TIMP-3 expression

Total RNA was isolated from *Xenopus* embryos at stages 8, 9, 11, 13, 15, 18, 20, 23, 25, 30, 33, 37 and 40, and LiCl and UV-treated embryos at stage 11, using the TRIzol reagent described above. First strand cDNA synthesis was carried out with 5 μg total RNA and 1 μl Oligo$(dT)_{12\text{-}18}$ (500 mg/ml) using the SuperScript™ protocol according to the manufacturer (Invitrogen). Following cDNA production RT-PCR was carried using 1 μl of the reverse transcription product in a 25 μl PCR reaction using HotStar Taq DNA polymerase (QIAGEN). Primers for the (700 bp) target TIMP-3 transcripts were 5'-ATGTCTGTGTGTGCTCTGAC-3' and 5'-GGGATCCGTGGTGTTTATTG-3'. Primers for detecting the control elongation factor-1 alpha (EF-1α) were 5'-CAGATTGGTGCTGGATATGC-3' and 5'-ACTGCCTTGATGACTCCTAG-3'. The expected amplicon is a 269 base pair product. Chordin primers also used were: 5'CAGTCAGATGGAGCAGGATC3' and 5'AGTCCCATTGCCCGAGTTGC3' producing a 303 bp product. TIMP-3 or chordin primers were used in a mutiplex-PCR reaction together with EF-1α primers. This would allow later semi-quantitative comparison of TIMP-3 or chordin expression levels with that of EF-1α. An annealing temperature of 50ºC and 30 amplification cycles were used for all reactions. Several reactions were run to ensure that 30 cycles was within the exponential linear growth phase for the PCR. PCR products were fractionated on a 1.2% agarose gel, ethidium bromide stained and photographed. Bio-Rad Quantity One 4.4.0 software was used to photograph and quantify the intensity of the PCR product bands. Relative levels of TIMP-3 expression are shown in relation to the level of EF-1α, whose expression remains relatively constant throughout development.

Photography

All micrographs of embryos were taken with a Photometrics Cool SNAPcf camera attached to a Leica (Model MZ FCIII) microscope, using a Chroma GFP filter to capture GFP fluorescence.

Sequence and secondary structure analysis

Amino acid identity/similarity searches were done using NCBI BLAST 2 at the Swiss Institute of Bioinformatics site http://au.expasy.org/tools/blast/. Secondary structure predictions were made using the PSIPRED method [32] at http://bioinf.cs.ucl.ac.uk/psipred/psiform.html. ClustalW (1.82) analysis was performed at the European Bioinformatics Institute site at http://www.ebi.ac.uk/clustalw/ [33]. All analyses were performed using default search setting.

RESULTS

Comparison of mammalian and non-mammalian TIMP-3 sequences

Although the *Xenopus* TIMP-3 amino acid sequence was previously published [3], a thorough analysis and comparison between *Xenopus* and its mammalian counterparts has not been performed. This analysis is particularly informative as several new TIMP-3 sequences have been identified since the publication of the *Xenopus* TIMP-3 sequence. To date there are eight full-length TIMP-3 sequences (Tab. 1). They are: *Xenopus laevis* [3]; *Gallus gallus* [17]; *Mus musculus* [18]; *Bos taurus* [20]; *Rattus norvegicus* [21]; *Homo sapiens* [23]; *Scyliorhinus torazame* [34]; and *Equus caballus* (direct submission, 2003).

ClustalW cladistical analysis revealed that *Xenopus* TIMP-3 is distantly related to mammalian TIMP-3 (Fig. 1A). However, an examination of the predicted secondary structures agrees with the BLAST similarity results showing that *Xenopus* TIMP-3 is more closely related in function to mammalian TIMP-3. When *Xenopus*, human and chicken amino acid sequences were placed in a PSIPRED analysis [32] to predict the secondary structure of each protein, *Xenopus* TIMP-3 is more similar to human than to chicken TIMP-3. Human and *Xenopus* TIMP-3 share an extended sheet structure at amino acids 60-70 that is

Tab. 1 Comparison of *Xenopus* TIMP-3 amino acid sequences with that of other known TIMP-3s.

	Overall Identity/ Similarity	Leader Peptide (AAs 1-24) Identity	Most Conserved (AAs 25-75) Identity/Similarity	Least Conserved (AAs 120-190) Identity/Similarity
Xenopus	100	100	100	100
Human	83/92	33.3	86/95	81/90
Bovine	83/92	27.8	88/95	80/89
Horse	83/93	27.8	86/95	80/91
Mouse	83/92	22.2	86/95	81/89
Rat	83/92	22.2	86/95	80/88
Chick	85/91	31.6	94/94	72/87
Cat shark	77/90	22.8	86/93	70/82

Percentage identity or similarity was determined by comparing (BLAST2) the entire *Xenopus* TIMP-3 amino acid sequence, or one of the three domains defined above, against the Swiss-Prot database. The most and least conserved regions were determined using ClustalW analysis [31]. Swiss-Pro accession numbers are as follows;
O73746|TIM3_XENLA *Xenopus laevis* [3]; P35625|TIM3_HUMAN *Homo sapiens* [23]; P79121|TIM3_BOVIN *Bos taurus* [20]; Q9TUL9|TIM3_HORSE *Equus caballus* (direct submission, 2003); P39876|TIM3_MOUSE *Mus musculus* [18]; P26652|TIM3_CHICK *Gallus gallus* [17]; P48032|TIM3_RAT *Rattus norvegicus* [21]; Q9W6B4|TIM3_SCYTO *Scyliorhinus torazame* [34].
AAs: amino acids. Numbers of amino acids correspond to the *Xenopus* sequence.

lacking is chicken. They also contain an extra alpha-helical domain at about amino acid residue 90 that is absent in chicken (Fig. 1B).

RT-PCR analysis of *Xenopus* TIMP-3 expression during development

TIMP-3 RNA was detected by RT-PCR at all stages examined ranging from stage 8 (pre-gastrulation) to stage 40 (free swimming tadpole) (Fig. 2). Yang and Kurkinen previously reported that TIMP-3 RNA was maternally expressed in oocytes with levels decreasing during early embryogenesis [3]. Our data support this finding as we see relatively high levels of TIMP-3 expression at stages 8 and 9, just at the time when the zygotic genome is activated and transcription is beginning. Following the mid-blastula transition at stage 9, TIMP-3 levels fall dramatically by stage 11 (Fig. 2). Following gastrulation, TIMP-3 levels increase in embryos from stage 13 onward, peaking at about stage 18. Past stage 18, TIMP-3 RNA levels are largely unchanged, with a relatively low level of expression seen until stage 37, then increasing slightly at stage 40 (Fig. 2). DNA band intensities used in semi-quantitative RT-PCR analyses were quantified using Quantity One 4.4.0 software (Bio-Rad laboratories). Relative levels of TIMP-3 expression are shown in relation to the level of EF-1α, whose expression remains relatively constant throughout development.

Having determined TIMP-3 expression levels during development, we then tested whether or not TIMP-3 levels could be altered by artificially increasing the amount

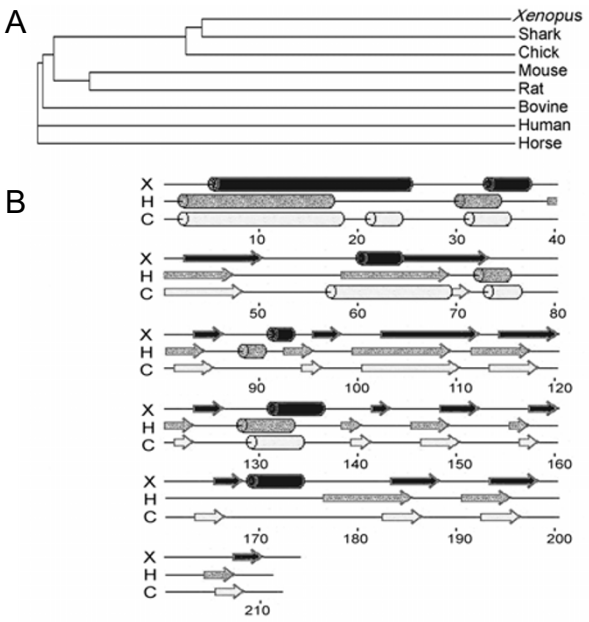

Fig. 1 Cladistical analysis of TIMP-3 and predicted secondary structures of *Xenopus* Human and Chicken TIMP-3. (**A**) Database searches identified 8 full-length proteins that were identified as TIMP-3 (see Tab. 1). ClustalW (1.82) analysis was performed at the European Bioinformatics institute web site at http://www.ebi.ac.uk/clustalw/ [33] using default search and comparison settings. The resulting ClustalW cladogram represents the relatedness of the various TIMP-3 proteins. (**B**) The *Xenopus* (X), human (H), and chicken (C), amino acid sequences were used to predict their proteins' secondary structures using the PSIPRED method [32] at http://bioinf.cs.ucl.ac.uk/psipred/psiform.html. Barrels represent alpha helical domains, arrows represent sheet domains, while lines denote random or undetermined structures. The numbers correspond to the numbering of the *Xenopus* sequence.

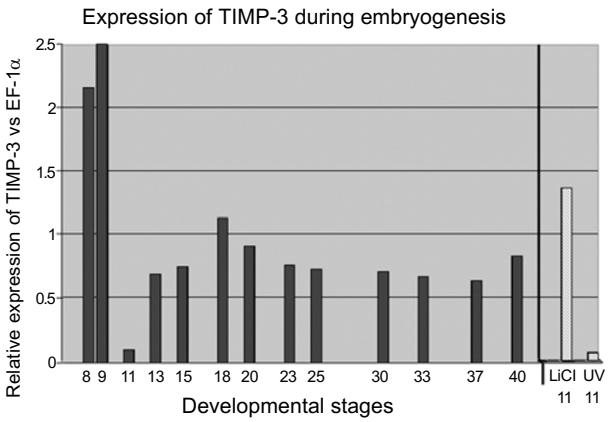

Fig. 2 Semi-Quantitative RT-PCR Analysis of TIMP-3 RNA Levels During Early Development. As compared to an internal control gene (*EF-1α*) TIMP-3 was abundantly expressed through all stages of embryogenesis. The stages examined included stage 8 (blastula), 9, 11, 13, 15, 18, 20, 23, 25, 30, 33, 37 and stage 40 (free swimming tadpole). TIMP-3 RNA levels peaked at stage 9 at the start of gastrulation but rapidly decreased during gastrulation reaching very low levels at stage 11. Levels increased during neurulation starting at stage 13 and peaked at stage 18. Levels decreased slightly until stage 37 at which point levels started to increase again. In embryos that were dorsalized (with LiCl) or ventralized (with UV light) shortly after fertilization, relative TIMP-3 levels increased in the dorsalized vs the ventralized embryos at stage 11. The data represent numerical values from one representative experiment. Four repeats were performed, all of which displayed consistent patterns and levels of expression.

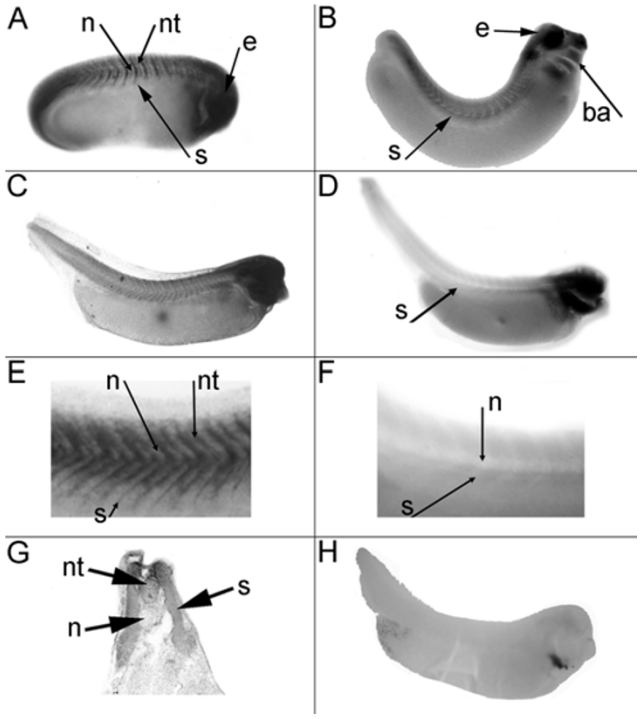

Fig. 3 Whole mount in situ localization of TIMP-3 RNA during early *Xenopus* development. TIMP-3 RNA was detectable through all stages of *Xenopus* development examined. (**A**) At stage 25, TIMP-3 message was abundant in dorsal structures such as the somites (s), neural tube (nt) and notochord (n) as well as head structures such as the eye (e), and in the tailbud. (**B**) By stage 30, TIMP-3 message was still abundant in the somites and further defined in head structures such as the branchial arches (ba). (**C**) At stage 35, relative signal intensity was strong in the neural tube (nt) and the somites (s), and weaker in the notochord (n). (**D**) By stage 38 signal was undetectable in the somites, neural tube and notochord, although TIMP-3 message was still abundant in head structures. (**E**) Higher magnification of the dorsal structures of the stage 35 embryo in C showing detectable signal in the neural tube (nt) but relative absence of signal in the notochord (n). (**F**) Higher magnification of the dorsal structures of the stage 38 embryo in D, showing the absence of TIMP-3 signal. (**G**) Transverse section of a stage 35 embryo demonstrating TIMP-3 signal in the somites (s) and neural tube (nt), but relative absence of signal in the notochord (n). (**H**) Expression of control stromelysin-3 RNA in the developing branchial arches (ba) and the posterior endoderm (pe).

of dorsal tissue at the expense of ventral tissue. Though relative TIMP-3 RNA levels are low at stage 11 during gastrulation, they are greatly elevated at this stage in embryos that have been dorsalized with LiCl. In contrast, TIMP-3 RNA levels are unaffected in stage 11 embryos that have been ventralized with UV light (Fig. 2). As expected, chordin RNA levels (used as a measure of dorsal character [35]) increased in LiCl treated embryos and decreased in UV treated embryos (data not shown).

In Situ hybridization detection of TIMP-3 RNA during *Xenopus* embryogenesis

TIMP-3 RNA was detected by whole mount *in situ* hybridization in all regions of the embryo from fertilization until gastrulation, at which time the signal began to be localized to dorsal structures (data not shown). In early stages (15, 22 and 25), TIMP-3 RNA was detectable in most dorsal structures such as the notochord (n), neural tube (nt) and somites (s) and in the head and eye (e) (Fig. 3A, and data not shown). At stage 30, TIMP-3 signals were still abundant in dorsal structures, but became more defined in the head, particularly the branchial arches (ba) and the eye (e) (Fig. 3B). By stage 35, staining intensity in the somites (s) decreases (relative to that in the head). A similar situation was true between the notochord (n) relative to the neural tube (nt) (Fig. 3C and E). By stage 38, staining was not detected in the somites (s), neural tube (nt) and notochord (n), although TIMP-3 expression was still abundant in the head (Fig. 3D and F). Mid-trunk transverse sections of whole mount embryos were performed to further characterize levels of TIMP-3 RNA in

the dorsal structures of the developing *Xenopus* embryo. Stage 35 *Xenopus* embryos showed TIMP-3 RNA signal in the somites (s), and the neural tube (nt), but not in the notochord (n), (Fig 3G). Expression of stromelysin-3 RNA [16], a message encoding a MMP known to be localized in the developing branchial arches (ba) and the posterior endoderm (pe) is shown in Fig. 3H.

Transgenic overexpression of TIMP-3 perturbs development and causes death

Xenopus TIMP-3 RNA, fused at its C-terminal end to GFP, was driven ubiquitously by a CMV promoter and overexpressed transgenically. All control transgenic embryos (vector alone) that completed gastrulation and expressed GFP protein developed normally regardless of the amount of GFP produced (data not shown). TIMP3-GFP expression (when detectable) always resulted in abnormal development leading to death (Fig. 4; Tab. 2). These TIMP3-GFP expressing embryos completed gastrulation but developed curved and truncated axes compared to normal uninjected embryos or those expressing GFP alone (Fig. 4; Tab. 2). In addition, TIMP3-GFP expressing embryos displayed abnormal eye and head development (Fig. 4). This effect was dose dependent, as assessed by the relative amounts of TIMP3-GFP observed in the embryo. Embryos expressing relatively high amounts of fluorescence from the fusion construct display severe developmental abnormalities including a truncated axis, an abnormal head, and a pronounced ventral curvature (Fig. 4). Developmental defects became more pronounced as development continued (as the transgene was continuously expressed) and all embryos expressing TIMP3-GFP died shortly after stage 40 (Tab. 2). The cavities seen on the embryo on the right in Fig. 4A exhibit GFP fluorescence (4B) indicating that the expressed chimeric TIMP protein was secreted. These cavities appear to be fluid-filled when examined at high magnification (not shown), and fluorescence staining within them is not punctate as is the case with cellular tissues, suggesting that they are extracellular cavities. Whether or not the chimeric TIMP protein is functional was not determined, despite its ability to be translated, processed and secreted.

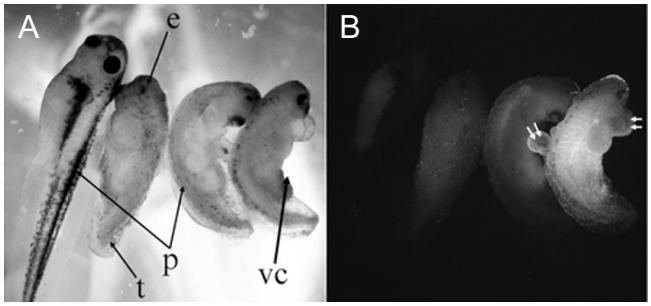

Fig. 4 Transgenic overexpression of TIMP-3 leads to abnormal phenotypes. Embryos that completed gastrulation normally were examined, at approximately stage 40, under visible light for morphology (**A**) and UV light for GFP expression (**B**). The left-most embryo displayed no detectable GFP and developed no visible abnormalities. All embryos expressing TIMP3-GFP showed a truncated axis phenotype (t), eye abnormalities, and reduced pigmentation (p) compared to the normal embryo on the left. The relative expression of TIMP3-GFP correlated with a dose dependent phenotype - embryos expressing a relatively high amount of GFP displayed more severe abnormalities, including a more severe ventral curvature (vc). TIMP3-GFP is present in extracellular cavities (B, double arrows). All embryos expressing detectable TIMP3-GFP died at, or shortly after stage 40.

Tab. 2 Survival of Embryos Following Transgenesis or Microinjection of TIMP-3 mRNA.

	Transgenesis – GFP alone		Transgenesis - TIMP3-GFP		Microinjection - 300 pg Truncated TIMP-3 mRNA		Microinjection - 300 pg Full-length TIMP-3 mRNA	
	Survival	Axial defects	Survival	Axial defects	Survival	Axial defects	Survival	Axial defects
Stage 30	85%	0%	65%	90%	100%	0%	100%	30%
Stage 40	75%	5%	40%	100%	100%	0%	90%	35%
Stage 45	75%	5%	0%	-	100%	0%	80%	35%

Following transgenesis or microinjection, only embryos that passed through gastrulation normally (as judged by closure of the blastopore) were examined. All transgenic embryos evaluated and scored displayed detectable (visible) levels of GFP expression when viewed with fluorescent light. The truncated TIMP-3 mRNA cannot be translated but can bind ribosomes. Each treatment included at least 30 embryos and the data represents one typical experiment. All treatments were repeated at least three times and all repeats showed similar trends and results.

Overexpression of sense TIMP-3 RNA intoTwo-Cell embryos causes head and axial defects

Having determined that deregulating TIMP-3 expression levels after the mid-blastula transition causes developmental abnormalities, and since TIMP-3 is normally maternally derived, we disrupted the levels of TIMP-3 in early embryos by microinjecting RNA into the 2-cell stage. Injections ranging from 10-900 pg gave phenotypes that were dose dependent, with higher doses leading to death shortly after gastrulation (data not shown). A dose of 300 pg was selected as representative as it resulted in a distinct phenotype and a good survival rate through most stages of development. Control microinjections of 300 pg of truncated TIMP-3 RNA (lacking the ATG start codon and the N-terminal-most 100 amino acids) resulted in relatively normal embryos developing to stage 41 (Fig. 5A; Tab. 2). Microinjecting 300 pg of capped and poly (A)-tailed TIMP-3 full-length sense RNA into one cell of a two-cell embryo resulted in severe head, axial or eye abnormalities. Head defects occurred in 31% of the embryos that had otherwise normal axes (data not shown), while an additional 35% of all embryos had both axial and head abnormalities (Fig. 5 B,C; Tab. 2). Abnormal head development included eye deformities as well as underdeveloped and improperly positioned cement glands with respect to the position of the eyes. The remaining 34% were relatively normal except that they exhibited various degrees of malformed, cyclopic or absent eyes (Fig. 5 B, C, E and F; and data not shown). Most injected embryos appeared to have completed gastrulation as judged by dorsal blastopore closure. Embryos that had axial defects were severely curved (Fig. 5 D, a) with an apparent failure of neural fold closure (Fig. 5 D, boxed area) resulting in a "twinned" tail phenotype in many embryos (Fig. 5 B--double arrows). These results would indicate that mis-expression of TIMP-3 by RNA injection has more severe consequences on early development than later stages as embryos survived well past stage 40 despite developmental abnormalities.

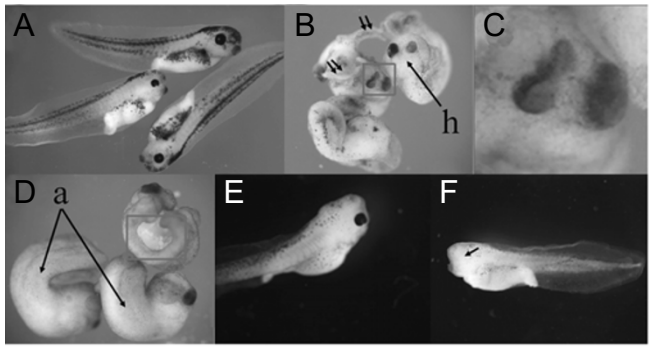

Fig. 5 Injection of sense TIMP-3 RNA into early blastomeres caused head and axis defects. (**A**) Injection of 300 pg of truncated TIMP-3 RNA (lacking the N-terminal-most 100 amino acids) into one cell of a two cell embryo resulted in relatively normal embryos developing to stage 41. (**B**) Microinjection of 300 pg of capped and poly (A)-tailed full-length sense TIMP-3 RNA resulted in severe head and axis abnormalities during development relative to sibling embryos. At approximately stage 30, embryos displayed severe head (h) and axis abnormalities, including curvatures, open neural folds, and twinned tails (double arrows). Embryos also displayed eye deformities where eyes were underdeveloped and/or improperly positioned relative to the cement glands (boxed area - higher magnification in **C**). (**D**) At approximately stage 26 embryos displayed a severely curved axis (a) with apparent failure of neural fold closure (box) and twinned tails (double arrows). Eyes were also often present on one side (**E**) but absent on the other side (**F**) of an embryo.

DISCUSSION

Conservation of TIMP-3 within vertebrates

Proteins that share common and important functions across a variety of species tend to have conserved amino acid sequences and domains [reviewed in 36]. The high conservation of amino acid sequence identity (83%) between *Xenopus* and mammals, and 90-93% conserved similarity amongst all vertebrate TIMP-3s also likely represents conservation of function.

N-terminal amino acids 25-100 are the most conserved peptide stretch amongst the TIMP-3s with *Xenopus* being 94% identical to chicken in this region. C-terminal amino acids 120-190 represent the least conserved peptide stretch as *Xenopus* shares only about 70% identity with chicken, but surprisingly about 80% identity with mammalian TIMP-3 in this region. Indeed, secondary structure analysis reveals that *Xenopus* TIMP-3 shares more domains in common with human than chicken TIMP-3. This conservation suggests that functions are also conserved between the human and *Xenopus* proteins. These data are somewhat in contrast to cladistical analysis using ClustalW [33] that places *Xenopus* and chicken TIMP-3 as more closely related sequences as compared to the mammals. The significance of this conservation is unknown.

As the TIMP-3 sequence is conserved in frog, human and chicken, then the conserved protein would serve a similar role with respect to regulating MMPs during developmental events shared in these animals. One such conserved series of developmental events found in all vertebrates is the formation and patterning of the dorsal axis. Though frog gastrulation is quite distinct from chicken and human gastrulation (*Xenopus* having a dorsal lip and blastopore, while chicks and humans have node and primitive streak structures); these structures have been shown to be homologous. In addition, though somitogenesis occurs in a distinct fashion in *Xenopus* versus human and chicken [37], the underlying molecular signals and

differentiation of muscle types is conserved. Interestingly, the most conserved processes in which TIMP-3 could play a common role in these three species is during neurulation, axis elongation and neural crest cell migration. The cell and tissue interactions and the cell movements involved are common, indeed they are shared by most vertebrates, and thus a function for TIMP-3 could also be shared. As discussed below, the expression patterns of TIMP-3 in *Xenopus*, and overexpression data suggest a role in events related to the dorsal axis formation following gastrulation, particularly related to neural structures.

TIMP-3 RNA is present throughout *Xenopus* embryogenesis

Using Northern analysis, Yang and Kurkinen [3] reported that TIMP3 mRNA is maternally inherited in eggs and midblastula (stage 8) embryos, and then down-regulated in gastrula [3]. Using semi-quantitative multi-plex RT-PCR we compared relative TIMP-3 expression levels to that of EF-1α in a number of developmental stages. We find relatively high levels of TIMP-3 RNA expression at stage 8 and 9, prior to the mid-blastula transition and the activation of the zygotic genome, as compared to later stages of development. Presence of TIMP protein was not assayed. If protein is produced, TIMP-3 function is unrelated to cell migration as there is no cell migration prior to gastrulation. Interestingly, TIMP-3 RNA levels dramatically reduce during gastrulation (stage 11) at which time cell migration and associated ECM remodeling events are occurring. Yang and Kurkinen also reported a processing of TIMP-3 RNA prior to gastrulation whereby maternal transcripts appear to be cleaved [3]. The rapid decrease in TIMP-3 RNA levels between stages 9 and 11 was not seen in LiCl treated embryos, (that have increased "dorsal" structures [38]) where TIMP-3 levels remained relatively high at stage 11. Presumably, the mechanisms that are responsible for lowering TIMP-3 RNA levels are inhibited in dorsalized embryos, or dorsalization increased the transcription of new TIMP-3 RNA. The mis-regulation of TIMP-3 in these dorsalized embryos (as well as the mis-regulation of a multitude of other genes) likely play a part in determining the overall abnormal phenotype of the dorsalized embryo.

Xenopus TIMP-3 RNA levels began to rise at stage 13 and were detectable through to stage 40. TIMP-3 RNA levels rise at a time when gastrulation has finished, neural structures are forming, and axis elongation is occurring. It has been demonstrated that several MMPs, including collagenase-4, are abundantly expressed along the entire dorsal axis [39]. TIMP-3 may be needed to regulate the balance of TIMPs and MMPs during this time to ensure that the proper components of the ECM are being remodeled to allow proper cell migration, proliferation, and function associated with the development of the dorsal axis following gastrulation. This is supported by the transgenic and RNA microinjection data (discussed later) where overexpression of TIMP-3 led to neural and axial defects.

TIMP-3 is transiently expressed in the dorsal tissues

Coinciding with the temporal studies, whole mount analyses demonstrated that TIMP-3 RNA was present in all stages of development until stage 38. Following gastrulation TIMP-3 RNA was present in most dorsal structures of the embryo, such as the notochord, neural tube and somites. This expression persisted until about stage 35 at which time relative expression within the notochord decreased. Expression of TIMP-3 within the somites during these late stages suggests that TIMP-3 plays a role in several aspects of late somitogenesis including differentiation (into dermatome, sclerotome and myotome) and innervation. Shortly after, at stage 38, expression within all dorsal structures was not detected, while expression within head structures was still strong.

TIMP-3 RNA expression within the notochord is more transient than in the somites, as expression deceases at a time when extensive remodelling events and axis elongation is occurring. As the notochord is supported by large bundles of collagen-II [40], this collagen must be remodeled, most likely by MMPs, for elongation to occur. As TIMP-3 is absent in the notochord following stage 35, another TIMP family member must presumably regulate these MMPs. This, together with the low overall level of TIMP-3 expression during gastrulation, suggests that expression of TIMP-3 within the notochord is associated with specific early developmental events, such as neurulation and neural crest formation, and not more general cell migration or cell rearrangement events. This is supported by the transgenic and RNA microinjection data (discussed later) where overexpression of TIMP-3 leads to failure of neural tube closure and decreased pigmentation.

High levels of TIMP-3 expression within head structures is seen at a time when extensive tissue remodeling is occurring as the embryo is preparing to filter feed. The gills are also developing, and the cartilage of the head is being remodeled as the mouth is about to open. Proper TIMP/MMP balance during head development is likely critical for these structures to form. Transgenic and overexpression studies, that disrupt development of these structures, support this role. TIMP-3 is also expressed in the developing optic vesicle and eye -- processes that require extensive cell movements, inductive interactions, and ECM remodelling [41,42]. Here we show that TIMP-3

overexpression often resulted in eye anomalies, supporting a role for TIMP-3 in normal eye development. In addition, TIMP-3 RNA is present in the branchial arches, an area through which neural crest cells migrate. The branchial arches are also associated with the branching morphogenesis that will result in the formation of the gills and their associated blood supply [43]. Interestingly, TIMP-3 is absent from other developing organs, such as the heart and the intestine, where extensive cell rearrangements and remodeling events are occurring.

Overexpression of TIMP-3 during embryogenesis disrupts post-gastrulation events

To elucidate a function for TIMP-3 during *Xenopus* embryogenesis, transgenic and RNA microinjections overexpression strategies were used. Both strategies resulted in similar phenotypes -- that of malformed embryos with shortened axes and a variety of head anomalies. High expression levels often resulted in death prior to gastrulation. However, most embryos injected with 300 pg of full length TIMP-3 RNA completed gastrulation. Overexpression of control truncated RNA had no effects on development. It should be noted that the two overexpression strategies resulted in differences in the timing of their respective overexpressed product. RNA injections result in the production of high levels of proteins shortly after the injection - thereby overexpressing high levels of TIMP-3 prior to and during gastrulation (injected RNA levels drop about 24 h after injection [44]). Transgenic overexpression would not result in production of overexpressed product until after activation of the zygotic genome, just before gastrulation. Once transgenic overexpression began, however, it would persist throughout development. Indeed, no transgenic embryo that displayed detectable levels of TIMP3-GFP survived past stage 40. This is consistent with results seen with the overexpression of MMPs [9]. Prolonged MMP or TIMP overexpression through transgenesis always leads to death, likely due to the long-term persistent disruption of the MMP/TIMP ratio that is normally found throughout development.

Such disruption would impact both the role TIMP plays in the inhibition of ECM proteolysis by MMPs, and the activation of pro-MMPs by TIMP/MT-MMP complexes. The deregulation of TIMP expression can affect cell-cell and cell-matrix remodelling events in a number of ways including MMP processing of cell surface molecules such as Fas-ligand and E-cadherins, and cleavage of a number of growth and angiogenic factors. In addition TIMP-3 has been demonstrated to both stimulate cell proliferation in non-transformed cells, as well as promoting apoptosis in transformed cells [45].

A severe abnormal phenotype, present in a majority of embryos following microinjection of TIMP-3 RNA but absent from the transgenic studies, was the failure of neural tube closure. This phenotype was due presumably to the high levels of TIMP-3 delivered prior to gastrulation at a time when endogenous TIMP-3 RNA levels are normally dropping. TIMP-3 could now be acting upon developmental events that it normally would not. This could interfere with proper notochord development and subsequent signalling. Notochord signaling to the neural ectoderm is required for tube closure [reviewed in 46,47-49]. The closing of the neural folds and the separation of the neural tube are mediated largely through the interaction of cells expressing specific cell adhesion molecules [reviewed in 46, 47]. Another possible role for TIMP-3 in the formation of the neural tube would be to regulate the MMPs responsible for ensuring that ectodermal cells are expressing the correct cell adhesion molecules. The closure of the neural folds and the separation of the neural tube are mediated largely through the interaction of cells expressing specific cell adhesion molecules [reviewed in 46,47]. A possible role for endogenous TIMP-3 in the formation of the neural tube would be to regulate the MMPs responsible for ensuring the cells are expressing the correct cell adhesion molecules and are in the proper location to ensure the closure of the neural tube. The presence of ectopic TIMP-3 early in development as a result of injected RNA could disrupt MMP mediated ECM remodeling as well as cell-cell signaling. As a consequence, inappropriate cell adhesion molecules may be expressed in the neural ectoderm and neural tube, and this may result in the failure of the neural tube to close.

This potential defect in tube closure would also affect future neural crest cell derivatives, some of which are involved in pigmentation, and others that play important roles in cranial structures. It has been demonstrated that proper ECM architecture is crucial for neural crest cell migration [50, 51]. Thus, TIMP-3 perturbation of neural crest (both through RNA injections and transgenic overexpression) could also explain head defects and reduced pigmentation seen in treated embryos. The perturbation of the ECM/neural crest interactions has been shown to result in abnormal development. Previous studies using antibodies have demonstrated that fibronectin, tenascin, laminin, and heparan sulphate proteoglycans play critical roles in chick cranial neural crest cell migration [50, 51].

In addition to cell adhesion molecules, the closure of the neural tube is also dependent on cell movements, such as convergent extension, that occur during neurogenesis. Wallingford and Harland [52] were able to show a strong correlation between the degree of axis elongation due to

convergent extension and the severity of the neural tube defect where embryos with severely open neural tubes elongated negligibly. It is possible that the inhibitor signal acting on axis formation in transgenic TIMP-3 embryos is not enough to interfere with neither tube closure nor convergent extension. In contrast, the RNA microinjections provide a high enough dose of TIMP-3 to prevent tube closure and convergent extension. Therefore, in normal development endogenous TIMP-3 could play a prominent role in neural tube closure by modulating notochord signals, altering cell adhesion molecules, and modulating convergent extension, or a combination of all three.

It is also possible that the resulting phenotypes seen here could be due to the fact that high expression levels could lead to non-specific binding of TIMP-3 protein to substrates that it would not normally act upon. In addition TIMP-3 has been shown to have roles unrelated to the ECM, that is, overexpression of TIMP-3 has also been shown to cause apoptosis of smooth muscle cells in mice [53]. Further studies are needed to investigate these possibilities.

The expression data and overexpression studies presented here demonstrate that the correct regulation of TIMP-3 during *Xenopus* embryogenesis is needed for the proper development of the dorsal axis and of head structures. The expression profile of TIMP-3, which demonstrates that TIMP-3 is expressed at lower levels during given events (such as gastrulation), or in given tissues (such as the dorsal axis after stage 35), draws attention to the importance of regulating TIMP-3 expression during development. As TIMP-3 is an MMP inhibitor, significance must be placed not only on its presence but also its absence in a given developmental process or disease. This is particularly important in situations where TIMP-3 is first abundantly expressed and then down regulated as part of its role and function-such as appears to be the case during *Xenopus* gastrulation. Given the importance of TIMPs in diseases and development, further work is required to delineate precise functions that TIMP-3 may be playing in cell-signalling and cell migration events, particularly during neurulation.

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