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Int J Dev Biol. Author manuscript; available in PMC 2008 April 15.

Published in final edited form as:
Int J Dev Biol. 2004 February ; 48(1): 57–61.

Identification of a second *Xenopus Twisted Gastrulation* gene

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Abstract

Twisted Gastrulation (Tsg) is a secreted molecule which regulates BMP signalling in the extracellular space as part of an evolutionarily conserved network of interacting proteins. In *Xenopus*, maternal *xTsg* mRNA can be found throughout the early embryo. After gastrulation, *xTsg* is expressed as part of the BMP4 synexpression group until late tadpole stages. Here we report the identification of a second *Xenopus Tsg* gene (*xTsg-2*). *Xenopus* Tsg-2 is highly homologous to xTsg. In particular, amino acid residues which have been shown to be required for the binding of xTsg to BMP and to Chordin are conserved. The expression of *Xenopus Tsg-2* mRNA was restricted to late stages of embryonic development; it was detected at tadpole stages in lateral plate mesoderm, neural crest, branchial arches and head mesenchyme. In microinjection experiments, the activity of *xTsg-2* mRNA was similar to that of *xTsg*. We conclude that two *Tsg* genes act in distinct temporal and spatial territories in the course of *Xenopus* embryonic development.

Keywords

twisted gastrulation; BMP; chordin; tolloid; Xenopus

The establishment of a BMP signalling gradient in the early embryo of vertebrates as well as invertebrates plays an important role in the determination of the dorsoventral (or back to belly) body axis. This gradient is generated in the extracellular space by an evolutionarily conserved system of interacting proteins involving the BMP antagonist Chordin that binds to BMP and prevents the binding of BMPs to their cognate receptors, metalloproteinases of the Xolloid/Tolloid family that cleave Chordin, and Twisted gastrulation (Tsg) a protein that binds both to BMP4 and Chordin (De Robertis *et al*., 2000). Several biochemical activities have been demonstrated for Tsg. First, Tsg facilitates the binding of Chordin to BMP leading to the formation of an inhibitory trimolecular complex (Oelgeschläger *et al*., 2000; Chang *et al*., 2001; Ross *et al*., 2001). Second, Tsg enhances the cleavage of Chordin by Xolloid/Tolloid, generating proteolytic fragments of Chordin that can still bind to BMP and retain some anti-BMP activity (Larraín *et al*., 2001). Third, Tsg competes with the binding of the proteolytic cleavage products of Chordin to BMP (Oelgeschläger *et al*., 2000; Larraín *et al*., 2001). In loss-of-function situations xTsg can cooperate with Chordin (Ross *et al*., 2000; Blitz *et al*., 2003) and with BMP4 (Zakin and De Robertis, 2003).

Multiple Tsg genes have been identified in fruit flies. In *Drosophila*, Tsg is required for the formation of a narrow stripe of peak BMP activity in the dorsal midline of the early fly embryo (Ross *et al*., 2001; Eldar *et al*., 2002).

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A second, highly homologous *Tsg* gene has been reported to be inactivated in the *crossveinless* (*cv*) mutant that lacks crossveins in the adult wing (Vilmos, 2001). The formation of crossveins is preceded by a localized peak of BMP signalling that can be visualized by phospho-MAD specific antibodies (Conley *et al*., 2000). A third Tsg-like gene has recently been identified in the *Drosophila* genome. *dTsg3* encodes a protein consisting exclusively of the carboxy-terminal cysteine-rich domain and maps close to the *shrew* locus (Vilmos *et al*., 2001). Shrew is the last of the *Drosophila* dorsal-ventral zygotic mutations that remains to be identified (De Robertis and Sasai, 1996). Interestingly, we have demonstrated that the ventralizing (pro-BMP) activity of Tsg resides in the domain encoded by dTsg3 (Oelgeschläger *et al*., 2003b). *shrew* mutant flies, like *tsg* mutants, are unable to form the amnioserosa, the dorsal-most tissue of the early fly embryo that requires maximum BMP signalling (Ferguson and Anderson, 1992; Arora *et al*., 1992).

In zebrafish, a second *Tsg* gene expressed at later stages of development has been reported (Ross *et al*., 2001). However, in *Xenopus*, mouse, and human only one *tsg* gene has been identified to date (Graf *et al*., 2001; Nosaka *et al*., 2003). In mouse, Tsg is required for the formation of bone and thymus, and has been implicated in the regulation of T-cell differentiation (Graf *et al*., 2002; Nosaka *et al*., 2003). $Tsg^{-/-}$ mice are viable and fertile, but are of small size and have kinky tails, osteoporosis, and other defects (Nosaka *et al*., 2003; Zakin and De Robertis, 2003).

Interestingly, when one copy of BMP4 is removed as well, $Tsg^{-/-}$; $BMP4^{+/-}$ embryos develop holoprocencephaly (Zakin and De Robertis, 2003). Since BMP4 becomes haploinsufficient in the absence of Tsg this shows that Tsg promotes BMP4 activity during forebrain development in the mouse. However, the overall phenotype of the mouse Tsg mutation was surprisingly mild. In this paper we report the isolation of a second *Tsg* gene in *Xenopus*.

Results and Discussion

A new *Xenopus* Tsg-like gene was identified in the public EST database (Accession # BJ075535; Fig. 1A) and sequenced on both strands. It encodes a 27 kD secreted protein with a potential signal peptide cleavage site at position 44 (VTG^{44}-$C^{45}N$) (Fig. 1B).*Xenopus laevis* is an allotetraploid species in which two pseudoalleles can be found for many genes (Kobel and Du Pasquier, 1986). However, the new gene, designated *xTsg-2*, does not appear to encode a pseudoallele of Tsg. *Xenopus* pseudoalleles share strong sequence identity at the nucleic acid level with over 90% amino acid identity in the coding region, although in the untranslated regions of the mRNA the sequence can be more diverse (Oelgeschläger *et al*., 2003b). For *Xenopus*, two *xTsg* pseudoalleles have already been reported (Oelgeschläger *et al*., 2000;Scott *et al*., 2001;Blitz *et al*., 2003). They share 90% amino acid identity including the signal peptides (Fig. 1B). The *xTsg-2* gene shares only 50% amino acid identity with the two *Tsg* pseudoalleles, even after excluding the signal peptide (Fig. 1B). The similarities are mainly found in the N- and C-terminal cysteine-rich domains that are required for the binding of xTsg to BMP and to Chordin (Oelgeschläger *et al*., 2000;Oelgeschläger *et al*., 2003a). We compared the xTsg-2 sequence to the Tsg proteins identified in other species and found that it is a distant member of the vertebrate Tsg family (Fig. 1C).

We recently identified amino acid residues required for the binding of Tsg to BMP and to Chordin (Oelgeschläger *et al*., 2003a). Mutations in the N-terminal domain of Tsg were found to enhance its ventralizing (BMP-promoting) activity and to interfere with BMP binding. As seen in Fig. 1D, these amino acids (indicated by arrowheads) are conserved in xTsg-2 as well as in Tsg proteins isolated from various vertebrate and invertebrate species. In the C-terminus, site-directed mutagenesis of C180 and C197 of xTsg into Alanine had the opposite effect, loss of the ventralizing activity, which correlated with a loss of the ability of xTsg to bind to Chordin

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(Oelgeschläger *et al*., 2003a). These residues are conserved in all Tsg proteins listed in Fig. 1D (arrowheads). We also identified an *in vivo* glycosylation site (S54) in xTsg that is conserved in xTsg-2 and most of the other Tsg proteins, with the exception of *Drosophila* and mouse Tsg (Fig. 1D, asterisk).

We next compared the expression patterns of *xTsg* and *xTsg-2. Xenopus Tsg* mRNA was detected in RT-PCR analysis from the onset of embryonic development until late tadpole stages while *xTsg-2* mRNA could not be detected until early tadpole stages (Fig. 2). In agreement with the RT-PCR analyses, *xTsg-2* transcripts became detectable by *in situ* hybridization at early tailbud tadpole stages, when transcripts could be detected in head mesenchyme, branchial arches and lateral plate mesoderm (Fig. 3 A,C). At later stages the signal increased significantly and expression became clearly visible in the head region, lateral plate mesoderm and neural crest (Fig. 3B). When embryos were cleared in Murray's solution, higher staining in the midbrain-hindbrain isthmus became apparent (Fig. 3D). In the branchial arches, *xTsg-2* was expressed in the neural crest derived central core, but not in the pharyngeal pouches and ectoderm (Fig. 4 A,B). The neural tissue showed weak signals in the dorsal margin of the retina, the ventricular (proliferative) zone of most of the hindbrain, and in the midbrain-hindbrain border (Fig. 4C). Expression in head mesenchyme extended throughout head structures, surrounding the eye (Fig. 4C) and otic vesicle, but excluding the cement gland (Fig. 4B). At the level of the trunk, *xTsg-2* was expressed in the lateral plate mesoderm (Fig. 4F), at weaker levels in the pronephros (Fig. 4D) and pronephric duct (data not shown). In addition, staining in neural crest cells that migrate into the dorsal fin was visible (Fig. 4F).

When compared to *xTsg-2*, *xTsg* was found to have a very different expression pattern. *xTsg* is expressed maternally and ventrally during gastrulation (Oelgeschläger *et al*., 2000). At early tadpole stages, strong expression of *xTsg* was found in the dorsal eye, the heart anlage and the peri-anal region (Oelgeschläger *et al*., 2000) (Fig. 3E). At later stages, the posterior expression domain becomes restricted to the posterior wall (ventral mesoderm, Gont *et al*., 1993) of the tip of the tailbud (Fig. 3F). We conclude that *xTsg-2* and *xTsg* are genes with distinct expression patterns. *xTsg-2* is expressed at tadpole stage in the mesenchyme of the head, branchial arches and lateral plate mesoderm. Expression of *xTsg* is more extensive in earlier development (Oelgeschläger *et al*., 2000), while xTsg-2 is expressed at tadpole stages.

The high homology between xTsg and xTsg-2, especially the conservation of amino acids involved in the interaction of Tsg with BMP and Chordin, implied that *xTsg-2* and *xTsg* might have similar biological activities. We tested *xTsg-2* activity by microinjecting synthetic mRNA and found that the phenotype observed was undistinguishable to that of *xTsg* (Fig. 5). Microinjection of *xTsg-2* mRNA induced the formation of an enlarged proctodeum that often detached from the trunk (Fig. 5C). This phenotype is seen in *xTsg* mRNA injections (Fig. 5B; Chang *et al*., 2001;Oelgeschläger *et al*., 2003a) and is also observed in embryos in which BMP signalling is modulated by a variety of agents (Beck *et al*., 2001;Oelgeschläger *et al*., 2003a). The results indicate that *xTsg-2* and *xTsg* have similar overexpression phenotypes in *Xenopus* embryos.

The existence of a second *Tsg* gene with redundant functions could help to provide an explanation for why the loss-of-function of mouse Tsg has only weak phenotypes (Nosaka *et al*., 2003; Zakin and De Robertis, 2003). We note that although the mouse and human genomes have been completely sequenced, some genes, particularly those consisting of small exons, are sometimes difficult to identify.

The expression of *xTsg-2* did not overlap at any time with the expression of *chordin*, whereas the broad early expression of *xTsg* and its specific expression later in the posterior wall of the tailbud may imply a functional interaction of xTsg with Chordin. *Chordin* is expressed in the

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posterior notochord or chordoneural hinge in the tailbud at early tadpole stages (Sasai *et al*., 1994). The expression domain of *xTsg-2* in lateral plate mesoderm and neural crest adjoins the expression of *Xolloid* in the inner layer of the epidermis, and in the head region *xTsg-2* overlaps with the expression of *xBMP1* (Goodman *et al*., 1998). A number of proteins that contain Chordin-like BMP-binding domains have been identified in vertebrates and for many of them a functional or biochemical interaction with TGF-β or BMP has been demonstrated (García Abreu *et al*., 2002). Thus, it seems possible that xTsg-2 may act together with BMP and members of the Tolloid family of metalloproteases on other Chordin-related proteins at later stages of development. In future it will be important to identify the extracellular proteins that interact with xTsg-2 in the course of embryonic development.

Experimental Procedures

DNA constructs

The constructs used for *xTsg* microinjections were previously described (Oelgeschläger *et al*., 2000). For *xTsg-2*, the coding region was amplified by PCR and cloned into the pCS2+ vector. For mRNA synthesis the pCS2-xTsg-2 construct was linearized with *Not*I and transcribed with SP6 RNA Polymerase using the mMessage mMachine Kit (Ambion). The sequence of the *xTsg-2* cDNA described here has been deposited in GenBank under accession number BK001651).

In situ *hybridization and RT-PCR*

Xenopus embryos obtained by *in vitro* fertilization were maintained in Barth Solution (Sive *et al*., 2000) and staged according to Nieuwkoop and Faber (1994). Whole-mount *in situ* hybridization for *xTsg* were performed as described (Oelgeschläger *et al*., 2000). For *xTsg-2* antisense probe, a full length EST (Accession number: BJ075535) was linearized with *Eco*RI, transcribed with T7 RNA polymerase and prepared as described (Oelgeschläger *et al*., 2000; and http://www.hhmi.ucla.edu/derobertis/index.html). Embryos were made transparent in Murray's Clearing Solution (2:1 benzyl benzoate: benzyl alcohol). Histological sections of *xTsg-2* stained embryos were embedded in paraplast, sectioned at 25 μm, dewaxed, dehydrated and embedded in Permount. RT-PCR conditions and primer sets were previously described (Oelgeschläger *et al*., 2000; and http://www.hhmi.ucla.edu/derobertis/index.html). For *xTsg-2*, the primer sets were 5′-ATG AAA TGG GGT CCT AC-3′ (forward) and 5′-AGT TTC CTT CTA TGG CTT GG-3′ (reverse).

Acknowledgements

We thank Drs. O. Wessely and J. Kim for comments on the manuscript. This work was supported by the National Institutes of Health (R37 HD21502-16 to E.M.D.R) and the Max-Planck Society. E.M.D.R. is an Investigator of the Howard Hughes Medical Institute.

Abbreviations used in this paper

BMP, bone morphogenetic protein; Tsg, twisted gastrulation gene; xTsg, Xenopus twisted gastrulation gene..

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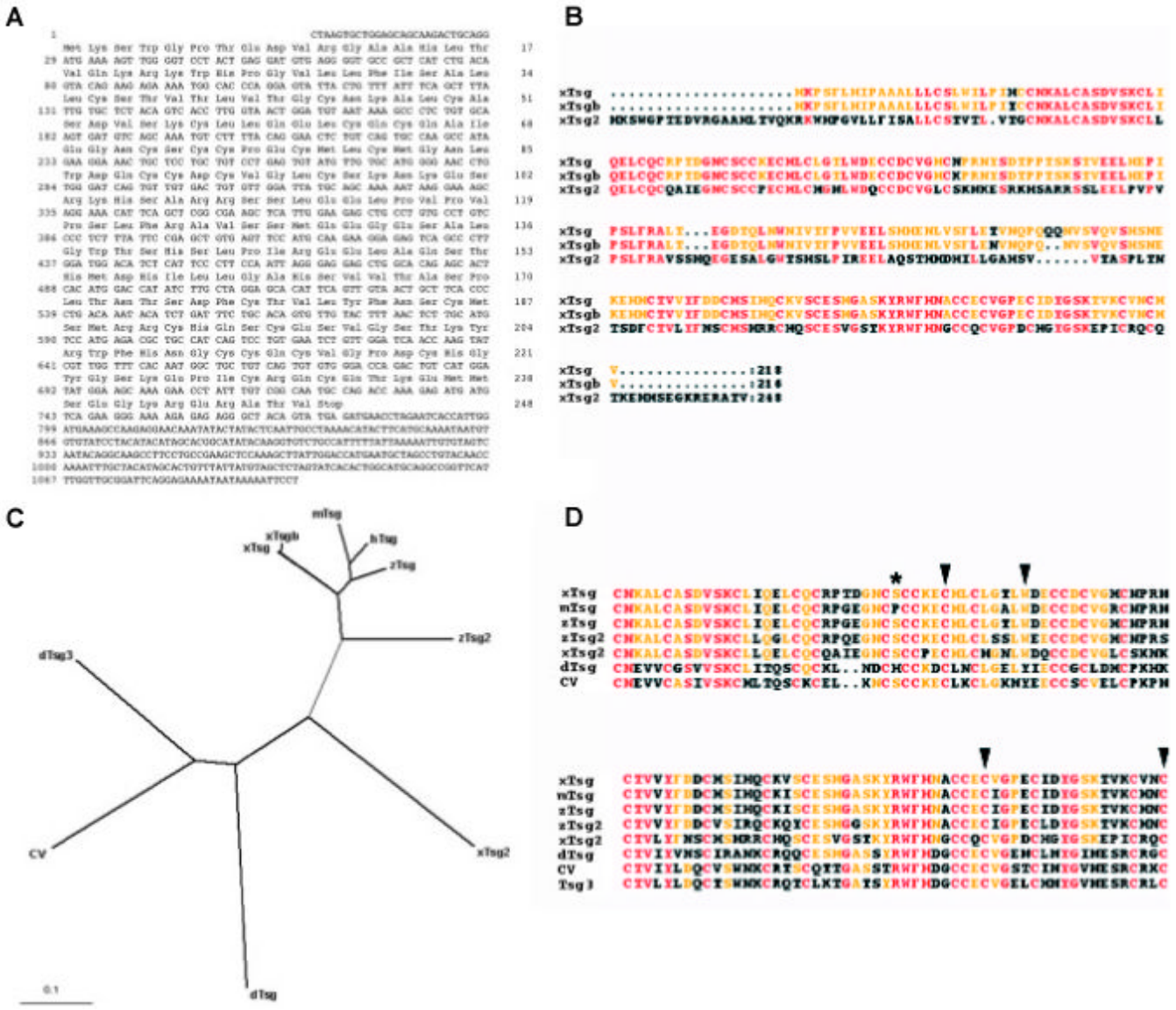

Fig. 1. Analysis of the xTsg2 cDNA and protein sequence

(A) Nucleotide and amino acid sequence of xTsg-2. **(B)** Alignment of the two pseudoalleles of *xTsg* with *xTsg-2*. **(C)** Dendrogram showing the phylogenic relations between the *Xenopus* (*xTsg*, *xTsgb*, *xTsg-2*), mouse (mTsg), human (hTsg), zebrafish (zTsg, zTsg-2) and *Drosophila* Tsg proteins (dTsg, cv/dTsg2, dTsg3). **(D)** ClustalW alignment of the indicated protein sequences demonstrating the conservation of amino acids required for ventralizing activity or chordin binding (arrowheads) and the position of an *in vivo* glycosylation site (asterisk). The accession numbers of the sequences used for the alignments were for xTsg, AF245221; xTsgb, AF279246; xTsg-2, BJ075535; mTsg, AAG00605; hTsg, NP_065699; zTsg, AAK27324; zTsg-2, AAK13255; dTsg, A53836; cv, CG12410; dTsg3, CG11582.

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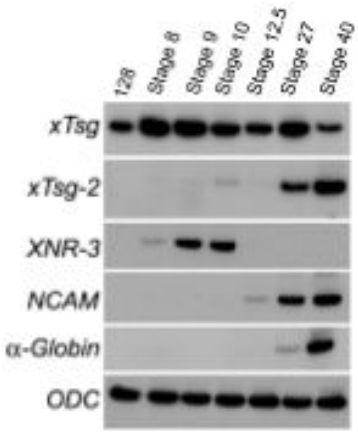

Fig. 2. Time course of *xTsg* and *xTsg-2* gene expression

mRNA samples were analyzed by RT-PCR at the indicated developmental stages. *Xenopus Tsg* mRNA can be detected throughout development, whereas xTsg-2 expression starts at tailbud stages. Other markers shown are XNR-3, which responds specifically to the early β-catenin signal in Xenopus; NCAM, which marks neural tissue; α-globin, which marks ventral mesoderm and blood differentiation; ODC (ornithine decarboxilase) serves as an mRNA loading control.

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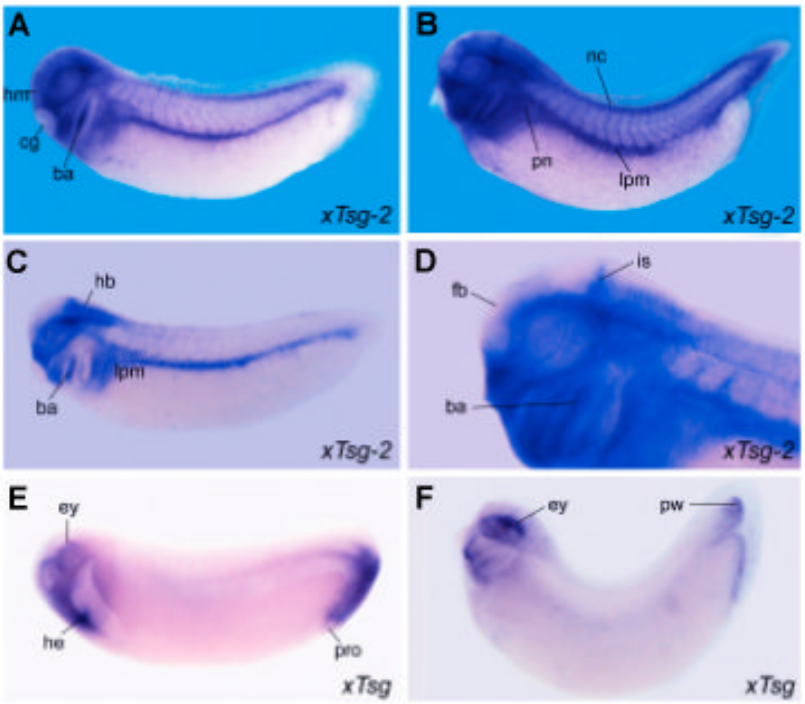

Fig. 3. Whole-mount in situ hybridization analysis of xTsg-2 and xTsg expression

(A,C)*xTsg-2* expression at stage 27 and **(B,D)** stage 33 embryos showing expression in the branchial arches (ba), neural crest (nc), lateral plate mesoderm (lpm) and head mesenchyme (hm). Embryos in C and D were made transparent in Murray's Clearing Solution (2:1 benzyl benzoate: benzyl alcohol) to visualize the expression of *xTsg-2* localized to the midbrain-hindbrain border, the isthmus (is). D is a close up of the embryo in B. **(E)** Stage 27 and **(F)** stage 33 embryos stained for *xTsg* and cleared in Murray's solution reveal expression in the dorsal eye (ey), heart anlage (he) and proctodeum (pro), as well as in the posterior wall (pw) of the tailbud. cg, cement gland; fb, forebrain; hb, hindbrain; pn, pronephros.

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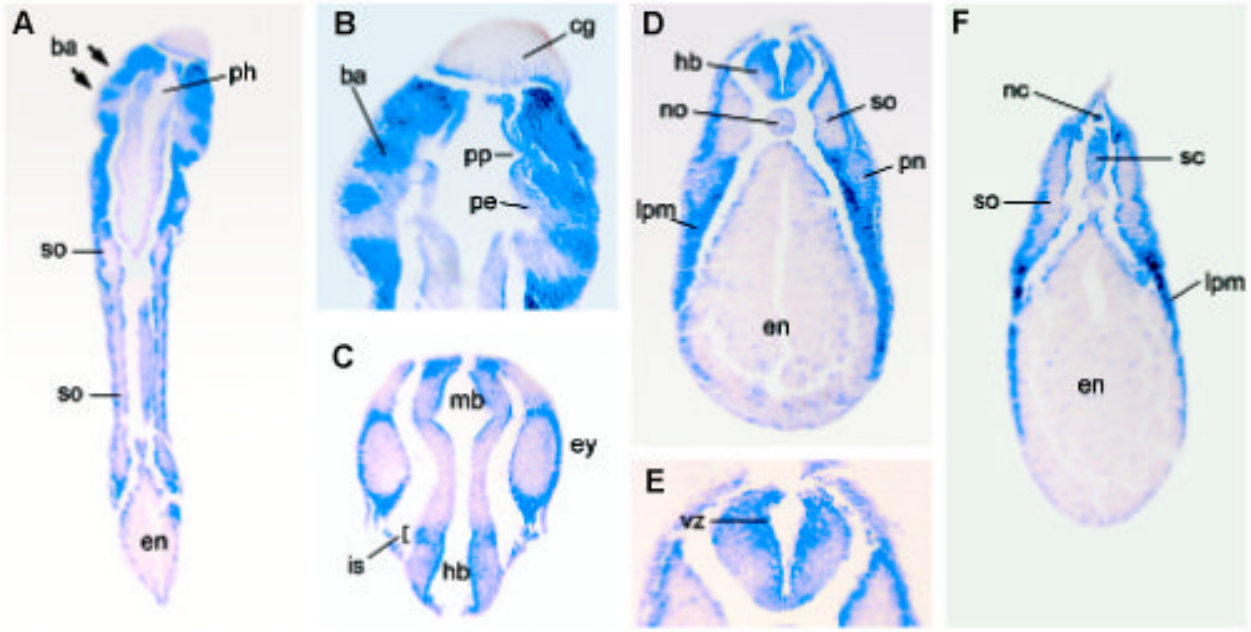

Fig. 4. *xTsg-2* is expressed in the mesenchyme of the branchial arches, lateral plate and other tissues

Histological sections of whole-mount in situ hybridization. **(A)** Coronal section of an embryo at stage 33 showing expression of *xTsg-2* in the branchial arches (ba), but not in the pharynx (ph). **(B)** Close up of (A) indicating the expression of *xTsg-2* is restricted to the branchial arches (ba), but devoid of detectable expression in the pharyngeal pouches (pp) consisting of pharyngeal endoderm (pe) as well as the cement gland (cg). **(C)** *xTsg-2* expression is observed at low amounts in the eye (ey), but strong in the surrounding head mesenchyme. The isthmus (is) is stained in this coronal section. **(D)** Transverse section of a stage 33 embryo at the level of the trunk showing *xTsg-2* expression in the lateral plate mesoderm (lpm) and hindbrain (hb) and low levels in the pronephros (pn). The somites (so), endoderm (en) and notochord (no) are not stained. **(E)** Close up showing *xTsg-2* expression in the ventricular (proliferative) zone (vz) of the hindbrain. **(F)** Transverse section showing *xTsg-2* expression in the dorsal fin neural crest cells and dorsal spinal cord (sc).

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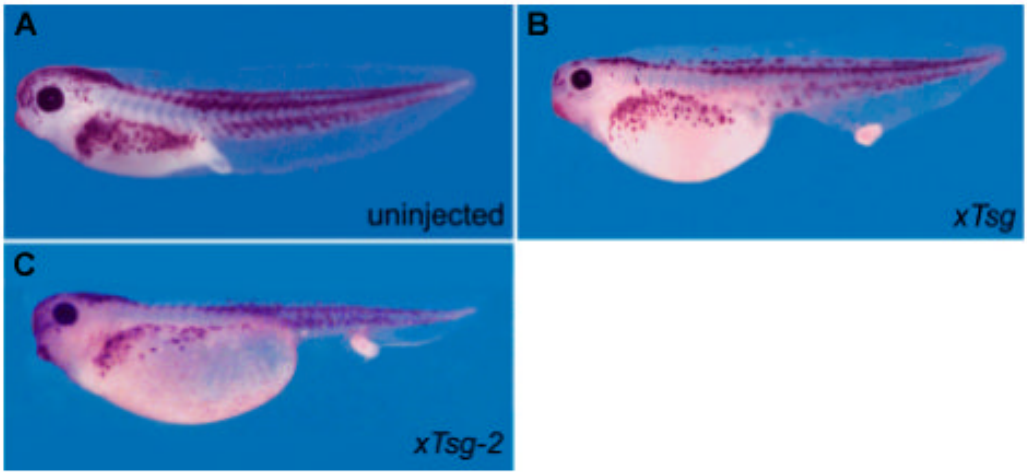

Fig. 5. Microinjection of *xTsg-2* mRNA generates phenotypes similar to those of *xTsg*

(A) Uninjected stage 42 embryo as control. **(B)** Embryo microinjected with 500 pg of *xTsg* mRNA or **(C)** 500 pg *xTsg-2* mRNA ventrally at the 4-cell stage.