Twisted gastrulation loss-of-function analyses support its role as a BMP inhibitor during early *Xenopus* embryogenesis

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Summary

BMP signals play important roles in the regulation of diverse events in development and in the adult. In amniotes, like the amphibian *Xenopus laevis*, BMPs promote ventral specification, while chordin and other BMP inhibitors expressed dorsally in the Spemann's organizer play roles in establishment and/or maintenance of this region as dorsal endomesoderm. The activities of chordin are in turn regulated by the secreted proteolytic enzymes BMP1 and Xolloid. Recently, we and others have identified the protein twisted gastrulation (TSG) as a soluble BMP modulator that functions by modifying chordin activity. Overexpression and genetic analyses in *Drosophila*, *Xenopus* and zebrafish together with in vitro biochemical studies suggest that TSG might act as a BMP antagonist; but there is also evidence that TSG may promote BMP signaling. Here we report examination of the in vivo function of TSG in early *Xenopus* development using a loss-of-function approach. We show that reducing TSG expression using antisense TSG morpholino oligonucleotides (MOs) results in moderate head defects. These defects can be rescued both by a TSG that cannot be inhibited by the MO, and by the BMP antagonists chordin and noggin. Furthermore, while neither the onset of gastrulation nor the expression of marker genes are affected in early gastrulae, dorsal marker gene expression is reduced at the expense of expanded ventral marker gene expression beginning at mid to late gastrula stage. TSG-MO and Chd-MOs also cooperate to strongly repress head formation. Finally, we note that the loss of TSG function results in a shift in tissue responsiveness to the BMP inhibitory function of chordin in both animal caps and the ventral marginal zone, a result that implies that the activity of TSG may be required for chordin to efficiently inhibit BMPs in these developmental contexts. These data, taken together with the biochemistry and overexpression studies, argue that TSG plays an important role in regulating the potency of chordin's BMP inhibitory activity and TSG and chordin act together to regulate the extent of dorsoanterior development of early frog embryos.

Key words: Twisted gastrulation, Bone morphogenetic proteins, Chordin, BMP1, BMP4, Tolloid, BMP signaling, *Xenopus*, CCN family

Introduction

Secreted signaling molecules are critical for coordinating cell fate specification and cell behavior. Bone morphogenetic proteins (BMPs) comprise the largest subgroup of the transforming growth factor beta (TGFβ) superfamily of secreted signaling molecules. Over 30 different members of the BMP family have been identified to date that function in a wide variety of developmental processes in organisms from basal metazoa to man (Hogan, 1996; Newfeld et al., 1999; Chang et al., 2002; Zhao et al., 2003).

BMPs bind to heteromeric transmembrane receptor complexes that function to directly phosphorylate the intracellular signal transducers Smads 1, 5 and 8 (reviewed by Itoh et al., 2000). These Smad proteins translocate to the nucleus upon activation where they function to regulate transcription of BMP target genes. The availability of BMP ligands is regulated in the extracellular environment by a variety of positive and negative factors. BMPs, like other members of the TGFβ superfamily, signal as obligate disulfide-linked dimers that are synthesized and proteolytically processed from precursor pre-proproteins. BMP dimers are secreted into the extracellular environment where they interact with cell surface proteoglycans and other secreted factors that act to modulate their behavior. Most secreted BMP modulators are inhibitors of ligand activity that act by sequestering BMP ligands from their cell surface receptors. These inhibitors include noggin, chordin, follistatin and members of the DAN family of proteins (Smith and Harland, 1992; Sasai et al., 1994; Hemmati-Brivanlou et al., 1994; Bouwmeester et al., 1996; Fainsod et al., 1997; Hsu et al., 1998). Among these, the regulation of BMP signaling by chordin appears the most complex.

Chordin is specifically expressed in the dorsal marginal zone (Spemann's organizer) of the *Xenopus* gastrula stage embryo and its ectopic expression in ventral mesoderm results in the

development of secondary dorsal axes (Sasai et al,1994). Chordin functions to oppose the action of BMPs, which are expressed broadly in the early gastrula and are ventralizing factors (Dale et al., 1992; Jones et al., 1992). BMPs induce the expression of ventral-specific transcription factors and chordin blocks ventralization by BMPs to maintain dorsal cell fates (Sasai et al., 1994; Piccolo et al., 1996). Chordin's activity, like its *Drosophila* counterpart encoded by *short gastrulation* (*sog*) (Zusman et al., 1988; Francois et al., 1994), is in turn opposed by secreted tolloid-related metalloproteases including BMP1 and Xolloid (Marques et al., 1997; Piccolo et al., 1997; Scott et al., 1999; Wardle et al., 1999; Blitz et al., 2000). These metalloproteases act by endoproteolytically cleaving chordin at several specific sites (Scott et al., 1999). Cleavage of chordin results in the separation of its two BMP binding domains onto separate cleavage products that appear to have 10-fold lower affinities for BMP ligands (Larrain et al., 2000). Whether or not the cleavage products have reduced half-lives in vivo is currently unclear, however, cleavage appears to function in the early embryo to either limit chordin's range of action or to alter the shape of a chordin protein gradient.

Recently it has become clear that the modulation of BMP signaling by chordin is more complex. We, and others, have identified the secreted protein twisted gastrulation (TSG) as a BMP modulatory protein (Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). TSG physically interacts with both BMP4 ligand and chordin. This interaction may either lead to stronger repression of BMP signaling through BMP sequestration (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001), or possibly to more readily release BMP4 ligand from chordin cleavage products after endoproteolysis by the metalloproteases (Oelgelschlager et al., 2000; Larrain et al., 2001). Finally, TSG also enhances the proteolysis of chordin at an underutilized site (Scott et al., 2001) and therefore may function to enhance chordin inactivation and hence up-regulate (de-repress) BMP signaling (Scott et al., 2001; Larrain et al., 2001). However, while the in vivo functional significance of this latter cleavage remains to be determined, it may also result in the production of a hyperinhibitory version of chordin, as has been suggested in the case of *Drosophila* SOG ['super-sog' (Yu et al., 2000)]. These observations have led to a complicated picture. One model suggests that TSG may function as both a BMP activator and also as a BMP inhibitor, and its mode of behavior depends on its coexpression with other factors (e.g. metalloproteases).

Superficially, some of these proposed mechanisms appear at odds with others. *Drosophila* genetics initially provided evidence consistent with the idea that TSG functions in the dorsal midline of the fly embryo (Zusman and Weischaus, 1985; Mason et al., 1994) as a BMP activator to enhance signaling by the *Drosophila* BMP encoded by *decapentaplegic* (*dpp*). The suggestion that *Drosophila* TSG functions as a BMP (DPP) activator was based on correlative evidence. TSG is required for proper amnioserosal development in the dorsal midline of the fly embryo (Zusman and Weischaus, 1985; Mason et al., 1994) and slight reductions in the level of DPP resulted in loss of amnioserosa (Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992). These data supported the notion that TSG might function to boost DPP signaling to higher levels in the dorsal midline. However, paradoxically, the *Drosophila* chordin homolog SOG, a known BMP inhibitor, is also required for amnioserosal development (Marques et al., 1997). To bring together these different observations, it has been proposed that the function of TSG as a BMP inhibitor (together with SOG) might facilitate higher level BMP signaling in the dorsal midline by 'transporting' BMPs to this region where they are then released by metalloprotease cleavage of SOG-TSG-BMP complexes (Ross et al., 2001). While it is clear that TSG is required both for BMP inhibition in dorsolateral regions and for high level BMP signaling in the *Drosophila* dorsal midline (Ross et al., 2001), this transport mechanism remains hypothetical. Finally, a recent study on mouse TSG, using a knockout strategy, demonstrated that TSG-deficient mice have thymocyte and bone defects (Nosaka et al., 2003). While the behavior of Smad1 phosphorylation in thymocytes is consistent with TSG functioning as a BMP inhibitor, the bone defects observed can be more easily explained, with current knowledge, if TSG is an activator (Nosaka et al., 2003). Therefore, the possibility that TSG can function directly to both antagonize and activate BMP signaling remains a viable model.

While the biochemistry has supported all of the multiple scenarios postulated, in vivo support for many of these conclusions have been primarily provided by forced overexpression studies. Therefore, we wished to examine the in vivo function of TSG using a loss-of-function approach in *Xenopus* embryos, the vertebrate system where the role of BMP signaling is most thoroughly studied, to attempt to provide evidence for a role for TSG as either a positive or a negative regulator of the BMP pathway. This would focus our attention toward certain models for further testing. Toward this end we have used morpholino antisense oligonucleotides (MOs) to inhibit the translation of endogenous TSG (and chordin) and have examined the phenotypes of *Xenopus* embryos during early development. We report that MO 'knockdown' of TSG expression on the dorsal side of the embryo results in a reduction of anterior head structures, and this phenotype can be rescued by either restoring TSG expression or by elevating chordin expression. Furthermore, we have found that loss of TSG function does not appear to significantly reduce dorsal marker gene expression in early gastrulae, but at late gastrula to early neurula stages markers of dorsal cell fates are reduced at the expense of expanded ventral marker gene expression, implying a role for TSG as a BMP antagonist. Using MOs to both TSG and chordin together we also show that embryos have stronger ventralization phenotypes consistent with previous suggestions that these molecules coordinately act to inhibit BMP signaling. Finally, we show that TSG is required for an efficient response of embryos or explanted animal caps to ectopically expressed chordin. These in vivo data support a model whereby TSG primarily functions to assist chordin as a BMP inhibitor to establish or maintain dorsal cell fates during gastrulation.

Materials and methods

Fertilizations, embryo culture and microinjections

Embryos were obtained by in vitro fertilization, de-jellied and maintained in 0.1× modified Barth's saline (MBS) using standard techniques (Sive et al., 2000). Embryos were microinjected in 1× MBS and then transferred and maintained in 0.1× MBS. Capped mRNAs for microinjection were prepared using the Ambion

mMessage Machine SP6 in vitro transcription kit according to the manufacturer's instructions.

Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed according to the method of Harland (Harland, 1991) using BM purple (Boehringer-Mannheim) as substrate. In situ probes were made as follows. Goosecoid probe was prepared by linearizing pBSSK*Xgsc*H43 with *Eco*RI and RNA was transcribed using T7 RNA polymerase (Cho et al., 1991). Otx2 and sizzled probes were prepared as previously described (Blitz and Cho, 1995; Blitz et al., 2000). MyoD and Sox2 probes were prepared from pBSSKII+ based clones from an arrayed library, and Krox20 was prepared from pGEM4-Krox20. All of these were digested with *Eco*RI and T7 RNA polymerase was used. HoxB9 probe was a kind gift from Bruce Blumberg.

Plasmid templates for in vitro transcription

Xenopus and human TSG mRNAs (Scott et al., 2001; Chang et al., 2001) were prepared from plasmid templates after cloning the XTSG coding region into pCS2+. To generate a *Xenopus* TSG rescue construct, PCR product was generated using the oligonucleotides 5′-CCCGCTAGCATGCAATAAGGCTCTCTGTGCTA-3′ and 5′-CCCCTCGAGTTAAACCATACAGTTCACGCACTT-3′ which was directionally cloned between the *Nhe*I and *Xho*I sites of pCS2+mTSG replacing the mouse TSG sequences (Scott et al., 2001). The resulting plasmid, pCS2+BM40-XTSG encodes the *Xenopus* TSG protein as a fusion to the signal peptide from the extracellular matrix protein BM40. Chd-MO rescue experiments utilized chordin mRNA synthesized from pCS2+Xchordin (kindly provided by Eddy De Robertis), which lacks its 5′ UTR and therefore lacks Chd-MO target sequences. pCS2+ template DNAs representing the two different chordin genomic copies, and containing Chd-MO complementary sequences, were reconstructed by substituting the 5′ *Eco*RI-*Bgl*II fragment of pCS2+Xchordin with DNA fragments produced by RT-PCR that contain its 'native' 5′ UTR or the 5′ UTR of the redundant pseudotetraploid copy (GenBank accession number AW460332). To generate these PCR products, the respective 5′ oligos 5′-GGGGAATTCTACGAGACAGAACGTTTGGAACCAC-3′ and 5′-GGGGAATTCAGCTTGGTTCGGGACAACCACAAA-3′ were used in combination with the 3′ oligo 5′-GTGCATAACTCCGAATGG-TTC-3′. The integrity of all constructs was verified by sequencing. Noggin RNA was synthesized with SP6 polymerase using *Eco*RI-linearized pSP64T-Noggin template. All chordin and TSG mRNAs were prepared using SP6 polymerase from pCS2+ based plasmids linearized with *Not*I.

RT-PCR

The protocol for RT-PCR was as previously described (Blitz and Cho, 1995) except that in some cases we used ethidium bromide-stained agarose gels to analyze the reaction products. Primers used to amplify otx2 and histone H4 were as previously described (Blitz and Cho, 1995). Msx1 primers: 5′-GATTCGTTGATAGGATCGCACT-3′ and 5′-GGTCTCTCCCAGGTTTCCTA-3′. Vent2 primers: 5′-AGGC-CATTTGTTAGATATTAATC-3′ and 5′-GTATTTTTCATAGAATAT-ACACGC-3′. Otx2, histone H4, vent2 and msx1 amplification reactions used 27, 24, 30 and 30 cycles respectively for agarose gels, and 21 or 25 cycles for 5% PAGE with radioactive PCR products. All RT-PCR reactions were done three times, with somewhat varying extents of shift in the response of the animal caps to chordin or noggin in the presence of TSG-MO (e.g. 2- to 5-fold shift in the dose requirement for noggin RNA to induce otx2 in the animal caps). Representative experiments are shown.

Morpholinos and in vitro translations

Capped mRNAs were synthesized using Ambion mMessage Machine T3 and SP6 in vitro transcription kits. Inhibition of translation of TSG and chordin mRNAs was performed as previously described (Taylor et al., 1996). Briefly, 1 μg of each capped mRNA was translated using the Promega Rabbit Reticulocyte System and [^{35}S]methionine in the absence or presence of 2.5 μM of the specified morpholino antisense oligonucleotide. Reactions were RNAse A treated, subjected to SDS-PAGE, and labeled proteins were detected by autoradiography. Two different morpholino oligos were tested for their efficacy in inhibiting TSG translation (see Fig. 1A). One TSG-MO (5′-AGGAAA-GAGGGCTTCATACTTGGCC-3′) hybridizes to the region around the translational start site of the published TSG sequences (GenBank accession numbers AF279246 and AF245221), and the other was designed against an unpublished allele of *Xenopus* TSG, which contains the sequence 5′-GCCAA**T**TATGAAGCCCTCTTTCCTT-3′ at the translation start (the polymorphic base is in bold and the ATG is underlined). The two TSG-MOs differ in their sequences by one nucleotide in the middle. Both TSG-MOs efficiently inhibited the synthesis of TSG protein containing either translational start sequences, and both efficiently induced similar phenotypes in vivo. We therefore refer to both morpholino oligos as TSG-MO and do not distinguish between the versions of these MOs used in the text. Two chordin MOs, designated ChdA-MO and ChdB-MO, were designed against the published chordin allele (Sasai et al., 1994) and a sequence for a chordin EST (GenBank accession number AW460332). These are 5′-CAGCATTTTTGTGGTTGTCCCGAAC-3′ and 5′-GGGA-CACTGCATTTTTGTGGTTCCA-3′, respectively. Throughout the text we designate an equimolar mixture of these simply as Chd-MO. Luciferase control RNA was provided with the Promega Rabbit Reticulocyte. 'Control morpholino' refers to Genetools 'Standard' MO. We find that injection of control MO even at concentrations as high as 60 ng/embryo has no observable effect on *Xenopus* development. The sequences of GDF6-MO and BMP7-MO used for control for blastopore closure are: GDF6-MO 5′-GCAGAGGGCTC-CTGTATGTATCCAT-3′ (GenBank accession number AF155125) and BMP7-MO 5′-CTGTCAAAGCATTCATTTTGTCAAA-3′ (GenBank accession number U38559).

Results

Specific inhibition of translation of twisted gastrulation and chordin using morpholino antisense oligonucleotides

To examine the role of twisted gastrulation (TSG) in BMP signal regulation in *Xenopus* embryos, we sought to inhibit endogenous TSG expression using morpholino antisense oligonucleotides ('morpholinos'; MOs). MOs complementary to the 5′ untranslated region (UTR) of mRNAs block translation initiation and are an effective means of reducing translation of endogenous mRNAs in *Xenopus* embryos (reviewed by Heasman et al., 2002). We compared the published cDNA sequences corresponding to the two functionally redundant pseudotetraploid copies of the *Xenopus* TSG (XTSG) gene (Oelgeschlager et al., 2000; Scott et al., 2001) and synthesized a MO (TSG-MO) that is complementary to both genomic copies (Fig. 1A). We first examined the efficacy and specificity of the TSG-MO to inhibit translation from XTSG mRNAs in in vitro translation reactions. The TSG-MO efficiently inhibited translation of XTSG protein (Fig. 1B, compare lanes 4 and 5). In contrast, the TSG-MO had no effect on translation of either a luciferase control mRNA (Fig. 1B, lanes 2 and 3), chordin mRNAs (Fig. 1C, lanes 7, 11 and 15) or XTSG mRNA in which the 5′ untranslated sequence complementary to the TSG-MO was modified (BM40-XTSG; Fig. 1B, lanes 8 and 9). In addition, two different MOs directed against chordin (discussed below) failed to inhibit XTSG

translation (Fig. 1B, lane 6 and 7) further demonstrating the specificity of the TSG-MO. These data taken together demonstrate that the TSG-MO specifically inhibits translation of *Xenopus* TSG protein in vitro.

As we also wished to examine the in vivo functional relationship between TSG and chordin, we designed MOs to inhibit chordin translation. Since it was not possible to easily make one chordin MO complementary to both pseudotetraploid isoforms, we designed two different Chd-MOs (ChdA-MO and ChdB-MO; Fig. 1A) each directed against one of the chordin isoforms and tested them either alone or in combination. In similar fashion to the TSG-MO, we first tested the efficacy of our chordin MOs in in vitro translations with various chordin mRNA templates. Both of the chordin MOs most efficiently inhibited translation of their respective target mRNAs ('ChdA' and 'ChdB' mRNAs) to which they were designed (Fig. 1C, lanes 4-11). Furthermore, each of the Chd-MOs also reduced the translation of the other chordin mRNAs. Finally, neither of the Chd-MOs inhibited the translation of luciferase (Fig. 1C, lanes 1-3), TSG (as discussed above; Fig. 1B, lane 6 and 7), or a chordin mRNA containing a 5′ UTR lacking MO recognition sequences (Δ5′-Chd; Fig. 1C, lanes 12-15). In conclusion, TSG and chordin protein translation in vitro is specifically inhibited by TSG-MO and Chd-MOs respectively.

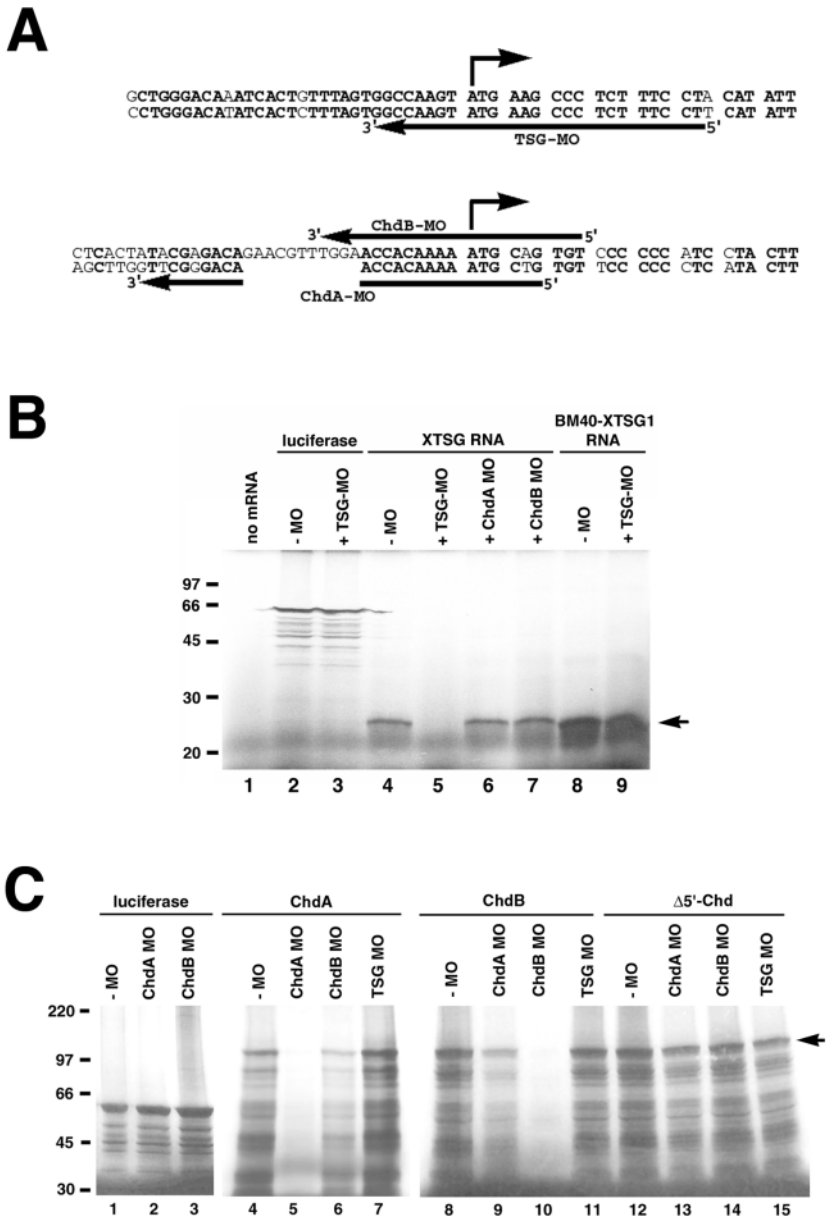

Fig. 1. Specific inhibition of twisted gastrulation and chordin translation using antisense morpholino oligonucleotides. (A) Sequences of the TSG-MO and two chordin MOs used in this study and their relative positions to translation start sites on their respective mRNAs. (B) The TSG-MO, but not chordin MOs, inhibits translation of TSG from the intact TSG mRNA, but not from a TSG mRNA that contains a modified sequence upstream of the TSG translation start site (BM40-XTSG1: see Materials and methods). (C) Both ChdA-MO and ChdB-MO completely block the translation of chordin RNA that contains their respective complementary starting sequences, and they also mutually reduce the translation of chordin from the other allelic RNA. Chordin MOs neither inhibit the translation of a chordin mRNA that lacks the 5′-UTR (Δ5′-Chd), nor do they interfere with the translation of TSG. Arrows in B,C indicate the XTSG1 and chordin proteins, respectively.

TSG-MO and Chd-MOs block TSG and chordin function in vivo

To further examine the specificity of the TSG-MO, we sought to inhibit TSG overexpression phenotypes by coinjecting the TSG-MO and TSG mRNA to which the TSG-MO was designed. Overexpression of TSG in *Xenopus* embryos results in a complex set of malformations in late tailbud and early tadpole stages including most prominently a reduction of head structures and defective ventral tail fin development (Chang et al., 2001; Scott et al., 2001; Oelgeshlager et al., 2000; Larrain et al., 2001). Head reduction and abnormal posterior morphogenesis first becomes visible during neurula stages (data not shown) and can readily be seen in 'pre-tailbud' stage 24 embryos (Fig. 2B). However, coinjection of the TSG-MO together with XTSG mRNA results in rescue of embryo morphology (Fig. 2C). Therefore, taking the in vitro translation data together with these in vivo observations, we suggest that the TSG-MO specifically inhibits XTSG function in vivo.

We also similarly tested the Chd-MOs by examining their ability to block the dorsalizing effects of chordin mRNAs. Ectopic expression of chordin in the ventral marginal zone, results (via its inhibition of BMP signaling) in the specification of a second Spemann organizer and therefore development of secondary dorsal axes (Sasai et al., 1994). We injected 5 pg of chordin mRNAs alone, or together with their respective Chd-MOs, into the ventral marginal zone and quantitated secondary axis formation. Chordin isoform A (ChdA; see Materials and methods) mRNA induced secondary axes in 98% of the injected embryos (n=43; Fig. 2D),

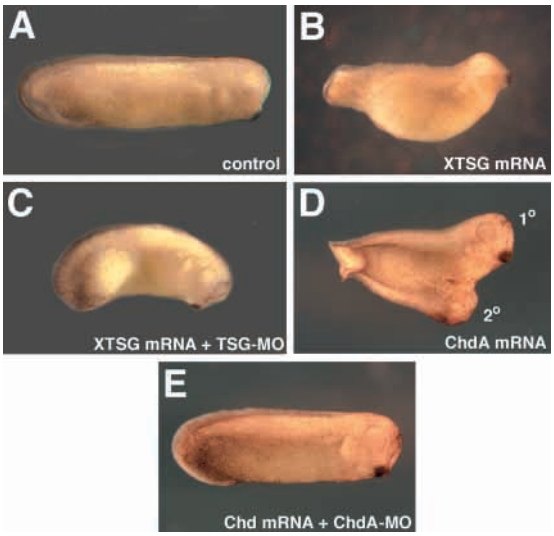

Fig. 2. Antisense TSG and chordin MOs block the in vivo activity of TSG and chordin respectively. (A) A stage matched uninjected control embryo. (B,C) Microinjection of 1 ng of *Xenopus* TSG mRNA per blastomere at the four-cell stage induces head and tail defects in early tailbud stage frog embryos (B), and this phenotype is inhibited by coinjecting 5 ng TSG-MO per blastomere together with the XTSG mRNA (C). (D,E) Similarly, 2.5 pg ChdA mRNA microinjected into the marginal zone of each ventral blastomere at the four-cell stage efficiently induces secondary axes (D) and these are completely blocked by ventral coinjection together with 5 ng/blastomere of ChdA-MO (E). Identical results were obtained for coinjections of ChdB-MO together with ChdB mRNA (data not shown). Coinjection of either XTSG or chordin mRNAs together with a control MO has no effect on the ability of these mRNAs to elicit these phenotypes. In all panels anterior is to the right and dorsal is to the top. 1° and 2° indicate primary and secondary axes.

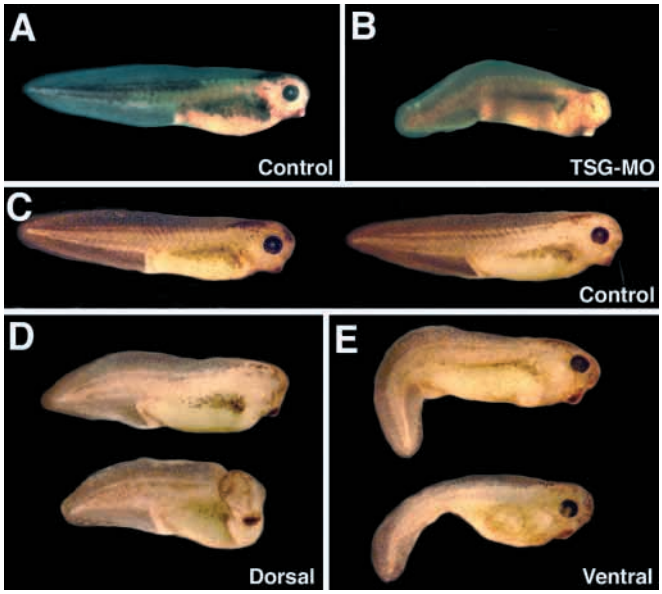

Fig. 3. Inhibition of endogenous TSG expression in early *Xenopus* embryos results in head and tail defects. (A) Control stage matched tadpole. (B) When 5 ng TSG-MO was injected into the marginal zone of each blastomere at the four-cell stage, the resulting tadpoles showed reduced head structures including defective eye formation together with mild tail defects and body curvature. (D,E) When the two dorsal (the region with low BMP signaling) or the two ventral (the region with high BMP signaling) blastomeres were injected with 10 ng TSG-MO per cell at the four-cell stage, the resulting embryos displayed head malformations and a bent body axis (dorsal injection, D) or reduction and ventralward bending of the tails (ventral injection, E) in the tadpoles. The control embryos for this experiment are shown in panel C. In all panels anterior is to the right and dorsal is up.

whereas coinjection together with the ChdA-MO efficiently blocked secondary axis formation (0%, *n*=36; Fig. 2E). Comparable results were obtained using the chordin isoform B mRNA, which induced secondary axes in 95% of the injected embryos (*n*=42), while coinjection together with the ChdB-MO similarly inhibited secondary axis formation (0%, *n*=42). Finally, a control MO failed to inhibit either the TSG or chordin overexpression phenotypes (data not shown). Therefore, we conclude that both the TSG and chordin MOs specifically inhibit translation of these mRNAs in vivo.

Embryonic TSG loss-of-function phenotypes

To determine how TSG regulates BMP signaling in vivo, we initially examined the TSG loss-of-function phenotype in TSG-MO microinjected embryos ('TSG-MO embryos'). As the consequences of overexpression (hyperventralization) and inhibition (hyperdorsalization) of BMPs in early *Xenopus* embryos have been well characterized, we wished to establish whether knockdown of TSG expression would result in either of the typical BMP overexpression or BMP inhibition phenotypes. Microinjection of TSG-MO into each blastomere at the four-cell stage resulted in defects in both head and tail development in frog tadpoles. The embryos show reductions in head size, a curved body axis and minor tail defects (Fig. 3B; for more detailed description see also below). To further dissect the function of TSG in dorsal (low BMP signals) and ventral (high BMP signals) regions, we also injected the TSG-MO into the dorsal or the ventral blastomeres only. Microinjection of TSG-MO into the ventral blastomeres resulted in a ventralward bending of the tail and abnormal tail fin development in 71% of the embryos (*n*=80; Fig. 3E), suggesting that TSG may be required on the ventral side in early frog development. Since TSG is expressed in the ventral region at gastrula stages and the ventral-posterior region of the trunk and the tail at tailbud stages (Oelgeschlager et al., 2000), the ventral injection phenotype may be the result of either an early or a late effect on TSG function in the ventral marginal zone derived tissues. Microinjection of the TSG-MO into each of the dorsal blastomeres at the four-cell stage resulted in reduction of head structures with many embryos displaying defective or even absent eyes (78%, *n*=181; Fig. 3D). This result suggests that TSG may play an important role(s) in dorsoanterior specification. In addition, a significant number of embryos (89%, *n*=181) show a sideways bending of the body axis and have smaller eyes (Fig. 3D). This reduction in anterior development suggests that the TSG-MO induces a 'moderate' elevation of BMP signaling. A similar result has also been recently reported when embryos are depleted of chordin (Oelgeschlager et al., 2003) and, therefore, these results imply that endogenous TSG, like chordin, may function to inhibit

BMP signaling on the dorsal side. We thus further characterized the phenotype of TSG-MO embryos in more detail.

The TSG loss-of-function phenotype can be rescued by restoration of TSG expression, or by overexpression of the BMP inhibitors chordin or noggin

To demonstrate that the TSG-MO phenotype is consistent with loss of TSG protein, we sought to rescue these defects by restoring TSG expression. Therefore, we coinjected the TSG-MO together with increasing doses of a human TSG mRNA that lacks the TSG-MO recognition sequence. Human and mouse TSGs have been shown to function similarly to *Xenopus* TSG in all the assays we have performed (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001) (data not shown). The head reduction and the bent body axis phenotypes induced by microinjection of TSG-MO into the dorsal blastomeres are increasingly suppressed by coinjection with human TSG mRNA (Fig. 4A); and similarly the tail defects induced by the ventral injection of TSG-MO are gradually rescued with the coinjected human TSG (Fig. 4B). Finally, injection of as little as 30 pg of a modified *Xenopus* TSG mRNA lacking the TSG-MO recognition sequence (BM40-XTSG; see Materials and methods) into each blastomere at the four-cell stage together with TSG-MO resulted in the rescue of normal development (data not shown). Therefore, we conclude that restoration of TSG expression can overcome the embryonic defects induced by the TSG morpholino oligo, suggesting that the TSG-MO is inhibiting translation of endogenous TSG and that the phenotype we observed above resulted from a reduction in levels of TSG protein.

We, and others, have previously demonstrated that TSG can enhance the ability of chordin to complex with BMPs in vitro and can enhance the induction of secondary axes by chordin in vivo, suggesting that TSG may function in vivo as a BMP inhibitor and that TSG may act to enhance the inhibitory activity of chordin on BMP (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). To determine whether TSG participates in BMP inhibition, we sought to examine whether the TSG loss-of-function phenotype could be rescued by coinjection of the TSG-MO with chordin mRNA. We microinjected the TSG-MO into each dorsal blastomere at the four-cell stage together with increasing doses of chordin mRNA and observed the embryonic phenotype at tadpole stages. We found that chordin can rescue the TSG-MO-induced head defects in a dose-dependent manner (Fig. 4C). These data are consistent with the hypothesis that TSG and chordin may cooperate to inhibit BMP signaling in vivo (see below). It is notable that the dose required for phenotypic rescue by chordin (0.5-1 ng) is much higher than that required for secondary axis induction by chordin (less than 10 pg; data not shown).

To determine whether other BMP antagonists can also rescue the TSG-MO phenotype, we coinjected the TSG-MO with increasing doses of noggin RNA into the dorsal blastomeres of four-cell stage embryos. We found that 5 pg to 10 pg of noggin RNA is sufficient to rescue the phenotype of TSG-MO embryos (Fig. 4D). As the TSG-MO phenotype can be rescued by coinjection with either chordin or noggin mRNAs, our results suggest that TSG may function as a BMP inhibitor in *Xenopus*. Loss of TSG expression may lead to

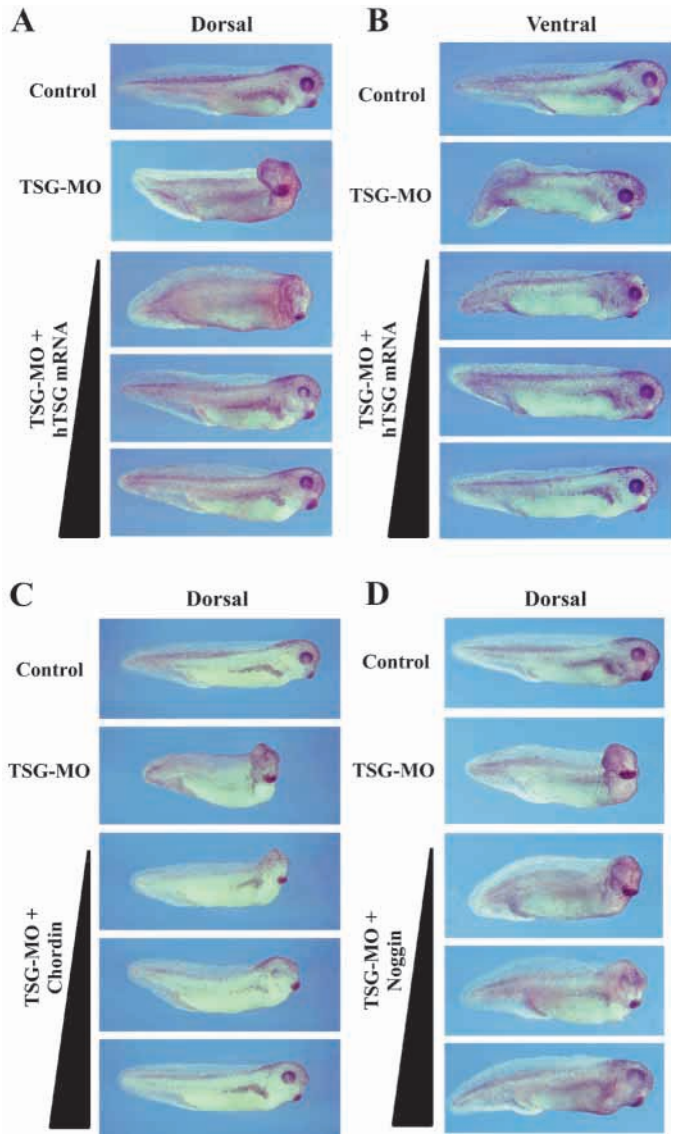

Fig. 4. Dose-dependent rescue of the TSG-MO phenotype by human TSG, chordin or noggin. (A) Rescue of TSG-MO phenotypes induced by dorsal MO injection, by coinjection with human TSG mRNA. 10 ng TSG-MO was injected into each dorsal blastomere of four-cell stage embryos, alone or with increasing doses (0.2-1 ng) of human TSG mRNA. While the TSG-MO induced a reduced head and bent body axis in frog tadpoles, these defects were rescued with human TSG, or *Xenopus* BM40-XTSG (data not shown). (B) Rescue of TSG-MO phenotypes induced by ventral MO injection, by coinjection with human TSG mRNA. TSG-MO and increasing doses of human TSG mRNA were injected as above, except ventral instead of dorsal blastomeres were injected. Human TSG rescued the tail defects induced by ventral expression of TSG-MO. (C) Rescue of dorsal defects by high doses of chordin. TSG-MO was injected with a range of between 0.2 ng and 1 ng of chordin RNA into the dorsal blastomeres of four-cell stage embryos. At high doses, chordin rescued the dorsal defects associated with the TSG-MO. (D) Rescue of the TSG-MO dorsal defects by noggin. TSG-MO was injected with between 2 pg and 10 pg noggin into the dorsal blastomeres of four-cell stage embryos. The head defect induced by TSG-MO was rescued by noggin overexpression. In all panels anterior is to the right and dorsal is up.

elevated BMP signaling in the embryo which can be suppressed by BMP inhibitors to restore normal development of early embryos.

The TSG-MO and Chd-MOs cooperate to inhibit dorsal specification

In zebrafish, TSG and chordin cooperate with each other to regulate dorsal development (Ross et al., 2001), however it is presently unclear whether TSG plays a similar role in dorsal specification in *Xenopus*. The rescue of the TSG-MO embryos with chordin mRNA (discussed above) suggested that TSG and chordin may cooperate to specify dorsal tissues in *Xenopus* as well. To further explore this possibility, we coinjected the TSG-MO and Chd-MOs and examined the morphology of the injected embryos.

We first examined the embryos at gastrula stages. While embryos injected with the TSG-MO into each four-cell stage blastomere had the normal onset of gastrulation, as judged by the timing of the appearance of the dorsal lip, reduction of TSG expression resulted in slowing of the progression of blastopore closure (data not shown). Interestingly, microinjection of the Chd-MOs has a similar effect in delaying blastopore closure (data not shown), however injection of the 'control' MO, or MOs to *Xenopus* GDF6 or BMP7, had little to no effect on the timing of blastopore closure (data not shown) demonstrating the specificity of the TSG-MO and Chd-MOs for this effect. Furthermore, we could partially rescue the blastopore closure defect induced by TSG-MO by coninjection of a wild-type TSG mRNA that cannot be inhibited by the MO (data not shown). The delay in blastopore closure mimics the effect of overexpression of low doses (20 pg mRNA/blastomere) of BMP2 (data not shown) suggesting that both TSG and chordin function to inhibit BMP signaling. Importantly, similar to what has been shown in the case of BMP overexpression (Jones et al., 1992), the blastopore does eventually close. At neurula stages, the embryos injected with the TSG-MO had recovered to a large extent, though they showed a shortened neural axis (data not shown). Taken together, these data suggest that the TSG-MO and Chd-MOs may induce embryonic defects after the onset of gastrulation (see also below).

We next sought to determine whether coinjection of the Chd-MOs together with the TSG-MO would result in an enhancement of the TSG-MO phenotypes. We first examined the phenotype of embryos coinjected dorsally with both TSG-MO and the Chd-MOs at tadpole stages. When injected into the dorsal side at 10 ng/blastomere, the TSG-MO and Chd-MOs each induced mild to moderate reductions in head (dorsoanterior) development (Fig. 5B,C); however when the two MOs were coinjected into the dorsal blastomeres, the resulting embryos had severely reduced or even missing heads and shortened anterior-posterior body axes (Fig. 5D; 84%, n=55, of co-injected embryos had barely discernible head structures). We also examined embryos that were injected with the MOs in all blastomeres. While dorsal injection of this 'high' dose of TSG-MO (10 ng/blastomere) causes head defects that are rescuable with TSG mRNA (Fig. 4A), injection of this dose into all blastomeres of four-cell stage embryos results in death of the embryos during late neurula stages (data not shown). We have not attempted to analyze the mechanisms responsible for this embryonic death. At lower doses of MO (5 ng MO into each four-cell stage blastomere), both TSG-MO and Chd-MOs each induced a 'weak' phenotype (Fig. 5F,G), while coinjection of these MOs together led to a stronger reduction of dorsoanterior structures concomitant with an apparent enlargement of the ventroposterior region (Fig. 5H). Finally, the combined MO phenotype we observed does not appear to be caused by a mere increase in the total amount of injected MOs, because (1) increasing the total dose of MO by coinjecting the TSG-MO together with a control MO did not result in more severe changes in embryo morphology than that of TSG-MO embryos (data not shown); (2) when the total amount of MO per blastomere was kept constant, the phenotype induced by the combination of low doses (5 ng of each MO/dorsal blastomere) of TSG-MO and Chd-MOs was always more severe than that when each individual MO was used at a higher dose (10 ng/blastomere; data not shown). Our

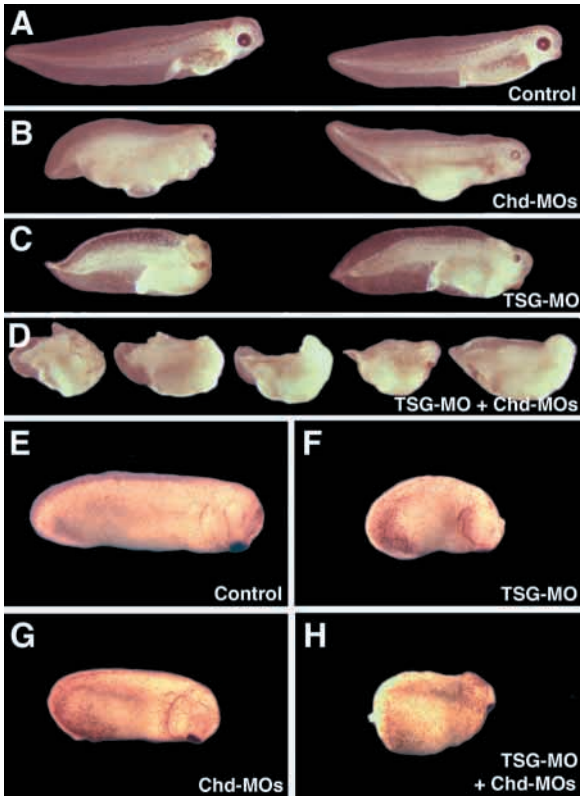

Fig. 5. (A-D) Frog tadpoles were injected [or not; (A) control] with 10 ng chordin-MOs (B), 10 ng TSG-MO (C) or both sets of MOs (D) into the two dorsal blastomeres at the four-cell stage. Dorsal injection of Chd-MOs or the TSG-MO leads to reduced heads in frog tadpoles (B and C), and the two sets of MOs cooperate to further reduce, and even eliminate, head structures in the resulting tadpoles (D). The length of the anterior-posterior body axis is also foreshortened. (E-H) Early tailbud stage embryos injected with 5 ng TSG-MO (F), or 5 ng of each chordin MO (G), or 5 ng TSG-MO + 5 ng of each chordin MO (H) into each blastomere at the four-cell stage. Inhibition of expression of TSG resulted in a mild head reduction (F) compared with control (E), whereas the Chd-MOs had a weaker effect (G). However, the TSG-MO and Chd-MOs strongly cooperate to produce significant head defects in conjunction with a shortening of the anterior-posterior body axis and apparent expansion of the ventroposterior region (H). In all panels anterior is to the right and dorsal is to the top.

results thus suggest that TSG and chordin cooperate in vivo to regulate dorsal development.

TSG and chordin cooperate in dorsal-ventral specification during gastrulation

To better understand the role of TSG in BMP regulation, we examined how TSG and chordin regulate dorsal specification by examining the expression patterns of various marker genes in TSG-MO and/or Chd-MOs injected embryos. First, we examined the expression of goosecoid and chordin [markers of Spemann's organizer (Blumberg et al., 1991; Cho et al., 1991; Sasai et al., 1994)], otx2 [a marker of Spemann's organizer and anterior neural ectoderm (Lamb et al., 1993; Blitz and Cho, 1995)], myf5 [a marker of dorsolateral marginal zone (Hopwood et al., 1991; Dosch et al., 1997)], Msx1, Wnt8 and Vent1 [markers of ventral and lateral tissues (Christian and Moon, 1993; Gawantka et al., 1995)], and sizzled [a marker of the ventral tissues (Salic et al., 1997)] at various gastrula stages. At early gastrula stage we found no remarkable differences in the levels or patterns of expression of any of these genes (Fig. 6A,B,E,F; data not shown). However, by midgastrula stages, we observed a weak reduction in the expression of goosecoid (compare Fig. 6D with C) and a more substantial reduction in the expression of otx2 (compare Fig. 6H with G). No obvious changes could be seen in any of the other above markers we examined (data not shown). These data suggest that reducing TSG expression levels results in a reduction in the maintenance of dorsal specification before or around midgastrula stage. It is interesting to note that a similar temporal pattern of regulation of dorsal gene expression after the onset of gastrulation has also been reported when using chordin antisense morpholino oligonucleotides (Oelgeschlager et al., 2003). We also examined the expression of the ventral sizzled domain at early neurula and early 'tailbud' stages in TSG-MO-injected embryos. The TSG-MO induced a strong expansion of sizzled toward the dorsal side (compare Fig. 6M-P with I-L). These observations also appear nearly identical to those reported previously for chordin MO-injected embryos (Oelgeschlager et al., 2003). Therefore, our data are consistent with the interpretation that TSG functions as a BMP inhibitor.

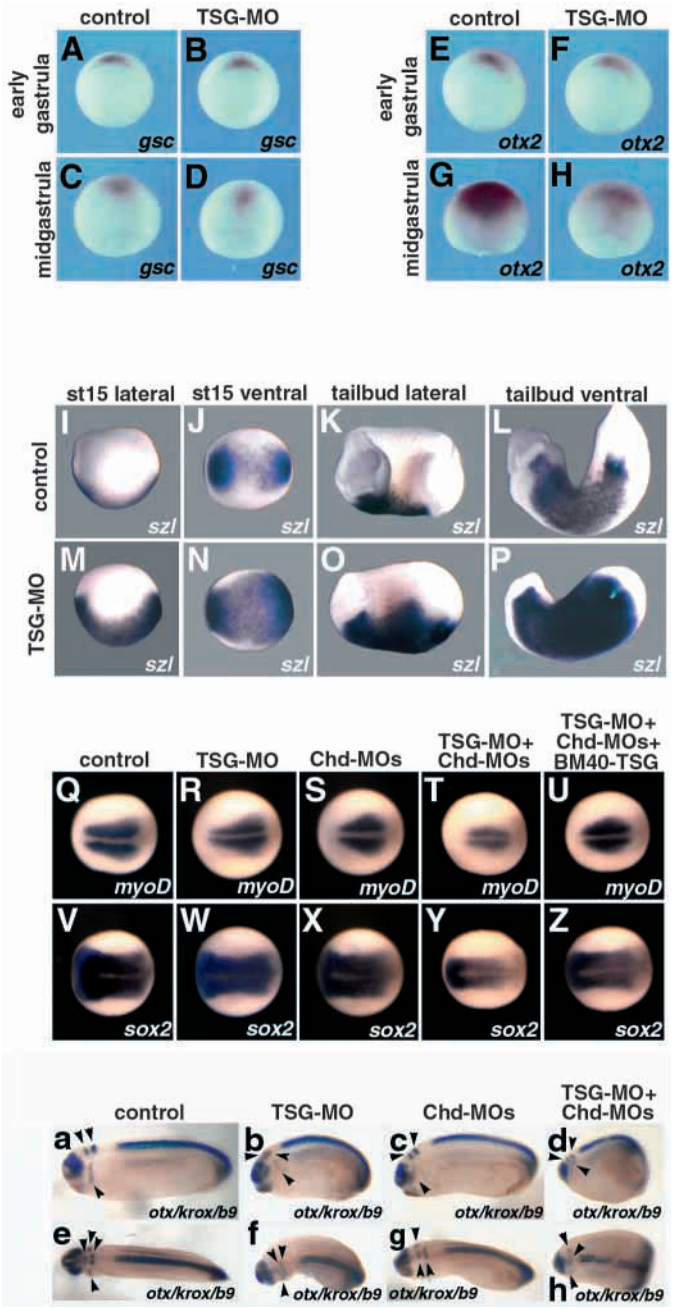

Fig. 6. The TSG-MO and Chd-MOs cooperate to reduce expression of dorsal-specific genes at the expense of expanded ventral-specific genes. (A-H) Embryos at early to mid-gastrula stages; uninjected control (A,C,E G) or injected with 5 ng TSG-MO into each blastomere at the four-cell stage (B,D,F,H). Expression of the dorsal markers goosecoid (gsc, A-D) and otx2 (E-H) were examined at early (A,B,E,F) or mid- (C,D,G,H) gastrula stages by whole-mount in situ hybridization. While no changes in expression of these dorsal markers were observed at early gastrula stages, by midgastrula both gsc and otx2 expression was reduced in TSG-MO embryos. A, B, E and F are viewed from the vegetal side with the dorsal region to the top. C, D, G and H are viewed from the dorsal side with the anterior to the top. (I-P) In situ hybridization with the ventral marker *sizzled* (*szl*) at early neurula (stage 15, I,J,M,N) and tailbud (K,L,O,P) stages. *szl* expression is expanded in TSG-MO embryos. Anterior is to the left in these embryos. (Q-Z) Neurula stage embryos injected with 5 ng TSG-MO (R,W), 5 ng Chd-MOs (S,X), 5 ng TSG-MO + 5 ng each Chd-MOs (T,Y), or 5 ng TSG-MO + 5 ng each Chd-MOs + 30 pg BM40-XTSG (a modified *Xenopus* TSG which is not inhibited by the TSG-MO; U,Z) into the marginal zone of each blastomere at the four-cell stage. The dorsal markers *myoD* (for paraxial mesoderm) and *sox2* (for neural tissues) were examined. While TSG-MO and Chd-MOs alone had minor effects on the expression of these markers (R,S,W,X), the MOs cooperate to greatly reduce their expression (T,Y). BM40-XTSG partially rescued the inhibitory effect by the MO combination (U,Z). The embryos are viewed from the dorsal side with anterior to the left. (a-h) TSG-MO and Chd-MOs cooperate to reduce anterior neural markers. 5 ng TSG-MO (b,f) or 5 ng each of Chd-MOs (c,g) were microinjected into each of the four-cell stage blastomeres, alone or in combination (d,h), and the embryos were examined at tailbud stages for expression (arrowheads) of the anterior and posterior neural markers *otx2* (fore- and midbrain), Krox20 (hindbrain) and HoxB9 (spinal cord) by in situ hybridization. While the TSG-MO and Chd-MOs mildly reduce the expression of *otx2* and *krox20* by themselves (b,c,f,g), they cooperate to further inhibit the expression of *krox20* and *otx2* (d,h). The embryos are shown in lateral view (a-d) or dorsal view (e-h), with the anterior towards the left.

We next examined the expression patterns of myoD [presomitic mesoderm (Hopwood et al., 1989; Harvey, 1990)], sox2 [neural plate (Mizuseki et al., 1998)] and sizzled [ventral (Salic et al., 1997)] in early neurula stage embryos. We found that the TSG-MO caused a slight reduction in the lateral width of the myoD and sox2 expression domains (compare Fig. 6R and W to Q and V, respectively). We observed similar effects on the expression of these markers in Chd-MO injected embryos (Fig. 6S and X) confirming the recent data of Oelgeschlager et al. (Oelgeschlager et al., 2003). Interestingly, when we coinjected Chd-MOs and the TSG-MO together, we found a substantial shrinkage in the domains of sox2 and myoD expression toward the dorsal midline (Fig. 6T,Y). Furthermore, we found that coinjection of the TSG-MO and Chd-MOs together with BM40-XTSG mRNA resulted in partial rescue of the myoD and sox2 expression domains toward the intermediate states observed in the TSG-MO or Chd-MOs only injected embryos (Fig. 6U,Z). Finally, we also examined the expression of the anterior and the posterior neural markers otx2 (fore- and midbrain), Krox20 (hindbrain) and HoxB9 (spinal cord) at the tailbud stages (Fig. 6a-h). We found that while the TSG-MO and Chd-MOs each mildly reduce the expression of otx2 and Krox20 (Fig. 6b,c,f,g), they cooperate to further reduce the expression of these two markers, though the effect on the expression of the spinal cord marker HoxB9 is weaker (Fig. 6d,h). These data taken together suggest that TSG and chordin are both required to maintain the wild-type 'level' of dorsal specification and both of these proteins act in BMP signal regulation in the same direction, to inhibit BMP signaling. Finally, the TSG-MO and Chd-MOs together result in a cooperative reduction in dorsal specification, consistent with the notion that these proteins interact to inhibit BMP signaling.

TSG is required for efficient inhibition of BMP signaling by chordin in animal caps and secondary axis induction

We, and others, previously suggested that TSG functions as a BMP inhibitor (Scott et al., 2001; Chang et al., 2001; Ross et al., 2001). These studies also provided in vitro and in vivo overexpression data supporting the notion that the inhibitory activity of TSG on BMP signaling might occur through its ability to enhance the interaction between chordin and BMP ligands. One prediction of this model is that reduction in the level of TSG expression would result in a decreased response to ectopic chordin expression. To test this hypothesis, we assayed the ability of chordin to induce secondary axes when overexpressed in the ventral marginal zone. We overexpressed chordin in the ventral marginal zone of embryos in the presence or absence of the TSG-MO and assayed for development of secondary axes at early tailbud stage. We found that, while chordin efficiently induced secondary axes (Fig. 7B; 95%, n=60), the TSG-MO completely inhibited chordin-mediated secondary axis formation (Fig. 7C; 0%, n=57). Furthermore, a control MO had no effect on chordin's activity in this assay (98%, n=58). To further rule out the possibility that this inhibition of chordin function might instead be due to more general 'non-specific' effects on secondary axis formation we sought to examine the effects of the TSG-MO on secondary axes induced by other secreted signaling molecules. Early Wnt signaling acts via a canonical β-catenin-mediated pathway to induce expression of *siamois*, *twin*, and *Xnr3* resulting in the formation of secondary axes. Furthermore, expression of these genes is not affected by elevating BMP signaling (Laurent et al., 1997). Therefore, we microinjected *Xwnt8* mRNA into the ventral marginal zone at the four-cell stage in the presence (Fig. 7E) or absence (Fig. 7D) of coinjected TSG-MO and examined the effects of TSG depletion on Xwnt8-induced secondary axis formation. We found that the frequency of formation of secondary axes by Xwnt8 was unaffected by reduction of TSG expression. Furthermore, we tested the effect of TSG-MO on secondary axis induction by noggin, another secreted BMP inhibitor. As shown in Fig. 7F-H, the ability of noggin to induce a secondary axis was not blocked by the TSG-MO either. These data suggest that the effect of TSG-MO on secondary axis induction by chordin are the result of chordin's BMP inhibitory function and that TSG is required for efficient BMP inhibition by chordin in vivo.

To further test this hypothesis, we examined the requirement for TSG in chordin-mediated BMP inhibition in explanted animal caps. Ventral animal pole tissue, under the influence of endogenous expression of BMPs 2, 4 and 7, is fated to become epidermis. Chordin overexpression in ectodermal explants ('animal caps') results in the respecification of this tissue to anterior neural cell fates (Sasai et al., 1994). Therefore, to compare chordin's ability to inhibit BMP signaling in the presence and absence of TSG, we expressed increasing amounts of chordin in animal caps in the presence or absence of TSG-MO. We performed RT-PCR on RNA derived from animal caps cultured to midgastrula stage 11 equivalent to examine gene expression patterns. In the absence of the TSG-MO, increasing doses of chordin mRNA resulted in increasing levels of expression of the anterior neural marker *Xotx2* (Lamb et al., 1993; Blitz and Cho, 1995) (Fig. 7I, lanes 6-8). Concomitant with the induction of *Xotx2*, chordin repressed expression of the ventral-specific direct BMP target genes *Xvent2* (Onichtchouk et al., 1996; Schmidt et al., 1996; Blitz et al., 2000) and *Msx1* (Su et al., 1991; Suzuki et al., 1997) (Fig. 7I, lanes 6-8). In contrast, in the presence of the TSG-MO, chordin was incapable of inducing *Xotx2* or repressing expression of either *Xvent2* or *Msx1* (Fig. 7I, lanes 9-11).

This inability of chordin to induce *Xotx2* or to repress *Msx1* or *Vent2* may explain why low doses of chordin could not rescue the phenotype induced by the dorsally injected TSG-MO (Fig. 4C). We did, however, observe suppression of the TSG-MO phenotype when chordin was injected at high doses, suggesting that chordin does not absolutely require TSG function. To test whether this is the case, we further analyzed the BMP inhibitory activity of chordin over a wider range of RNA doses and with a more sensitive detection method using radioactive nucleotides in the PCR reaction. As shown in Fig. 7J, we observed a dramatic shift in the caps' response to chordin in the absence as compared to the presence of TSG-MO. While 10 pg chordin mRNA alone induced high levels of *Xotx2* and repressed *Msx1* efficiently, it appears that approximately 20- to 50-fold higher doses of chordin were required to achieve a similar level of induction/repression when TSG-MO was present (Fig. 7J, compare lane 4 to lanes 16 and 17). To address whether the TSG-MO blocks BMP antagonists other than chordin in animal caps, we performed

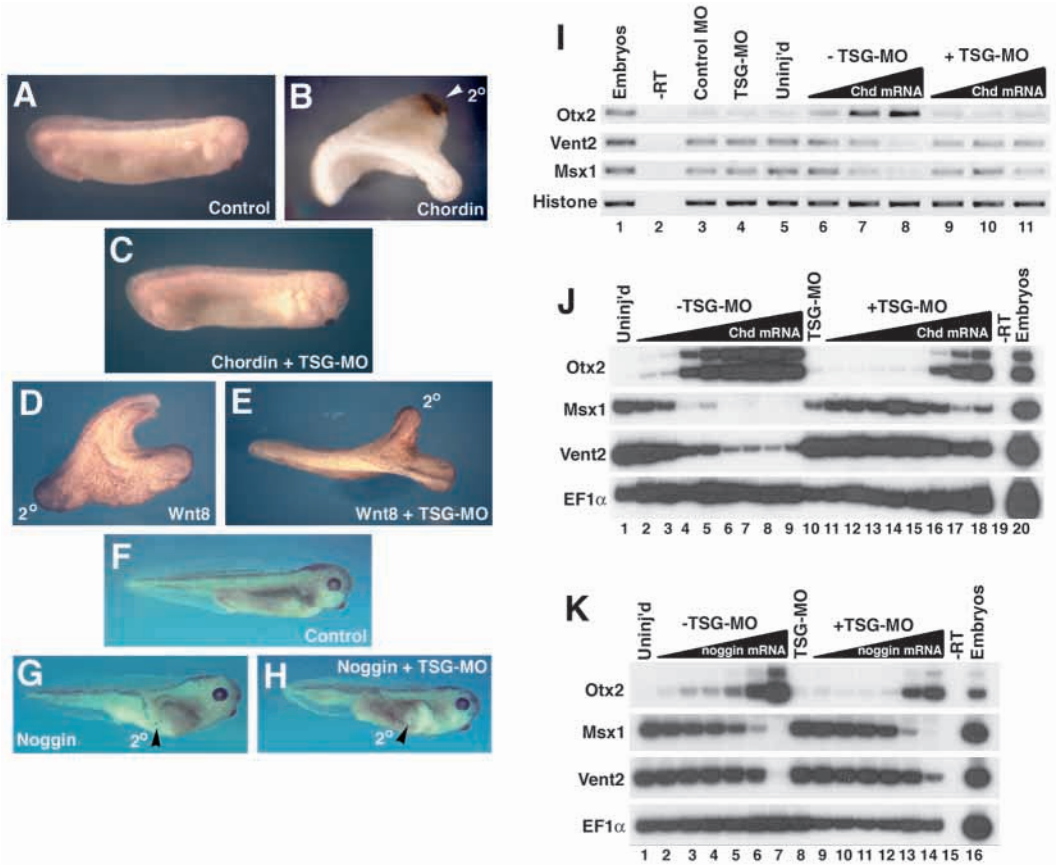

Fig. 7. TSG is required for efficient inhibition of BMP signaling by chordin. (A-H) The TSG-MO blocks secondary axis induction by chordin, but not by wnt8 or noggin. 0.6-6 pg of Δ5′-Chd mRNA, 0.5-1.5 pg of *Xenopus* wnt8 mRNA, or 5-10 pg of *Xenopus* noggin mRNA was microinjected into the ventral marginal zone of four-cell stage embryos, without or with 10 ng TSG-MO. While the TSG-MO had no effect on the secondary axis induction by wnt8 (D,E) or noggin (G,H), it inhibited secondary axis induction by chordin (B,C). Control embryos were uninjected embryos from each experiment. Coinjection with 10 ng control MO per embryo has no effect on secondary axis induction by any of these RNAs (data not shown). '2°' designates the induced secondary axis. All embryos are oriented with their 1° axes to the right. (I) The TSG-MO blocks chordin function in explanted animal caps. Increasing doses (lanes 6-8, and 9-11, are derived from embryos injected with 0.6, 2 and 6 pg, respectively) of Δ5′-Chd mRNA, with or without 20 ng TSG-MO, were injected into the animal poles of four-cell stage embryos. The ectodermal explants (animal caps) were removed at blastula stages and incubated until the sibling control embryos reached the midgastrula stages (stage 11). Total RNA was then extracted from the caps and RT-PCR was performed to assay for gene expression. While chordin induced the anterior neural marker otx2 and repressed the direct BMP target genes *vent2* and *msx1* in a dose-dependent fashion (lanes 6-8), chordin lost its activity in the presence of the TSG-MO (lanes 9-11). (J) The TSG-MO significantly shifts the response to ectopically expressed chordin in animal caps. Increasing doses of chordin RNA (2, 5, 10, 20, 50, 100, 500 and 1000 pg in lanes 2-9 and 11-18) were injected, without or with 20 ng TSG-MO, into animal poles of two-cell stage embryos. Animal caps were obtained and processed as described above and assayed for gene expression by radioactive RT-PCR. While 10 pg chordin was sufficient to induce *otx2* and repress *msx1* (lane 4), a 10- to 50-fold higher dose of chordin (lanes 16 and 17) was required to achieve comparable levels of induction/repression in the presence of TSG-MO. (K) The TSG-MO has a minor effect on the activity of noggin in gastrula animal caps. Increasing doses of noggin (0.2, 0.5, 1, 2, 5 and 10 pg in lanes 2-7 and 9-14) were injected, without or with 20 ng TSG-MO, into animal poles of two-cell stage embryos. Animal caps were obtained and processed as above and gene expression was assayed by RT-PCR. TSG-MO has a minor influence on repression of *msx1* and *vent2* and shifts the induction of *otx2* by noggin slightly.

a similar assay using the unrelated BMP inhibitor noggin. While the TSG-MO had an effect on induction of *Xotx2* and repression of *Msx1* and *XVent2* by noggin, this reduction in noggin's BMP inhibitory behavior appears to be weaker, perhaps as little as two- to fourfold (Fig. 7K). From these experiments, we conclude that, while the TSG-MO can reduce the BMP inhibitory behavior of noggin in these overexpression experiments, its ability to alter chordin function appears to be significantly stronger. Finally, while it has previously been shown that expression of chordin itself can be induced by overexpression of BMP inhibitors (Blitz et al., 2000) (suggesting that TSG might indirectly influence BMP inhibition by noggin through noggin's induction of chordin), significant levels of chordin were not induced at these doses of noggin (data not shown). Therefore, we conclude that endogenous TSG is important for determining the strength of the inhibitory activity of chordin towards BMP, but TSG may also function indirectly, in a chordin-independent manner, to establish appropriate levels of BMP signaling in vivo.

Discussion

Xenopus TSG loss-of-function phenotypes suggest that TSG is a BMP inhibitor

Strict temporal and spatial regulation of BMP signaling is critically important to ensure the 'proper' allocation of tissues to different domains along the dorsal-ventral axis of early vertebrate embryos. Twisted gastrulation (TSG) is a secreted molecule involved in the modulation of BMP signaling which functions through its direct physical interactions with both BMP ligands and the secreted BMP inhibitor chordin (Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). However, unlike all other extracellular BMP regulators, TSG has been reported to be both a BMP antagonist and a BMP agonist, and therefore may have dual functions depending on other factors (Yu et al., 2000; Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2003). Interpretation of the in vivo mode of action of TSG vis-à-vis BMP signaling derives mainly from comparisons of phenotypes produced by forced TSG overexpression with BMP overexpression and inhibition phenotypes. High-level overexpression of BMPs in early *Xenopus* embryos results in 'Baushtück' embryos – radially ventroposteriorized embryos that develop as 'a belly piece' completely lacking dorsal structures (Dale et al., 1992; Jones et al., 1992). However, lower-level overexpression of BMPs results in varying degrees of (dose-dependent) loss of dorsal structures, beginning with a reduction of the head. Concomitant with head truncations, it is common to observe an increase in the size of the ventroposterior region of the trunk. In contrast, a reduction of BMP signaling below endogenous levels (in the mesoderm) results in the opposite effect: varying (dose-dependent) degrees of hyper-dorsoanteriorization.

High-level TSG overexpression results in head truncations (Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001) consistent with models that it might promote BMP signaling. Furthermore, when chordin is coexpressed with high levels of TSG, secondary axis formation is inhibited (Oelgeschlager et al., 2000; Ross et al., 2001) (our unpublished data), consistent with the notion that TSG can inhibit chordin function. These observations suggest that 'high-level expression' of TSG promotes BMP signaling. However, other experiments lead to opposite conclusions. For example, when low doses of ectopic chordin are expressed ventrally, in amounts incapable of inducing secondary axes, the addition of low doses of overexpressed TSG together with chordin resulted in secondary axis induction (Scott et al., 2001). These and other results of TSG overexpression (Chang et al., 2001; Ross et al., 2001) provided in vivo evidence that TSG can also function as a BMP inhibitor. Finally, curiously certain TSG loss-of-function phenotypes in zebrafish embryos show similarities to some of the phenotypes achieved by TSG overexpression in *Xenopus* (Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). This raises the possibility that TSG may function differently in *Xenopus* and zebrafish embryos. As it remains unclear whether endogenous TSG indeed has dual activities in regulating BMP signaling, we have undertaken a loss-of-function approach in *Xenopus* embryos to examine the role of in vivo levels of TSG to determine whether TSG might function as a BMP inhibitor or activator, or both.

In the present study, loss of TSG function, by inhibition of its translation using morpholino oligonucleotides, resulted in a reduction of head structures. The eyes are often defective or even missing in TSG knockdown embryos. Since head formation requires inhibition of the BMP pathway (Glinka et al., 1997; Piccolo et al., 1999; Bachiller et al., 2000), this observation is consistent with the notion that BMP signals may be elevated when levels of TSG expression are reduced. This hypothesis is supported by our analysis of marker expression in TSG-MO embryos. At neurula and tailbud stages, expression of several dorsal and anterior markers are moderately reduced, while the expression of the ventral marker *sizzled* is expanded. Additionally, phenotypes of head reduction and expansion of the *sizzled* domain were found when chordin expression is reduced by chordin antisense MOs (Fig. 6) (Oelgeschlager et al., 2003). Furthermore, we observe that chordin or noggin, like wild-type TSG, rescues the head defect induced by the TSG-MO. These data suggest that TSG functions in vivo to inhibit BMP signaling.

TSG inhibits BMP signalling during early to midgastrulation to maintain dorsal cell fates

TSG loss-of-function embryos do not show any obvious defects at early gastrula stages. Both dorsal and ventral markers are expressed at apparently normal levels and in the correct spatial patterns, and initiation of dorsal blastopore lip formation is normal. The effects of reduced TSG expression arise at midgastrula stages, when changes in some markers of dorsal-ventral patterning become apparent. Later in gastrulation we observed a slowing in the progression of blastopore closure in TSG-MO embryos. The lack of an early effect by the TSG-MO can be explained in two ways. Either maternally deposited TSG protein, which is not affected by the antisense oligonucleotides, may be present to function at blastula to early gastrula stages and the residual low level of TSG may therefore be sufficient for early embryogenesis; or loss of TSG function may only affect embryo development after the onset of gastrulation. We favor the latter hypothesis as we have found that knockdown of chordin expression with antisense morpholino oligos similarly induces defects in frog embryos after the initiation of gastrulation, but not earlier. The fact that both chordin and TSG loss-of-function embryos show a delayed effect on marker expression and embryo morphology suggests that reduced BMP inhibition at early gastrula stages may reveal its effects only during later development of *Xenopus* embryos. This observation is also consistent with a previous report that ectopic BMP overexpression alters the embryo development after the onset of gastrulation (Jones et al., 1996). It has been shown that, when overexpressed at high doses, BMPs can arrest gastrulation without influencing the initial formation of the dorsal lip or marker expression (Dale et al., 1992). At low to intermediate doses of BMP, which mimic TSG-MO or Chd-MOs injections, formation of the dorsal lip is still normal and the blastopore eventually closes (Jones et al., 1992; Jones et al., 1996), but the closure is also delayed (our unpublished observations). The slow progression through gastrulation in both TSG-MO and Chd-MO embryos may therefore be due to enhanced BMP signaling as well. These observations are also reminiscent of a recent report of BMP regulating cell movements during gastrulation in the zebrafish embryo (Myers et al., 2002). Therefore, consistent

with these previous observations, it is perhaps not surprising that altering the levels of expression of BMP regulators with MOs (as we have performed in the present study) might also not reveal phenotypes until midgastrulation.

TSG and chordin cooperate to inhibit BMP signaling in dorsal cell fate specification

We also examined the relationship between TSG and chordin in dorsal specification by coinjecting the TSG-MO together with Chd-MOs. While inhibition of translation of each of these genes individually resulted in relatively weak reductions in the extent of dorsal development, simultaneous inhibition of both TSG and chordin resulted in moderate-to-strong ventralization. These data further support the notion that both TSG and chordin act in the same direction; they both inhibit BMP signaling. TSG on its own is a weak BMP inhibitor as it is incapable of inducing secondary axes (Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001) unless it is expressed at high doses as a membrane-tethered protein (Chang et al., 2001). These observations are consistent with the finding that TSG has a ten-fold lower binding affinity for BMP ligands than chordin (Oelgeschlager et al., 2000). These observations are also consistent with the previous biochemical evidence that chordin and TSG physically interact with one another. TSG can promote chordin's BMP binding and BMP inhibitory activities (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001) and therefore cooperative effects of BMP inhibition by both TSG and chordin on dorsal cell fate specification in the present study are in good agreement with the suggestion that these proteins function together to form an inhibitory complex with BMP4. Finally, a loss-of-function study in zebrafish has also suggested that TSG acts in partnership with chordin to inhibit the activities of BMPs (Hammerschmidt et al., 1996; Ross et al., 2001). An antisense TSG MO induces a ventralized phenotype that is similar to moderate chordin loss-of-function mutants, and chordin and TSG MOs synergistically enhance the expansion of blood islands, a ventral tissue type. Our results thus bolster the idea that the function of vertebrate TSGs may be conserved in that TSG cooperates with chordin to inhibit BMP signaling.

Are there two distinct modes of chordin-mediated BMP inhibition?

An unexpected result from our studies suggests that TSG not only assists chordin in inhibition of BMP signaling, but may be required for efficient BMP inhibitory activity of chordin. This result is surprising since purified chordin can directly bind to BMPs in vitro to prevent their association with their cognate receptors, thus inhibiting BMP signaling (Piccolo et al., 1996). As the chordin-BMP interaction is enhanced by TSG, we and others suggested that TSG enhances chordin's BMP binding activity to increase BMP inhibition (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). Our morpholino coinjection experiments with chordin (Fig. 7) are consistent with this idea. Furthermore, the expression patterns of TSG and chordin suggest that the embryo might utilize TSG as a cofactor to enhance the activity chordin only when higher level BMP inhibition cannot be achieved by chordin alone.

In the early stages, the expression domain of chordin overlaps with two regulators of chordin, TSG and BMP1/Xolloid (Goodman et al., 1998; Scott et al., 2001), and their expression segregates into different regions as development proceeds. TSG is ubiquitously distributed during early gastrulation (Scott et al., 2001) and overlaps chordin in Spemann's organizer during late blastula and early gastrula stages. The presence of the negative chordin regulator BMP1/Xolloid in all cells during early *Xenopus* development, and the early expression of BMPs 2, 4 and 7 in the organizer itself may necessitate a requirement for TSG to enhance chordin's BMP inhibitory activity in order to permit dorsal gene expression and establishment of an 'appropriately sized' organizer (Stewart and Gerhart, 1990). The intricate balance between the levels of TSG, BMP1/Xolloid, BMP ligands and chordin (and other BMP inhibitors) at these stages may thus determine the level of BMP inhibition by chordin in early gastrulae.

By late gastrulation, TSG expression is excluded from the dorsal side of the embryo (Oelgeschlager et al., 2000; Scott et al., 2001), and Xolloid is detected only in posterior ectodermal patches (Goodman et al., 1998). Chordin at these stages is expressed in the dorsal mesoderm underlying the neural plate (Sasai et al., 1994), a TSG-free zone. It is thus likely that at these later stages, conditions are such that the embryo no longer requires a reliance of chordin on TSG to enhance its ability to bind and inhibit BMPs. At these later stages, chordin may therefore block BMPs appropriately even in the absence of TSG in these developmental contexts. It is thus conceivable that there may be two distinct modes of chordin-mediated BMP inhibition, TSG-dependent and TSG-independent.

It is also interesting to speculate that TSG may interact with other proteins in vivo to regulate early frog development, as ventrally injected TSG-MO induces ventroposterior defects, even though chordin expression is absent from the ventral region and Chd-MOs have no effect on early frog embryogenesis when expressed ventrally (data not shown). Further studies are required to understand all the partners of TSG and how they regulate early *Xenopus* development.

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