Sirenomelia in *Bmp7* and *Tsg* compound mutant mice: requirement for Bmp signaling in the development of ventral posterior mesoderm

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Summary

Sirenomelia or mermaid-like phenotype is one of the principal human congenital malformations that can be traced back to the stage of gastrulation. Sirenomelia is characterized by the fusion of the two hindlimbs into a single one. In the mouse, sirens are observed in crosses between specific strains and as the consequence of single one. In the mouse, sirens have been observed in crosses between strains carrying mutations at the *Twsg1*-like loci, thus establishing that sirenomelia can have a genetic basis. The phenotype was not described again in the mouse until 1970 (Hoornbeek, 1970). More recently, two groups linked retinoic acid to sirenomelia in gene knockouts of the retinoic acid-degrading enzyme, CYP26 (Abu-Abed et al., 2001; Sakai et al., 2001). Here, we report the appearance of the sirenomelia phenotype in *Tsg/Bmp7* compound mutants.

Dorsoventral patterning in vertebrate and invertebrate embryos is determined by a gradient of Bmp activity controlled in the extracellular space by a network of secreted proteins. Tsg (*Twsg1* – Mouse Genome Informatics) is one of these Bmp regulators and can act both to promote and inhibit Bmp activity. When Tsg forms a stable ternary complex with Bmp and Chordin (Chd), Bmp signaling is prevented (Oelgeschläger et al., 2000; Chang et al., 2001; Larrain et al., 2001; Ross et al., 2001; Scott et al., 2001). However, the presence of Tsg in the complex facilitates cleavage of Chd by the metalloprotease Tld, enabling Bmp to signal again, and thus promoting Bmp activity (Oelgeschläger et al., 2000; Larraín et al., 2001; Oelgeschläger et al., 2003a; Oelgeschläger et al., 2003b). Tsg loss-of-function experiments in several species have resulted in various phenotypes interpreted as pro-Bmp or anti-Bmp effects. In *Drosophila*, Tsg mutation results in the loss of the amnioserosa, a tissue that requires the highest levels of Bmp/Dpp activity (Mason et al., 1994), and absence of Tsg

Introduction

Sirenomelia is a human developmental malformation that has been a source of enduring interest. The abnormal fetuses are characterized by the fusion of both legs into a single member (Geoffroy Saint-Hilaire, 1837; Kampmeier, 1927). Descriptions of sirenomelia in human fetuses can be traced back to the 16th and 17th centuries, although these first reports were still a mixture of reality and mythical imagination. In addition to the hindlimb deformity, sirenomelic fetuses display a single umbilical artery (instead of two) and atrophic, cystic or absent kidneys (Feller and Sternberg, 1931). Several mechanisms have been proposed to explain sirenomelia: (1) deficiencies in caudal mesoderm, (2) mechanical defects resulting from lateral compression by amniotic folds and (3) trophic defects due to a deficient blood supply in the posterior region (Padmanabhan, 1998; Sadler and Langman, 2004).

Gluecksohn-Schoenheimer and Dunn published the first study on sirens in the mouse, in these terms: ‘Animal with fused or ur loci, thus establishing that sirenomelia can have a genetic basis. The phenotype was not described again in the mouse until 1970 (Hoornbeek, 1970). More recently, two groups linked retinoic acid to sirenomelia in gene knockouts of the retinoic acid-degrading enzyme, CYP26 (Abu-Abed et al., 2001; Sakai et al., 2001). Here, we report the appearance of the sirenomelia phenotype in *Tsg/Bmp7* compound mutants.

Dorsoventral patterning in vertebrate and invertebrate embryos is determined by a gradient of Bmp activity controlled in the extracellular space by a network of secreted proteins. Tsg (*Twsg1* – Mouse Genome Informatics) is one of these Bmp regulators and can act both to promote and inhibit Bmp activity. When Tsg forms a stable ternary complex with Bmp and Chordin (Chd), Bmp signaling is prevented (Oelgeschläger et al., 2000; Chang et al., 2001; Larrain et al., 2001; Ross et al., 2001; Scott et al., 2001). However, the presence of Tsg in the complex facilitates cleavage of Chd by the metalloprotease Tld, enabling Bmp to signal again, and thus promoting Bmp activity (Oelgeschläger et al., 2000; Larraín et al., 2001; Oelgeschläger et al., 2003a; Oelgeschläger et al., 2003b). Tsg loss-of-function experiments in several species have resulted in various phenotypes interpreted as pro-Bmp or anti-Bmp effects. In *Drosophila*, Tsg mutation results in the loss of the amnioserosa, a tissue that requires the highest levels of Bmp/Dpp activity (Mason et al., 1994), and absence of Tsg...
also impairs Bmp/Dpp diffusion in the embryo (Eldar et al., 2002). In Xenopus, microinjection of Tsg antisense morpholino oligos (MO) has been proposed to cause phenotypes consistent with an increase in Bmp signaling (Blitz et al., 2003). In zebrafish, by contrast, knockdown of Tsg dorsalizes the embryo, indicating that Tsg has a pro-Bmp effect in development (Little and Mullins, 2004; Xie and Fisher, 2005). In the mouse, Tsg has been shown to play a role in thymus, foregut, craniofacial and skeletal differentiation; Tsg appears to be a Bmp antagonist during T-cell development, yet it foregut, craniofacial and skeletal differentiation; Tsg appears to be a Bmp antagonist during T-cell development, yet it

in the absence of Tsg, half a dose of Bmp4 is insufficient for head development (Zakin and De Robertis, 2004).

Studies on Tsg have focused on interactions with Bmp4, but, as shown here, Tsg also binds to Bmp7. In Xenopus, Tsg and Bmp7 were knocked down using antisense MO oligos; the injected embryos displayed tail truncations and loss of the ventral fin. In the mouse, we performed crosses between Tsg and Bmp7 mutant lines (Dudley et al., 1995; Zakin and De Robertis, 2004). As Bmp7 mutant mice are perinatal lethal (Dudley et al., 1995) and Tsg mutants are viable (Zakin and De Robertis, 2004), increased or new phenotypes could be readily scored in this experimental setting. We found that Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> and Tsg<sup>+/−</sup>;Bmp7<sup>−/−</sup> compound mutants displayed sirenomelia. The appearance of this rare, severe developmental phenotype revealed that in the absence of Bmp7, two functional alleles of Tsg are required for dorsoventral patterning of posterior mesoderm in mammals.

**Materials and methods**

**Mouse strains and genotyping**

Mice carrying the Tsg mutation (Zakin and De Robertis, 2004) were crossed to the Bmp7 mutant strain (Dudley et al., 1995) and backcrossed into the hybrid strain B6SJL/F1 (Jackson Laboratories). Genotyping of pups or embryos was performed as described (Dudley et al., 1995; Zakin and De Robertis, 2004).

**Mouse in situ hybridization, histology and skeletal preparations**

Mouse in situ hybridization on whole mounts were performed (Henrique et al., 1995) using the following probes: Bmp4 (Winnier et al., 1995), Bmp7 (Lyons et al., 1995), brachyury (T) (Herrmann et al., 1990), Fgfl8 (Crossley and Martin, 1995), Shh (McMahon et al., 1998), Tsg (Zakin and De Robertis, 2004) and Wnt3a (Roelink and Nusse, 1991). Procedures for Alcian Blue and Alizarin Red skeletal staining (Belo et al., 1998), and β-gal staining of whole embryos (Zakin and De Robertis, 2004) were as described.

**Western blot analysis**

Immunoprecipitation of pure recombinant human Bmp7, TGFβ2 and Bmp4 (from R&D Systems) was performed as described (Oelgeschläger et al., 2000), using mouse Tsg-Flag pre-bound to anti-Flag agarose beads (M2, Sigma). Secreted proteins for crosslinking studies were harvested from conditioned medium from 293T cells transfected with human Tsg-HA (Tsg containing a hemagglutinin epitope tag at the C-terminus) or mouse Tsg-Flag (Flag epitope tag at the N-terminus), or human Bmp7. Before chemical crosslinking, samples were dialyzed into PBS, then incubated together and treated with DSS (disuccinimidyl suberate) as previously described (Oelgeschläger et al., 2000; Larrain et al., 2001). An immunopurified goat antibody specific for human Bmp7 (R&D systems) was used for western blot analyses.

**Tsg-MO and Bmp7-MO injections and in situ hybridization**

Xenopus microinjection and in situ hybridization were performed as described (Piccolo et al., 1997). Tsg-MO was as previously described (Blitz et al., 2003). Bmp7-MO sequence is as follows 5’-TTA-CTGTCAAAAGCATTCTTTTGTC-3’ (underline indicates AUG start codon) and was designed using sequences identical in the two pseudo-alleles (Heasman et al., 2000; Oelgeschläger et al., 2003b). In vitro, Bmp7-MO efficiently and specifically inhibited translation of Xenopus Bmp7 mRNA (B.R., E.M.D.R. et al., unpublished). Tsg-MO (6 ng per embryo) or of Bmp7-MO (12 ng per embryo) were injected.

**Ventral marginal zone (VMZ) assay and RT-PCR**

VMZ explants from stage 10 embryos were cultured in 1× Steinberg solution until tailbud stage 20 and processed for RT-PCR or phenotypical analysis. The conditions and primer sequences were as described (http://www.hhmi.ucla.edu/derobertis).

**Results**

**Tsg and Bmp7 bind to each other and have overlapping expression domains**

Tsg is known to interact biochemically and genetically with Bmp4 (Oelgeschläger et al., 2000; Zakin and De Robertis, 2004). To test whether Tsg could also interact directly with Bmp7, we analyzed their biochemical interaction by co-immunoprecipitation and chemical crosslinking with DSS (Fig. 1A,A′). For co-immunoprecipitation, mouse Tsg-Flag pre-bound to agarose beads was incubated with 15 nM human recombinant Bmp7, with or without the addition of recombinant TGFβ2 or Bmp4 (Fig. 1A). Tsg was able to pull down Bmp7, and this binding was specific because it was not affected by a six-fold excess of TGFβ2. Bmp4 in equimolar amounts was able to reduce Bmp7 binding by about half, suggesting that Tsg binds both Bmps with comparable affinities (Fig. 1A, compare lanes 2 and 4). When Tsg and Bmp7 proteins from 293T cell culture supernatants were incubated together and chemically crosslinked (see Materials and methods), a bimolecular complex corresponding to a homodimer of Tsg bound to a homodimer of Bmp7 was formed (Fig. 1A′, lane 3).

Tsg and Bmp7 expression domains overlapped in multiple tissues during embryogenesis (Fig. 1B-D′). In particular, histological sections showed that Tsg and Bmp7 are co-expressed in ventral mesoderm and endoderm (Fig. 1E-G). The expression patterns of Bmp7 and Tsg have been reported previously (Solloway and Robertson, 1999; Graf et al., 2001). At 8.25 dpc, Tsg and Bmp7 were expressed diffusely in axial mesoderm and lateral mesoderm, gut endoderm and allantois (Fig. 1B,B′,E). At later stages, co-expression persisted in gut endoderm and overlapping or adjacent expression domains were found in limb bud mesenchyme, optic and otic vesicles, first branchial arch and posterior ventral mesoderm (Fig. 1C,C′,D,D′,F,G).

We conclude that Tsg and Bmp7 proteins are capable of establishing direct biochemical interactions, and that the
two genes share overlapping expression domains during embryogenesis. To investigate whether genetic interactions existed, we next crossed mice carrying mutations in the Tsg and Bmp7 genes (Dudley et al., 1995; Zakin and De Robertis, 2004).

**Sirenomelia and embryonic lethality in Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> and Tsg<sup>−/−</sup>;Bmp7<sup>+/−</sup> compound mutants**

Tsg<sup>−/−</sup> and Bmp7<sup>−/−</sup> mutant strains were mated to generate Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> double heterozygotes. Table 1 summarizes the results obtained from a total of 197 neonates from Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> intercrosses. Interestingly, only 0.5% (n=1) of Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> mutants and 2.5% (n=5) of Tsg<sup>−/−</sup>;Bmp7<sup>+/−</sup> mutants were observed, instead of the expected 6.25% and 12.5%, respectively. This suggested embryonic lethality associated with these genotypes. Embryonic lethality required the loss of both copies of Bmp7; for example, the number of Tsg<sup>+/−</sup>;Bmp7<sup>−/−</sup> mutants recovered was normal (Table 1). Phenotypes previously known to occur when only Bmp7 is mutated (microphthalmia, hindlimb polydactyly, sternebral defects) (Dudley et al., 1995) were examined, and most appeared enhanced in Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> and Tsg<sup>−/−</sup>;Bmp7<sup>+/−</sup> animals (Table 1). Two of these neonates displayed a striking sirenomelia phenotype, characterized by the presence of a single hindlimb. Examination of embryos recovered between 9.5 dpc and 12.5 dpc confirmed that all 23 cases with clear sirenomelia had either the Tsg<sup>−/−</sup>;Bmp7<sup>−/−</sup> or Tsg<sup>−/−</sup>;Bmp7<sup>+/−</sup> genotypes. These results indicate that in the absence of Bmp7 there is a dose-dependent requirement of Tsg for mouse development.
The sirenomelia phenotype can be seen in Fig. 2. In ventral view, a single medial hindlimb was present in pups that had otherwise normal forelimbs (Fig. 2A,B). Alcian blue/Alizarin red staining of the skeletons of Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– pups showed that the single lower limb consisted of the fusion of two independent hindlimbs (Fig. 2C-F). Most skeletal elements were duplicated, but the two limbs were fused at the pelvis and at the heel of the foot (Fig. 2F), and lacked the fibula. The Tsg–/–;Bmp7–/– mutant had only one foot (designated symphus monopus) (Kampmeier, 1927) with only five digits and a bifurcation of the first digit (Fig. 2D; inset). The Tsg–/–;Bmp7–/– mutant had two fused feet (symphus dipus) (Kampmeier, 1927) with nine digits, a single first digit and a bifurcation of one of the second digits (Fig. 2F; inset). The sirenomelia phenotype in the Tsg–/–;Bmp7–/– neonate is considered more severe due to the presence of only one foot. The tail was shorter, with fewer vertebrae and delayed and reduced bone deposition with respect to the wild-type (Fig. 2C,D). As Bmps and Tsg promote chondrogenesis and bone deposition, this is consistent with the notion that the compound mutants have less Bmp signaling. The thoracic region was also affected in Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– mutants (see Fig. S1 in the supplementary material).

In conclusion, the analysis of the skeletons of Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– compound mutants revealed phenotypes indicative of reduced Bmp signaling, not observed in either mutant alone. These results suggest that in a Bmp7-null background the lack, or haploinsufficient doses, of Tsg affects the activity of other Tsg-interacting Bmps, which in vivo would be

<table>
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<th>Observed percentage</th>
<th>Anophthalmia microphthalmia (%)</th>
<th>Hindlimb polydactyly (%)</th>
<th>Sirenomelia (%)</th>
<th>Sternebral defects (%)</th>
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<td>4 (80%)</td>
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<td>Tsg+/–;Bmp7+/–</td>
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<td>0.5</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
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<tr>
<td>Total</td>
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N/A, not assessed.
*A determined by skeletal staining.

Fig. 2. Sirenomelia in Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– compound mutants. (A,B) Comparison of a wild-type neonate with a Tsg–/–;Bmp7–/– mutant displaying sirenomelia. The mutant appears normal up to the level of the hindlimbs which are fused. (C-F) Alcian Blue (cartilage) and Alizarin Red (bone) preparations of wild-type and Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– mutants. (C,D) Lateral view of the posterior region of a wild-type and Tsg–/–;Bmp7–/– skeleton. In the mutant, the tail is shorter and has fewer vertebrae. The single hindlimb contains two femurs (fe), two tibias (ti), a single foot and lacks both fibulae (fi). (E,F) Ventral views of the hindlimbs and pelvic region of a wild-type and Tsg–/–;Bmp7–/– neonate. The mutant hindlimb is formed by the fusion of two hindlimbs at the level of the ischium (is); two feet are present, fused at the level of the heel. Insets in D and F show higher magnification views of the feet of Tsg–/–;Bmp7–/– and Tsg+/–;Bmp7–/– neonates, respectively. Digits are indicated by numbers and digit duplications by letters. il, ilium; is, ischium; p, pubic bone; ps, pubic symphysis.
functionally redundant with Bmp7. In Bmp7+/− embryos, these other Bmps would compensate for the loss of Bmp7; however, when Tsg function is reduced, the activity of these other Bmps is lowered to levels incompatible with normal development.

**Knockdown of Tsg and Bmp7 affects ventral development in Xenopus embryos**

These interactions were also observed in Xenopus loss-of-function experiments using antisense MO oligonucleotides. Tsg-MO (Blitz et al., 2003) and Bmp7-MO, were injected independently and in combination, into Xenopus embryos (Fig. 3). Injections were performed at the site of Tsg and Bmp7 expression, in the ventral side. Injection of Bmp7-MO at the four-cell stage produced a mild phenotype characterized by a partial loss of the ventral fin and a posteriorized anus (Fig. 3D,I,J; arrowhead in J). Similar injections of Tsg-MO produced a phenotype characterized by a bent tail and reduced ventral fin tissue (Fig. 3N,O). The ventral fin tissue is an indicator of ventral mesoderm development (Hammerschmidt and Mullins, 2002) and in Xenopus is derived from the ventral-most mesoderm (Tucker and Slack, 2004). At earlier stages, Tsg-MO or Bmp7-MO caused a reduction of the ventrally expressed gene sizzled (Fig. 3A,F,K), a marker of high Bmp signaling (Collavin and Kirschner, 2003; De Robertis and Kuroda, 2004). The anterior marker Otx2, which is negatively regulated by Bmp, was unaffected or slightly expanded by injection of the individual morpholinos (Fig. 3B,G,L). When Tsg-MO and Bmp7-MO were injected together, sizzled expression was almost completely eliminated, indicating a loss of Bmp signaling (Fig. 3P). Otx2 expression domain was significantly expanded at the neurula stage, also indicating decreased Bmp signaling (Fig. 3Q). However, the head region marked by Otx2 and somitic mesoderm marked by Myod1 appeared normal by the early tailbud stage (Fig. 3R). When Bmp7-MO and Tsg-MO were co-injected, the ventral fin phenotype increased markedly, causing the development of tadpoles with a truncated tail and virtually no ventral fin (Fig. 3S,T).

One of the most sensitive assays for Bmp signaling levels is provided by the Xenopus ventral marginal zone (VMZ) explant (Fig. 4A). When such explants were prepared from embryos injected ventrally with Bmp7-MO or Tsg-MO, they resembled lateral marginal zone (LMZ) explants containing polarized patches of unpigmented cells (Fig. 4B-D). When Bmp7-MO and Tsg-MO were co-injected, the explants elongated and adopted the external appearance of dorsal marginal zone (DMZ) explants (Fig. 4E). As shown by the RT-PCR gene marker analyses of Fig. 4F, both Tsg-MO and Bmp7-MO had anti-Bmp effects in Xenopus. VMZ explants expressed the ventral mesodermal marker β-globin (lane 4), while Tsg-MO (lane 5) or Bmp7-MO (lane 9) expressed markers present in more lateral LMZ, explants (lane 3), such as α-actin, the neural crest marker slug, and the neuronal marker N-tubulin. When Tsg and Bmp7 were knocked down simultaneously (lane 8), strong induction of somitic mesoderm (α-actin), neurons (N-tubulin), cement gland (marked by XAG) and even the dorsal midline markers pintallavis/HNF3β and sonic hedgehog (Shh) was observed. This pattern of expression of dorsoventral markers in double knockdowns was very similar to that of uninjected DMZ (also known as Spemann organizer) explants.

![Fig. 3. Tsg and Bmp7 cooperate in ventral development in Xenopus embryos.](image-url)
Importantly, we also injected several dosages of Bmp7-MO (lanes 6-8), showing that Bmp7 and Tsg interacted also at intermediate Bmp7 levels in Xenopus embryos. As the siren phenotype in mouse was only observed on a Bmp7−/− background, these epistatic experiments provide evidence that Bmp7 and Tsg interact with each other in vivo.

These results show that the wild-type function of Tsg in Xenopus is to promote Bmp signaling, as is the case in zebrafish (Little and Mullins, 2004; Xie and Fisher, 2005). They also show that Bmp7 and Tsg cooperate in the formation of ventral tissues, suggesting that the siren phenotype observed in mouse could be due to a loss of Bmp signaling in ventral mesoderm.

**Tsg−/−;Bmp7−/− and Tsg+/−;Bmp7−/− embryos lack ventral mesodermal structures**

We next dissected embryos from Tsg+/−;Bmp7+/− heterozygous matings at different stages. Most Tsg−/−;Bmp7−/− and Tsg+/−;Bmp7−/− compound mutants recovered after 9.5 dpc were found dead. The likely cause of lethality is related to defects in chorioallantoic fusion (discussed below). At 10.5 dpc, compound mutant embryos appeared developmentally delayed compared with their littermates (Fig. 5A-C). The morphology of embryos displaying sirenemia, in which the hindlimb buds are fused along the ventral midline into a single bud is shown in Fig. 5B (arrow); this fusion can be best seen in ventral views such as the one shown in Fig. 7D′ below. We found that 100% of Tsg−/−;Bmp7−/− (11 embryos out of a total of 140) and 75% of Tsg+/−;Bmp7−/− (12 embryos) were abnormal. The siren phenotype was observed in 75% of Tsg−/−;Bmp7−/− mutants and in 88% of Tsg+/−;Bmp7−/− mutants. In addition, kinked neural tubes were often seen (data not shown). In rarer cases, 25% of Tsg−/−;Bmp7−/− and 12% of Tsg+/−;Bmp7−/− mutants were severely underdeveloped and displayed heart edema (Fig. 5C).

Serial histological sections were performed on 9.5 dpc embryos from the hindlimb bud region to the tip of the tail to examine the anatomy of embryos with sirenomelia (Fig. 5D-G′). At the level of the hindlimb buds in the normal embryos four blood vessels are seen. Two dorsal aortae, which after they curve back into the tailbud, form ventrally the recurved part of the distal aortae separating two distinct lateral coelomic cavities (Fig. 5D,E). Two thickenings of the lateral plate mesoderm mark the forming of the left and right hindlimb buds (Fig. 5E). In mutant embryos, a single coelomic cavity was observed (Fig. 5E′; see Fig. S2 in the supplementary material), and the recurved ventral region of the posterior aorta was absent (Fig. 5D′,E′; see Fig. S2 in the supplementary material). In compound mutants, the posterior arterial system had a single abnormally placed umbilical artery directly arising from the dorsal aorta (Fig. 5D′), and a single thickening of the mesoderm was seen in the ventral hindlimb buds (Fig. 5E′). In mutant embryos, a single coelomic cavity was observed (Fig. 5E′, see Fig. S2 in the supplementary material), and the recurved ventral region of the posterior aorta was absent (Fig. 5D′,E′; see Fig. S2 in the supplementary material). In compound mutants, the posterior arterial system had a single abnormally placed umbilical artery directly arising from the dorsal aorta (Fig. 5D′), and a single thickening of the mesoderm was seen in the ventral hindlimb buds (Fig. 5E′). Most defects were ventral in Tsg−/−;Bmp7−/− compound mutants (Fig. 5E′; see Fig. S2 in the supplementary material) and, more posteriorly, tailbud mesoderm cells were sparser (Fig. 5G′). In summary, the sirenemia phenotype is caused by a reduction of ventral mesodermal tissue that results in the fusion of the hindlimb buds and coelomic cavities. The results indicate a requirement in the mouse for Bmp signaling in ventral mesoderm formation and/or survival.

**Onset of ventral defects in Tsg−/−;Bmp7−/− and Tsg+/−;Bmp7−/− compound mutants**

To determine the onset of this ventroposterior mesodermal...
phenotype, mesodermal markers were analyzed at 8.25 dpc and 9.0 dpc (Fig. 6). Brachyury is a notochord and primitive streak marker required for axial elongation and formation of posterior mesoderm (Herrmann et al., 1990; Wilson et al., 1995). Its expression was not significantly changed at 8.25 dpc (Fig. 6A,A′). Fgf8 is required for cell migration and patterning of the primitive streak and the mesodermal cells exiting from it (Crossley and Martin, 1995; Sun et al., 1999) and for formation of posterior mesoderm (Draper et al., 2003). Its expression was reduced at 8.25 dpc in mutant embryos (Fig. 6B-C′). Expression of Wnt3a, which regulates paraxial mesoderm fates at the expense of neuroectodermal fates (Yoshikawa et al., 1997), was unchanged in the primitive streak and ectoderm (data not shown). Thus, at 8.25 dpc, mesodermal cells appear properly specified (as shown by the normal expression of Brachyury and Wnt3a), but posterior mesoderm cells may be reduced in number, as suggested by the decrease in Fgf8 staining.

After the embryo has turned at 9.0 dpc, most mutants displayed a small and poorly developed allantois delayed in its fusion to the chorion (Fig. 6E,E′). At this stage, expression of Brachyury in tailbud mesoderm was moderately reduced in the ventral mesoderm close to the tip of the tail (Fig. 6D,D′). Normally at 9.0 dpc, Bmp4 expression is found in posterior lateral plate mesoderm and extra-embryonic mesoderm of the allantois (Fujiwara et al., 2001). In mutant embryos, the Bmp4 expressing region closest to the tip of the tail was reduced and the allantois failed to fuse to the chorion (Fig. 6E,E′).

We found that the onset of ventral mesoderm defects could be traced prior to the turning of the embryo and was initially characterized by a deficit in posterior mesoderm cells expressing Fgf8. Posterior mesodermal cells appear to be specified normally in the posterior primitive streak, but are unable to either proliferate or survive. The lack of ventral posterior mesoderm could also help explain the defects observed in allantoic growth and fusion to the chorion, as the allantois increases in size by the recruitment of cells from the posterior primitive streak (Fujiwara et al., 2001). The defect is not due to a failure of cells exiting the primitive streak as no ectopic neural structures were observed in Tsg+/–;Bmp7+/– and Tsg+/–;Bmp7–/– mutants, which would have indicated transformation of axial mesoderm into neural fates (Yoshikawa et al., 1997; Abu-Abed et al., 2001; Sakai et al., 2001). We conclude that Tsg and Bmp7 appear to be required for the proper survival and/or proliferation of posterior ventral mesoderm cells and their derivatives.

**Development of fused hindlimb bud territory in siren mutants**

We next examined the development of the hindlimb buds and posterior ventral structures (Fig. 7). At 9.5 dpc Bmp4 marks the ventrolateral part of the embryo in ectoderm, limb mesenchyme and lateral plate mesoderm (arrow in Fig. 7A) (Ahn et al., 2001). In Tsg+/–;Bmp7+/– embryos, Bmp4 staining in limb bud mesoderm spans the embryo transversely (90° from the normal orientation of the hindlimb bud), while the ventralmost mesoderm forms a separate domain (Fig. 7A′,B′). The apical ectodermal ridge marker Fgf8 (Crossley and
Martin, 1995) is also expressed in a transverse domain in the mutant instead of in two arches seen on each side of a wild-type embryo at 10.5 dpc (Fig. 7C-D′). These observations suggest that, as early as 9.5 dpc the hindlimb buds are fused in the ventral and posterior region. Expression of Shh in hindgut endoderm (Gofflot et al., 1997) was reduced in mutant embryos (Fig. 7E-F′), which confirms the reduction in hindgut structures observed in the histological analysis (Fig. 5E,E′ and see Fig. S2 in the supplementary material). Expression of Shh was normal in the notochord, but was delayed in the zone of polarizing activity (Fig. 7E-F′). In the tailbud, the number of cells expressing brachury (Gofflot et al., 1997) at 9.5 dpc was reduced in ventral mesoderm but not in the notochord of mutant embryos (Fig. 7G-H′). We also note a ventral indentation continued posteriorly by a narrower tail in Tsg−/−;Bmp7−/− embryos (arrow in Fig. 7H′). We conclude that in mutant embryos the hindlimb bud territory is improperly specified as early as 9.5 dpc. The sirenomelia phenotype results from an early fusion of the posterior hindlimb buds that correlates with a deficit of ventral posterior mesoderm.

Discussion

Previous work has shown that Tsg is not essential for embryogenesis, as Tsg−/− mice are viable and fertile, but are smaller and exhibit skeletal defects (Nosaka et al., 2003; Petryk et al., 2004; Zakin and De Robertis, 2004). However, when one copy of Bmp4 was removed in certain genetic backgrounds, Tsg was shown to exhibit a genetic interaction involved in forebrain formation (Zakin and De Robertis, 2004). As Tsg is broadly expressed throughout the embryo and many members of the Bmp family exist (Hogan, 1996; Zhao, 2003), we investigated whether genetic interactions with Bmps were a general feature of Tsg function by analyzing Tsg;Bmp7 compound mutants. Our results show that in the absence of Bmp7, Tsg is required for the formation of posterior ventral structures.

Genetic interactions between Tsg and members of the Bmp family

Tsg interacts biochemically with Bmp4 (Oelgeschläger et al., 2000; Chang et al., 2001). In the mouse, Tsg interacts with Bmp4 in a dose-dependent fashion; in the absence of Tsg, the loss of one copy of Bmp4 results in holoprosencephaly and branchial arch defects (Zakin and De Robertis, 2004). In the present study, we found that the combined functions of Tsg and Bmp7 are required in a different aspect of embryogenesis. Tsg−/−;Bmp7−/− and Tsg−/−;Bmp7−/− compound mutants displayed sirenomelia. Embryonic lethality and sirenomelia were observed only in Bmp7−/− embryos in which one or two copies of Tsg were also missing. Thus, the dose of Tsg is the limiting factor. Tsg was shown to bind directly to Bmp7 (Fig. 1A) but could also interact with other Bmps, which could be functionally redundant to Bmp7 and compensate for its loss. When one copy of Tsg is removed in Bmp7−/− mutants, the levels of these other Bmp signals may fall beneath the
Bmp and Tsg signaling in ventral mesoderm

Development and disease

threshold required for ventral mesoderm development. It is also possible that Bmp7 may actually modulate Tsg function; however, this seems unlikely in view of previous biochemical work showing that Tsg regulates the ability of Bmps to signal through Bmp receptors (Larrain et al., 2001; Oelgeschläger et al., 2003a).

One candidate for Bmp7 compensation is Bmp4, as both Bmp7 and Bmp4 are expressed in ventroposterior mesoderm (Lawson et al., 1999) and Bmp4 is required for tailbud formation in the frog (Beck et al., 2001). In mouse, Bmp4;Bmp7 double heterozygotes display increased defects in the rib cages and limbs, supporting a cooperation between Bmp4 and Bmp7 (Katagiri et al., 1998).

In addition, tetraploid chimeras lacking Bmp4 in inner cell mass derivatives have impaired allantois differentiation, absence of the vitelline artery at 8.25 dpc and kinked neural tubes (Fujiiwara et al., 2001). Because similar phenotypes are seen in our sirenomelic mutants, the defects observed could be the result of a deficit in Bmp4 activity. Another candidate for an interaction with Tsg is Bmp5. Embryonic lethality is observed in Bmp5;Bmp7 double mutants but not in each individual mutant (Solloway and Robertson, 1999), and they display, among other defects, failure of chorioallantoic fusion and kinked neural tubes (Solloway and Robertson, 1999), also seen in our sirenomelic mutants.

**Tsg and Bmp7 are involved in posterior ventral mesoderm formation**

When Tsg-MO and Bmp7-MO were co-injected into frog embryos, the loss of ventral fin and of tail structures was observed (Fig. 3). In Xenopus, the ventral mesoderm has been shown to be required for ventral fin formation (Tucker and Slack, 2004). In zebrafish, the loss of ventral fin is associated with decreased Bmp signaling (Bauer et al., 2001; Hammerschmidt and Mullins, 2002). For example, in the mini fin mutants, increased Bmp inhibition by Chd occurs, because Tolloid, the metalloprotease that cleaves Chd, is mutated (Connors et al., 1999). Moreover, knockdown of Tsg in zebrafish results in dorsalization consistent with a loss of Bmp signaling, and a genetic interaction between swirl (Bmp2b) and Tsg loss-of-function was observed; thus, Tsg functions in zebrafish to promote Bmp signaling in dorsoventral patterning (Little and Mullins, 2004; Xie and Fisher, 2005). In Xenopus, ventral mesodermal explants co-injected with Tsg-MO and Bmp7-MO become dorsalized (anti-Bmp phenotype) in a dose-dependent way (Fig. 4). Thus, as in zebrafish, but unlike other reports (Ross et al., 2001; Blitz et al., 2003), the function of Tsg protein in the Xenopus embryo is to promote the formation of ventral mesoderm by increasing the activity of Bmp7 and other Bmps.

In the mouse a loss of ventral mesoderm is also observed:
sirenomelia in Tsg\textsuperscript{−/−};Bmp7\textsuperscript{−/−} and Tsg\textsuperscript{−/+};Bmp7\textsuperscript{−/+} compound mutants is characterized by a reduction of Fgf8 staining at 8.25 dpc, deficiencies of posterior lateral plate mesoderm expressing Bmp4, and a reduction of tail bud mesoderm expressing brachyury at 8.5 dpc. In tetraploid chimeras that lack Bmp4 in the inner cell mass, a reduction of Fgf8 in the primitive streak has been reported (Fujinawa et al., 2001). That study showed that extra-embryonic Bmp4 was required for survival and differentiation of the allantois.

Mutations in the retinoic acid degrading enzyme Cyp26 cause sirenomelia (Abu-Abed et al., 2001; Sakai et al., 2001). They differ from our Tsg\textsuperscript{−/+};Bmp7\textsuperscript{−/−} and Tsg\textsuperscript{−/+};Bmp7\textsuperscript{−/+} sirens in that they also display severely truncated tails and spina bifida. Cyp26 knockouts showed a reduction of Wnt3a and brachyury expression, associated with impaired mesoderm proliferation and the mesoderm adopting neural fates (Sakai et al., 2001). In our mutants, we did not observe changes in the expression of Wnt3a nor excessive neural structures, but a reduced number of cells expressing brachyury in the tailbud mesoderm was seen. Preliminary results indicate that cell proliferation was not significantly changed, but apoptosis was increased in ventral posterior regions in compound mutants (L.Z. and E.M.D.R., unpublished). In the mouse, other work has implicated low Bmp signaling both in decreased cell proliferation and/or increased apoptosis (Solloway and Robertson, 1999; Fujinawa and Hogan, 2001). Although sirenomelia has variable phenotypic traits and multiple causes (Wei and Sulik, 1996; Padmanabhan, 1998), its occurrence is always associated with defects in posterior primitive streak. The sirenomelia phenotype observed here is consistent with a defective differentiation of the posterior and ventral mesoderm caused by decreased Bmp signaling.

A link between Tsg, Bmp7 and the original sirens?

Sirens were discovered in the mouse (Gluecksohn-Schoenheimer and Dunn, 1945) among the progeny of parents carrying various combinations of the Short-tail (T locus), anury (ur\textsuperscript{−/−}), Fused and ur mutations. The siren pups obtained had no tail, various degrees of reduction and fusion of elements of the hindlimbs, abnormalities of the spine, and fusion of ribs. Even though Tsg\textsuperscript{−/+};Bmp7\textsuperscript{−/−} and Tsg\textsuperscript{−/+};Bmp7\textsuperscript{−/+} sirenomelic pups do form tails (albeit shorter), the limb bud phenotypes we observe are very similar to those of Gluecksohn-Schoenheimer and Dunn. Could the old and new mutations be linked in any way? We note that the T locus (including brachyury), Fused (corresponding to Axin) (Zeng et al., 1997) and Tsg are all located on chromosome 17. The ur mutation (urogenital syndrome) (Lyon and Searle, 1989), which is phenotypically identical to the now extinct ur (urogenital) mutant, and Bmp7 are both located on chromosome 2. Although the respective locations of these genes on these chromosomes are distant from each other, mutations at the T locus correspond to important chromosomal rearrangements, often leading to duplications and deficiencies of chromosome segments upon cell division (Gluecksohn-Schoenheimer and Dunn, 1945). Thus, it is conceivable, although perhaps unlikely, that the occurrence of sirens in the initial description was associated with disruptions of the Tsg and/or Bmp7 genes. Unfortunately, some of the original mutations have been lost, so this is not a testable proposition.

In subsequent work, Hoornbeek found sirenomelic neurones in crosses between SM/J and BUA strains studied for the incidence of the ‘careener’ phenotype (Hoornbeek, 1970; Schreiner and Hoornbeek, 1973). These sirens have the same phenotype as ours (fused hindlimbs, a tail, an abnormal umbilical artery). The genes affected in these crosses are not known, but the carriers of the ‘siren’ mutation (Hoornbeek, 1970) had tightly twisted tails, which is of relevance because Tsg\textsuperscript{−/+} or Bmp7\textsuperscript{−/+} mutants also have kinked tails (Jena et al., 1997; Zakin and De Robertis, 2004).

In conclusion, in the absence of Bmp7, two copies of Tsg are required for the proper differentiation of ventral and posterior structures. In the mouse, when Tsg and Bmp7 are mutated, the siren phenotype results from the fusion of the limb buds in the ventroposterior midline owing to a paucity of posterior ventral mesoderm. In Xenopus, knockdown of Tsg and Bmp7 results in an analogous phenotype: loss of posteroventral cell fates associated with decreased Bmp activity. These results demonstrate a common mechanism, mediated by Bmp signaling, in mouse and frog in the patterning of the dorsoventral axis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/10/2489/DC1

References


Development and disease


Geoffroy Saint-Hilaire, I.


Gluecksohn-Schoenheimer, S. and Dunn, L. C.

Grauwiler, S., Boorla, S., Frendo, J. L., Hogan, B. L. and Karsenty, G.

Harmscmidt, M. and Mullins, M. C.


Katagiri, T., Boorla, S., Frendo, J. L., Hogan, B. L. and Karsenty, G.


Sirenomelia in Bmp7 and Tsg compound mutant mice: requirement for Bmp signaling in the development of ventral posterior mesoderm
Development Zakin et al. 132: 2489

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Files in this Data Supplement:

- **Supplemental Figure 1**

**Fig. S1.** Phenotypic defects in the thoracic region of Tsg−/−;Bmp7−/− and Tsg+/-;Bmp7−/− neonate skeletons stained with Alcian Blue (cartilage) and Alizarin Red (bone). (A and B) View of the vertebral bodies showing the presence of two ossification centers in the Tsg;Bmp7 double mutant instead of the normal single one (white arrow). (C-F) Ventral view of the sternum of wild-type, Bmp7−/−;Tsg−/− and Tsg or Bmp7 single mutants. Numbers I to VI designate the sternebrae and numbers 1 through 7 the ribs. Note that Bmp7−/− mutants often lack the fifth sternebra (asterisk in panel E) (Dudley et al., 1995) while in the Tsg−/−;Bmp7−/− mutant the fourth and fifth sternebrae are incomplete or missing (bracket in panel F). In the Tsg−/−;Bmp7−/− mutant the fourth, fifth, and sixth ribs are fused to each other (black arrow). These results showed that in the spinal column of the compound mutant the vertebral bodies were smaller, ossification was delayed and chondrogenesis was incomplete in the region of the centrum, with the formation of two ossification centers instead of a single central one in compound mutants (panels A,B). Since Bmps are well-known inducers of chondrogenesis, the defects observed in the Tsg−/−;Bmp7−/− mutant are consistent with a role for Tsg in promoting Bmp activity. The sternum of Tsg−/− pups was unaffected (panels 1D), but much more severe additional defects were observed in Tsg−/−;Bmp7−/− rib cages (panel F).
**Supplemental Figure 2**

**Fig. S2.** Anatomical analysis of the siren phenotype. Serial transverse histological sections of the posterior region of wild-type, $Tsg^{+/+};Bmp7^{+/+}$, $Bmp7^{-/-}$, and $Tsg^{+/+};Bmp7^{-/-}$ embryos. Hematoxylin and Eosin staining.