The secreted Frizzled-related protein Sizzled functions as a negative feedback regulator of extreme ventral mesoderm

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SUMMARY

The prevailing model of dorsal ventral patterning of the amphibian embryo predicts that the prospective mesoderm is regionalized at gastrulation in response to a gradient of signals. This gradient is established by diffusible BMP and Wnt inhibitors secreted dorsally in the Spemann organizer. An interesting question is whether ventrolateral tissue passively reads graded levels of ventralizing signals, or whether local self-organizing regulatory circuits may exist on the ventral side to control cell behavior and differentiation at a distance from the Organizer. We provide evidence that sizzled, a secreted Frizzled-related protein expressed ventrally during and after gastrulation, functions in a negative feedback loop that limits allocation of mesodermal cells to the extreme ventral fate, with direct consequences for morphogenesis and formation of the blood islands. Morpholino-mediated knockdown of Sizzled protein results in expansion of ventral posterior mesoderm and the ventral blood islands, indicating that this negative regulation is required for proper patterning of the ventral mesoderm. The biochemical activity of sizzled is apparently very different from that of other secreted Frizzled-related proteins, and does not involve inhibition of Wnt8. Our data are consistent with the existence of some limited self-organizing properties of the extreme ventral mesoderm.

Key words: Sizzled, Wnt signaling, Ventr al blood islands, sFRP, Embryonic patterning, Xenopus

INTRODUCTION

The concept of the Spemann organizer has dominated our understanding of early vertebrate development. In amphibians, the organizer corresponds to a small dorsal sector of the gastrula marginal zone (~60°); if experimentally transplanted to the ventral region, these cells induce a complete ectopic axis in the recipient tissue (Spemann and Mangold, 1924). In molecular terms, the organizer functions by secreting diffusible inhibitors of the Wnt and BMP pathways (De Robertis et al., 2001; Harland and Gerhart, 1997). Therefore, in a model similar to the original gradient proposal of Wolpert (Wolpert, 1989), the marginal zone of the gastrula would manifest extreme dorsal cell fates at low levels of BMPs and Wnts, and extreme ventral cell fates at high levels of these signaling molecules. Intermediate fates may be elicited by intermediate levels.

Although the dominance of the dorsal signals is obvious (transplanting ventral cells to the dorsal side does not produce an ectopic ventral axis), it does not follow that all of the remaining tissue merely responds to levels of signals dictated by the organizer. It is possible that patterning in the ventral and lateral domains is only weakly dependent on the dorsal signals, although still subservient to them. The ventral region may rely on self-organizing processes to regulate behavior and fate of the cells at a distance from the organizer. When Wnt and BMP inhibitors diffusing from the dorsal side fall below a certain threshold, these ventral organizing activities would be activated and much of the pattern would be generated by them, rather than by the concentration of the inhibitors. Most phenomenological experiments, such as those of Spemann and Mangold do not distinguish between these possibilities.

There is much complexity to control on the ventral side, including the allocation of cells to the lateral plate mesoderm, muscle, pronephros and blood (Davidson and Zon, 2000; Hemmati-Brivanlou and Thomsen, 1995), and there is some evidence for processes of ventral organization being more complex than simply reading out a gradient of BMP and Wnt signals created by inhibitors secreted from the organizer (Munoz-Sanjuan and Hemmati-Brivanlou, 2001). For example, not all mesoderm is converted to blood in UV-irradiated embryos. Analogously, in embryos ventralized by dorsal injection of BMP4 or DNsTCF3, it is possible to distinguish a defined domain of globin expression in the absence of the organizer (Kumano and Smith, 2000). The BMP and Wnt pathways themselves interact and are capable of generating considerable complexity (Hoppler and Moon, 1998; Marom et al., 1999). Part of the patterns of ventrolateral mesoderm undoubtedly originates from reciprocal modulation of these two signals (Dale and Jones, 1999; De Robertis et al., 2001). But are different ventrolateral fates determined simply by the distance from the dorsal organizer, or are there self-organizing
activities that generate semi-independent patterns? If so, what role do they play in regulating cell differentiation and cell behavior on the ventral side?

Exactly 180° opposite the organizer at early gastrulation is a restricted expression domain for the gene szl (Szl), encoding a secreted Frizzled-related protein similar to Wnt inhibitors expressed in the dorsal mesendoderm (Salic et al., 1997). Secreted Frizzled-related proteins (sFRP) contain a conserved cysteine rich domain similar to the ligand-binding domain of the Wnt receptors (Frizzleds), and can inhibit signaling by directly competing for secreted Wnt proteins (Wang et al., 1997; Wodarz and Nusse, 1998; Xu et al., 1998). Szl is the only known sFRP expressed ventrally during and after gastrulation, eventually overlapping with the ventral blood islands at tailbud stages (Bradley et al., 2000; Salic et al., 1997). In embryos where the balance of mesoderm is shifted from ventral to dorsal by early exposure to Li+, expression of Szl is abolished. Reciprocally, when embryos are ventralized by UV irradiation of the egg cortex, Szl expression extends around the entire marginal zone (Salic et al., 1997). Additional studies have shown that Szl RNA is induced by BMP4 in a dose-dependent manner in animal caps, whereas it is downregulated in embryos injected with a truncated BMP receptor (Marom et al., 1999). Therefore, it is likely that Szl expression depends on BMP4 signaling, either directly or indirectly. Based on its behavior and its structural features, we had previously proposed that Szl generates a domain in the extreme ventral side with high BMP and reduced Wnt signals (Salic et al., 1997).

In the present work, we have attempted to define the biological role of Szzled by examining the effects of morpholino-mediated suppression of Szl translation, and by studying the effects of Szl on Wnt8 activity. The results suggest that Szl is not an inhibitor of Wnt8, and that compared with other sFRPs its activity may be unique. The data further show that Szl is required for proper development of ventral posterior mesoderm, and in particular of the ventral blood islands (VBI). Specifically, Szl knockdown expands ventral posterior mesoderm and the VBI, whereas its overexpression restricts the ventral mesoderm and the VBI. Therefore, Szl appears to function in a negative feedback loop regulating allocation of cells to the most ventral fate. These observations suggest the existence of some limited self-organizing properties of the extreme ventral mesoderm, and reveal unexpected complexity within the ventral marginal zone.

MATERIALS AND METHODS

Embryo manipulation and injection

Xenopus laevis embryos were obtained by in vitro fertilization, dejellied in 2% cysteine and cultured in 0.1× Marc’s Modified Ringer’s (MMR) according to standard procedures (Sive et al., 2000). Staging was carried out according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For injections, DNA or RNA samples were dissolved in water and injected at a volume of 10 nl per blastomere. Embryos were injected in 0.4× MMR + 5% Ficoll and subsequently transferred to 0.1× MMR. Samples were injected in the marginal region of all four blastomeres at the four-cell stage, unless otherwise specified. When required, the ventral side was identified at the four-cell stage based on size and pigmentation of the blastomeres, but preferentially on location of the sperm entry point. Dorsal and ventral explants were separated from devitellinized stage 15 embryos using an eyebrow knife as described (Larkin and Danilchik, 1999). Explants were dissected in 1× MMR and incubated overnight in 0.5× MMR containing 10 μg/ml gentamycin.

Design and characterization of morpholino oligos and a rescue Szl cDNA

The morpholino oligo used to knockdown Szl expression, MOSZL (5’-GAGGAGGAAAGACCTCCCATG-3’), was purchased form Gene-Tools LLC. It spans nucleotides ~1 to +24 with respect to the ATG of Xenopus szl, and anneals to each paralog of the gene with a single mismatch. Two different morpholino oligos were used as negative controls: MOC, which is the standard control provided by Gene-Tools; and MOC2 (5’-GCGAGCTTGTATGAACCTCA-AC-3’), which was designed within the coding region of szl. MOC2 anneals perfectly to each szl paralog but, being downstream of the ATG, has no effect on translation. As morpholino oligos diffuse very rapidly in Xenopus blastomeres (Nutt et al., 2001), we always injected MOSZL in both blastomeres at the two-cell stage for even distribution to the entire embryo. The construct pCS2-wSzl-MT used in rescue experiments contains appropriate conservative mutations so that the resulting mRNA has nine mismatches to MOSZL. Efficacy of MOSZL was tested in vivo according to the following protocol: 10 ng of MOSZL were injected in each blastomere of two-cell embryos; subsequently, 200 pg of an expression plasmid encoding Myc-tagged versions of either Szl (pCS2-Szl-MT) or wSzl (pCS2-wSzl-MT) were injected in the marginal zone of the same embryos at the four-cell stage. Expressed proteins were detected at different developmental stages by immunoblotting. Under these conditions, MOSZL efficiently blocked szl translation up to 3 days after injection (stage 30/35), while expression from the rescue construct pCS2-wSzl-MT was only minimally affected (data not shown).

Benzidine and o-dianisidine staining for blood cells

Both methods stain erythrocytes by forming a precipitate upon oxidation by the heme group of hemoglobin in the presence of hydrogen peroxide. Benzidine staining was performed as described (Hemmari-Brivanlou and Thomsen, 1995); reactions were monitored closely, and embryos were promptly photographed before significant damage by corrosion. o-dianisidine staining was performed as described previously (Huber et al., 1998). Reactions were terminated by transferring the embryos in ethanol.

In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Harland, 1991; Salic et al., 1997) using digoxigenin- or fluorescein-labeled antisense RNA probes. Details on the constructs and probes used in the present work are available from the authors. For double in situ hybridization, the two probes were detected closely, and embryos were promptly photographed before significant damage by corrosion. o-dianisidine staining was performed as described previously (Huber et al., 1998). Reactions were terminated by transferring the embryos in ethanol.

Plasmids

The coding regions of Xenopus Frzb1, Crescent and Dkk1 were PCR amplified from corresponding plasmids using high-fidelity PfX Polymerase (Invitrogen/Life Technology) and cloned in pCS2-MT so that 6xMyc epitope tags were added at the C terminus. pCS2-DNxWnt8 has been described previously (Hoppler et al., 1996), as well as pCS2-DNxWnt11 (Tada and Smith, 2000).

RT-PCR

Blood induction in animal caps was performed as described (Huber et al., 1998; Mead et al., 1998). Animal caps were dissected at stage...
8-9, and incubated in 0.5× MMR until sibling embryos reached stage 30-35. When indicated, 50 ng/ml of recombinant human bFGF (Life Technology/Invitrogen) were added to the incubation medium. Total RNA was extracted using the RNAeasy procedure with an additional DNaseI step to remove contaminant genomic DNA (Qiagen).

Radioactive semi-quantitative RT-PCR was performed on random primed cDNA using previously described primers for xGata1, αT3 globin (Kelley et al., 1994) and EF1α. (Agius et al., 2000). The primers used to amplify endogenous szl in embryos injected with wSzL were EndoSzl-U 5′-CATGTCCGGAGTCTTCTGC-3′ and EndoSzl-L 5′-GGATGAAACGGTCCAGCCG-3′.

Immunoblotting and luciferase assays

For immunoblotting, embryos were crushed by pipetting in low salt buffer (25 mM HEPES pH 7.6, 50 mM NaCl, 0.2% Tween-20, 2mM PMSF, protease inhibitors). Lysates were cleared by centrifugation and 2× Laemmli sample buffer was added to the supernatant. A volume corresponding to one quarter of an embryo was separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Myc-tagged proteins were detected using the 9E10 monoclonal antibody (Santa Cruz). Actin was detected using a polyclonal antibody (Sigma). For luciferase assays, embryos were crushed by pipetting in Luciferase Lysis Buffer (20 mM Tris pH 7.5, 125 mM NaCl, 1 mM MgCl2, 1% Triton-X100, 1 mM DTT, protease inhibitors) and lysates were cleared by centrifugation. A volume of the supernatants corresponding to one half embryo was added directly to Luciferase Assay Reagent (Promega) and light emission was measured in a Turner Design luminometer.

RESULTS

Effects of altered Szl levels on development of Xenopus embryos

_Xenopus laevis_ is a functional diploid, but most of the genes are present in two copies as a heritage of its allotetraploid origin (Graf and Kobel, 1991). Both copies (paralogues) of _Xenopus szl_ have been described, encoding very similar proteins (95% identity) with indistinguishable expression patterns (Bradley et al., 2000; Salic et al., 1997). However, the mRNAs diverge significantly in their 5′ untranslated sequence; we therefore designed an antisense morpholino oligo complementary to the first nucleotides of the coding region, common to both paralogues. This oligo, named MOSZL, blocked in vitro translation of _szl_, and efficiently prevented expression of Szl-MT from an injected plasmid in embryos (see Materials and Methods for details).

As shown in Fig. 1A, injection of increasing amounts of MOSZL produce increasingly severe morphological defects. The affected embryos develop normally through gastrulation, with the first defects being visible after the neurula stage, when they appear enlarged ventrally around the remnant of the blastopore. At tailbud stage, Szl knockdown embryos have a significant expansion of the ventral posterior tissue; in fact, high doses of MOSZL induce a dramatic enlargement of the caudal region, a shortened tailbud and often a bent axis. These embryos develop into tadpoles with an excess of posterior ventral tissue, very often with edema (not shown). Embryos injected with similar or greater amounts of two different control morpholino oligos (MOC and MOC2) are indistinguishable from uninjected siblings (Table 1). The morphological effects of _szl_ knockdown are opposite to those of Szl injection, which results in a dramatic reduction of the ventral posterior tissue. This can be readily appreciated in Fig. 1A, where Szl overexpressing embryos are directly compared with Szl knockdown siblings. Anterior structures are moderately affected; Szl knockdowns have smaller heads, whereas Szl-injected embryos have larger heads. Yet, heads in both cases are fully formed and appear normal in all of their features. Normal, beating hearts develop both in Szl knockdown and overexpressing tadpoles.

To understand better the basis of these phenotypes, we...
followed the development of sibling embryos injected with Szl RNA or with the antisense morpholino oligo by time lapse video microscopy. As shown in Fig. 1B, embryos are indistinguishable up to the neurula stage. Subsequently, when embryos lengthen along the anteroposterior axis, those with reduced levels of Szl protein retain most of the ventral tissue in the posterior region. At the same time, embryos overexpressing Szl accumulate ventral tissue in the anterior region, assuming a characteristic shape with large heads and reduced posterior ventral structures. Although superficially one could refer to these as dorsalized or ventralized phenotypes, they appear to involve a redistribution of cells from posterior ventral in embryos with low Szl levels, to anterior ventral in embryos with high Szl levels.

**Szl affects ventral extension during the neurula to tailbud transition**

Characteristic morphogenetic movements occur in the ventral mesoderm and ectoderm during the transition from neurula to tailbud, when the shape of the embryo changes from spherical to elongated. Evidence that these movements are autonomous and not passive has come from studies of isolated ventral explants, which lengthen as much as the ventral sides of intact embryos. Ventral elongation is primarily due to morphogenetic rearrangements of mesodermal and ectodermal cells (Larkin and Danilchik, 1999). We asked whether the morphological aberrations of szl knockdown or overexpressing embryos derived from defects in dorsal or ventral elongation. Embryos were injected with MOSZL or with Szl RNA, and dorsal and ventral explants were dissected at neurula stage. As shown in Fig. 2, ventral explants from uninjected and Szl-injected embryos elongated to similar lengths, while ventral explants from MOSZL-injected embryos did not lengthen at all. There was a subtler anomaly in the elongation of ventral explants from Szl-overexpressing embryos, where the bulk of ventral tissue remained anterior. As there were no significant differences in lengthening of the corresponding dorsal explants (not shown), these results suggest that the morphological features of affected embryos arise primarily from altered morphogenetic processes of the ventral tissue.

**Szl negatively regulates development of the ventral blood islands**

In *Xenopus*, the primitive site of embryonic hematopoiesis is in the ventral blood islands (VBI), which are located along the ventral midline of tailbud embryos. Blood is therefore a marker of the most ventral mesoderm, independent of its pre-gastrula origins (Davidson and Zon, 2000; Kumano et al., 1999; Lane and Sheets, 2002). As shown in Fig. 3A, embryos injected with increasing amounts of MOSZL developed abnormally large VBIs. Correspondingly, embryos injected with increasing amounts of Szl RNA had severely reduced or absent VBI (see also Table 1). To examine specific stages of the blood differentiation pathway, we analyzed expression of xSCL and xGata2 (not shown) as markers of hematopoietic precursors, and xGata1 and αT3 globin as markers of the committed erythroid lineage (Davidson and Zon, 2000; Mead et al., 2001; L. Collavin and M. W. Kirschner)

![Fig. 2. Elongation of isolated ventral halves from Szl knockdown and overexpressing embryos.](image)
Feedback inhibition of ventral fate (Mead et al., 1998; Zon et al., 1991). Using in situ hybridization, we found that all of these markers were clearly expanded in embryos injected with the Szl morpholino, when compared with control embryos (Fig. 3B). In some instances, we analyzed the same markers in sibling embryos injected with Szl RNA, and we found that levels of xSCL, xGata1 (not shown) and αT3 globin were all significantly reduced (Fig. 3B, parts c,d). Notably, Szl restricted the VBI when injected at levels that have only a mild effect on the overall morphology of the embryo. Taken together these results indicate that Szl inhibits development of the ventral blood islands; expansion of the VBI in Szl knockdown embryos indicates that this inhibitory activity is actually required for proper formation of this ventral derivative.

Szl does not directly affect hematopoietic differentiation

To understand whether Szl might affect blood differentiation per se, we took advantage of a well-established experimental system whereby embryonic hematopoiesis is recapitulated in animal pole explants, and expression of blood markers is monitored by RT-PCR (Huber et al., 1998). Large numbers of blood cells are formed in animal caps after treatment with basic fibroblast growth factor (bFGF), which induces mesoderm, while injection of a moderate amount of BMP4 specifies such mesoderm to ventral fate (Huber et al., 1998; Mead et al., 2001). Alternatively, blood differentiation can be induced in animal caps by injection of high levels of BMP4 RNA even in the absence of bFGF treatment (Xu et al., 1999). Finally, it has been shown that bFGF treatment alone can induce some blood differentiation in animal caps, albeit producing few erythrocytes (Miyanaga et al., 1999). As shown in Fig. 4A, injection of Szl RNA had no detectable effect on xGata1 and αT3 globin expression in animal caps treated with any of the methods described above. Given that Szl transcription is induced by BMP4 in animal caps (Marom et al., 1999), we also injected MOSZL in these experiments. As shown in Fig. 4A, preventing Szl translation had no detectable effect on BMP4-induced erythropoiesis. However, in agreement with the in situ experiments, Szl depletion or overexpression had dramatic effects on the same blood markers in the context of the whole embryo (Fig. 4B). Within the limitations of the experimental system, these results suggest that Szl does not directly interfere with the blood differentiation program.

Szl protein levels regulate the expansion of ventral mesoderm at post gastrula stages

The effects of Szl protein levels on the hematopoietic lineage prompted us to look at its effects on a variety of genes expressed at gastrula stage. First, we analyzed Brachyury (Xbra), a T-box transcription factor expressed in the prospective mesoderm during gastrulation (Smith et al., 1991). As shown in Fig. 5A, both the ring of Xbra expression and the rim of cells not expressing Xbra just above the blastopore lip (leading edge mesoderm) appeared normal in morpholino injected embryos. As a marker of dorsal mesoderm, and particularly of the Spemann organizer, we analyzed the secreted BMP inhibitor Chordin (Sasai et al., 1994); at the same time we analyzed Sox17b, a transcription factor involved in specification of the endoderm (Hudson et al., 1997). As markers of ventral mesoderm, we analyzed the transcription factors Vent2/Xom (Rastegar et al., 1999) and Xpo (Sato and Sargent, 1991). As shown in Fig. 5A, there was no difference
in the expression of any of these markers in MOSZL injected or uninjected gastrulae.

We also analyzed expression of xMyoD, a lateral mesoderm gene which was shown to be downregulated upon Szl overexpression (Salic et al., 1997), and we analyzed szl itself, the most restricted marker for the most ventral sector of the prospective mesoderm at gastrula stage. As shown in Fig. 5A (parts i,j), Szl expression was increased dramatically in MOSZL-injected gastrulae, while xMyoD was not significantly affected. At later stages, Szl RNA was even more dramatically expanded both laterally and anteriorly on the ventral side in embryos treated with the Szl morpholino (Fig. 5A, parts k,l), while xMyoD was correspondingly reduced in its lateral extension at the posterior end of the embryo. szl expression was also dramatically increased at stage 20 (Fig. 5A, parts m,n) in the knockdown experiments, similar to what observed for xSCL (Fig. 3B, part a).

Based on this analysis, szl itself is an early marker of the extreme ventral mesoderm that is expanded when Szl protein levels are reduced by morpholino knockdown. If this is true, szl RNA should be in turn downregulated by Szl overexpression. To test this hypothesis, we used a modified version of Szl designed for rescue experiments (wSzl, see below). We injected wSzl RNA in the marginal zone of four-cell embryos, and monitored expression of endogenous szl at gastrula stage by RT-PCR using primers that do not recognize wSzl. As shown in Fig. 5B, injection of wSzl RNA in the marginal zone of four-cell embryos, and monitored expression of endogenous szl at gastrula stage by RT-PCR using primers that do not recognize Szl mRNA. Both repression and accumulation of endogenous szl are more dramatic at stage 12 than at stage 10.5.

In situ hybridization analysis of two markers expressed in lateral mesoderm after gastrulation further supported the notion that decreased Szl protein levels result in expansion of the most ventral mesoderm. For example, when we analyzed expression of Xlim-1 (Taira et al., 1994) in MOSZL-injected embryos, we found the two characteristic lateral stripes demarcating the future pronephros to be weaker and closer to the dorsal midline in the posterior part of the embryo (Fig. 5C, part a). Similarly, when we analyzed Flk-1/VEGF-R2, a marker of vascular endothelial precursors (Cleaver et al., 1997), we noticed that the vitelline veins that run parallel along the ventral side of tailbud embryos are more dorsal in Szl knockdowns. This means that more ventral mesoderm separates the vitelline veins, while less lateral mesoderm divides the vitelline veins from the cardinal veins located at the base of the somites (Fig. 5C, part c,d).

**Ventral injection of exogenous Szl fully rescues the phenotype of Szl knockdown embryos**

To prove that the observed phenotype is specifically due to knockdown of Szl translation, we asked whether MOSZL-injected embryos could be rescued by exogenous Szl expression. As MOSZL anneals to the coding region, we built a rescue construct in which the corresponding nucleotides were mutated preserving the amino acid sequence. This construct was dubbed wSzl, as ‘wobble’ bases of codons were changed (see Materials and Methods). In rescue experiments, MOSZL was injected in both blastomeres at the two-cell stage, and wSzl RNA was injected in the ventral marginal zone (VMZ) of the same embryos when they reached the four-cell stage (Fig. 6). The same amount of wSzl RNA was also injected in the VMZ of control embryos. Under these conditions, wSzl rescued both the morphological abnormalities and expansion of the VBI in Szl knockdown embryos (Fig. 6, part d). Notably, injection of MOSZL reciprocally rescued the phenotype induced by wSzl overexpression. In fact, control embryos injected with the same amount of wSzl RNA as MOSZL-injected embryos displayed the expected Szl phenotype (Fig. 6, part c; Table 2).

**Increased Wnt expression does not recapitulate the effects of Szl knockdown**

Given that Szl has the structural features of a secreted Wnt inhibitor, we asked whether overexpression of Wnts could produce an effect similar to injection of the antisense
MOSZL, or increasing amounts of wSzl RNA. Endogenous szl was selectively amplified with primers that do not recognize the injected wSzl RNA. EF1α served as an internal control for RNA levels. (C) In situ hybridization of lateral mesoderm markers in embryos injected with 20 ng of MOSZL. (a) Expression of Xlim-1 at stage 20 in lateral (top) and dorsal view (bottom). Arrowheads indicate the point where expression is shifted dorsally in szl morphants. (b-d) Expression of Flk-1 at stage 26 in lateral view and corresponding transverse section. Arrowheads indicate the presumptive vitelline (yellow) and cardinal (black) veins.

Table 2. Rescue of the morphant phenotype by injection of wSzl mRNA

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<th>Morphology (MOSZL-wSzl phenotype)</th>
<th>Blood staining (o-dianisidine)</th>
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<tr>
<td>% healthy embryos with</td>
<td>% healthy embryos with</td>
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<tr>
<td>Weak* MOSZL phenotype</td>
<td>Reduced VBI or absent VBI</td>
</tr>
<tr>
<td>Strong† MOSZL phenotype</td>
<td>Other defects</td>
</tr>
<tr>
<td>Sizzled phenotype (dorsalized)</td>
<td></td>
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<tr>
<td>Other defects</td>
<td></td>
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<tr>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>0.0</td>
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<tr>
<td>MOSZL (10 ng)</td>
<td>22.2</td>
</tr>
<tr>
<td>MOSZL+wSzl (40 pg)</td>
<td>0.0</td>
</tr>
<tr>
<td>wSzl (40 pg)</td>
<td>0.0</td>
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Embryos were injected at the two-cell stage with the morpholino oligo MOSZL and subsequently injected with wSzl RNA in the ventral marginal zone at the four-cell stage. Phenotypes were scored at stage 30/35.

*Weak phenotype: expansion of ventral-posterior tissue with normal tailbud.
†Strong phenotype: dramatic expansion of ventral-posterior tissue with short tailbud and kinked axis.
RNA was detected by in situ hybridization at tailbud stage. As shown in Fig. 7, the VBI were not expanded upon zygotic overexpression of xWnt8 or xWnt11. Specifically, xWnt8 induced severe head truncations and dose-dependent reduction of the VBI, both consistent with previously published observations (Hoppler and Moon, 1998; Kumano et al., 1999). Injection of xWnt11 also caused reduction of the VBI, along with dose-dependent gastrulation defects (Fig. 7). At the same time, injection of RNAs encoding dominant negative Wnt8 (Hoppler et al., 1996) or Wnt11 (Tada and Smith, 2000) failed to reproduce the effects of Szl overexpression. In fact, DNxWnt8 induced a dorsalized phenotype with smaller but clearly detectable VBIs, while injection of DNxWnt11 induced characteristic morphogenetic defects (Tada and Smith, 2000), but no suppression of the VBI.

Other sFRPs cannot phenocopy Szl activity: evidence that Szl function does not involve inhibition of the canonical Wnt pathway

Given the complexity of the Wnt signaling pathway and the many effects that Wnts have on cell differentiation and behavior, overexpression of xWnt8 and xWnt11, or their dominant-negative versions, may induce pleiotropic effects that are unrevealing. We therefore asked whether other sFRPs could recapitulate the activity of Szl in the ventral marginal zone. We injected Crescent, which is closely related to Szl (44% identity) (Pera and De Robertis, 2000; Shibata et al., 2000) and Frzb1, which is perhaps the best characterized sFRP (Leyns et al., 1997; Wang et al., 1997). We also injected Dkkp1 (xDkk1), which is not a sFRP but is a potent and specific inhibitor of the canonical Wnt pathway (Bafico et al., 2001; Glinka et al., 1998). To verify that proteins were expressed at comparable levels, we used epitope-tagged versions that could be detected by immunoblotting.

In experiments analogous to those described in Fig. 6, none of the tested Wnt inhibitors could rescue the szl knockdown. Morphologically, the injected embryos often displayed a complex phenotype, with defects induced by the sFRPs superimposed on those induced by MOSZL (not shown). More easily measurable, expansion of the VBI was not rescued by injection of any of these genes (Table 3).

Next, we asked whether these Wnt inhibitors could inhibit formation of the VBI. Increasing amounts of wSzl-MT, Frz-MT, xCrescent-MT and xDkk1-MT were injected in the marginal zone of four-cell stage embryos, and expression of blood markers was analyzed by RT-PCR at stage 30/35. Some embryos were collected at gastrula stage to verify the levels of injected proteins by immunoblotting (Fig. 8B). As shown in Fig. 8A, only Szl repressed blood markers under these conditions. Embryos

![Fig. 6. Rescue of the Szl knockdown phenotype by ventral injection of szl RNA. The experimental procedure is schematically illustrated. (A) Uninjected embryos. (B) Embryos injected with 10 ng of MOSZL. (C) Embryos injected ventrally with 40 pg of wSzl RNA. (D) Embryos injected with 10 ng of MOSZL and subsequently injected ventrally with 40 pg of wSzl RNA. Each panel also shows a representative embryo stained with benzidine to reveal hemoglobin-containing cells.](image)

![Fig. 7. Effects of Wnt8 and Wnt11 on development of the VBI. Embryos were injected in the marginal zone at the four-cell stage with the indicated amounts of expression vectors encoding xWnt8 or xWnt11 (pCS2-xWnt8 and pCS2-xWnt11), or the indicated amounts of RNA encoding dominant-negative xWnt8 or xWnt11. Embryonic aT3 globin was detected by in situ hybridization at stage 30/35.](image)
not used for RNA extraction were fixed and stained for αT3 globin by in situ hybridization to evaluate morphological phenotypes; again, only Szl-injected embryos showed efficient loss of the VBI (Fig. 8C). Interestingly, embryos dorsalized by Frzb appeared morphologically similar to embryos dorsalized by Szl; however, analysis of erythroid markers uncovered a critical difference in their ability to affect development of the VBI.

To verify that Myc-tagged Wnt inhibitors were biologically active, we used a Luciferase reporter controlled by a TCF/β-catenin-responsive promoter (Korinek et al., 1997). We co-injected the reporter construct with xWnt8 and different amounts of wSzI-MT, Frzb-MT, Crescent-MT or Dkk1-MT into the animal pole of two-cell stage embryos, and assayed luciferase activity at early gastrula (stage 10+ to 10.5). RNA dilutions were calibrated to express comparable levels of proteins. Under these conditions, Szl had no detectable effect on the canonical Wnt pathway and did not inhibit luciferase activation even when injected at an RNA concentration 100-fold greater than xWnt8 (400 pg, Fig. 8D). By contrast, xDkk1

Table 3. Rescue of the morphant phenotype by injection of different secreted Wnt inhibitors

<table>
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<tr>
<th>Blood staining (o-dianisidine)</th>
<th>% healthy embryos with</th>
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<tr>
<td></td>
<td>Enlarged VBI</td>
</tr>
<tr>
<td>Uninjected</td>
<td>1.8</td>
</tr>
<tr>
<td>MOSZL (10 ng)</td>
<td>95.7</td>
</tr>
<tr>
<td>MOSZL+wSz (40 pg)</td>
<td>28.0</td>
</tr>
<tr>
<td>MOSZL+Cres-MT (40 pg)</td>
<td>95.0</td>
</tr>
<tr>
<td>MOSZL+Frzb-MT (80 pg)</td>
<td>90.0</td>
</tr>
<tr>
<td>MOSZL+Dkk1-MT (40 pg)</td>
<td>94.1</td>
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Embryos were injected at the two-cell stage with 10 ng of MOSZL and subsequently injected with the indicated RNAs encoding for secreted Wnt inhibitors in the ventral marginal zone at the four-cell stage. At stage 30/35, embryos were treated with o-dianisidine to stain the ventral blood islands (VBI).

Fig. 8. Comparative analysis of szl and other secreted Wnt inhibitors. (A-C) Increasing amounts of RNA encoding Myc-tagged versions of Xenopus Szl, Frzb-1 (Frzb), Crescent (Cres) and Dikkopf1 (Dkk1) were injected in the marginal zone of four-cell stage embryos. (A) Expression of the erythroid markers xGata1 and αT3 globin was analyzed by RT-PCR at stage 33/34. EF1α was used as an internal control. (B) Expression of the injected Myc-tagged proteins was analyzed by immunoblotting at stage 11 on embryos from the same round of injections. The same blot was probed for actin as a loading control. (C) At the same time, morphology of the embryos and shape of the VBI were visualized by whole-mount in situ hybridization for embryonic αT3 globin. (D) Activity of the injected proteins as inhibitors of the canonical Wnt pathway was assessed in a Luciferase reporter assay. Embryos were injected in the animal pole with 200 pg of pTOP-FLASH, 4 pg of xWnt8 RNA, and increasing amounts of RNA encoding Myc-tagged Wnt inhibitors. Relative LUC activity is normalized to activation of the reporter by xWnt8 alone, and amounts of injected sFRPs are plotted on a logarithmic scale. For each RNA dilution, injected proteins were expressed at comparable levels; i.e. Frzb-MT RNA was injected at a higher concentration to compensate for poor translation.
and the other sFRPs efficiently suppressed luciferase activation when injected at much lower doses (4 pg and 40 pg respectively). The minimal effective concentration of Szl RNA that completely inhibited formation of the VBI in whole embryos was 80 pg. Therefore, levels of Szl that were much higher than needed to completely suppress the blood islands had no effect on the canonical Wnt pathway.

**DISCUSSION**

**A knockdown approach to understand the function of a putative secreted Wnt inhibitor**

We have used an antisense morpholino oligonucleotide to investigate the function of Szl, a secreted Frizzled related protein (sFRP) expressed in the ventralmost mesoderm of gastrula and post-gastrula *Xenopus* embryos. Translational knockdown offered a unique opportunity to study this putative Wnt antagonist, for which it is not obvious how to construct a dominant-negative mutant. We designed an antisense morpholino oligo (MOSZL) that would target both szl paralogs, and used two different morpholino oligos (one of which complementary to sequences in the coding region of szl) as negative controls. We verified specificity of the knockdown by demonstrating that all aspects of the phenotype are fully rescued by ventral injection of small amounts of exogenous Szl [for considerations on specificity of morpholino knock-down see Heasman (Heasman, 2002)].

We found that Szl plays a crucial and surprisingly specific role in coordinating development of specific ventral posterior structures, in particular of the ventral blood islands (VBI). Given its similarity to secreted Wnt inhibitors expressed in the Spemann organizer, we had previously proposed that Szl protects the extreme ventral marginal zone from Wnt signals inducing lateral mesoderm, so that this region could assume a ventral fate. Antagonism toward xWnt8 satisfactorily explained the paradoxical evidence that Szl overexpression actually dorsalizes the embryo (Salic et al., 1997). Based on this model, we had predicted that inhibiting Szl expression would reduce ventral-most derivatives. Instead, we observed that Szl knockdown expanded ventral-posterior tissue and the VBI. Such a complete disparity between prediction and experiment clearly demonstrates the crucial importance of combining loss-of-function with overexpression approaches to understand gene function.

**The morphological effects of Szl**

Time lapse recordings and dissection of ventral explants indicate that Szl levels affect lengthening of the ventral side of the embryo between neurula and tailbud stage. It is possible that Szl directly or indirectly regulates cell behavior, but it is also possible that these defects arise from altered competence of the ventral cells to undergo extension in response to other signals. In the intact embryo, lengthening reflects both the capacity of the tissue to elongate and the number of cells in the elongating population. There was ample evidence that Szl affects the recruitment of cells into a specific population, as we observed a larger region of ventral mesoderm expressing Szl RNA and early blood markers when Szl translation was inhibited. However, it is possible that cell behavior and cell recruitment reinforce each other, with cell movements required to place cells in a spatial context where they can be properly exposed to inductive signals. Such a linkage between morphogenesis and cell differentiation is observed in the dorsal mesoderm (Sive et al., 1989; Winklbauer and Keller, 1996). It is not easy to distinguish regulation of cell behavior from changes in cell identity, as the two are tightly correlated; but if we accept Szl itself as a marker of ventralmost mesoderm, the fact that its expression is affected long before the ventral cell movements during the neurula to tailbud transition would support a patterning phenomenon.

**Ventral mesoderm patterning or differentiation of the VBI?**

Careful lineage tracing studies have shown that the marginal zone at gastrula stage is divided in two domains along the animal-vegetal axis: the animal marginal zone gives rise mostly to muscle, while the vegetal marginal zone (leading edge mesoderm, or LEM) gives rise to blood (Keller, 1991; Kumano et al., 1999; Lane and Sheets, 2002). The two domains are distinguished by differential expression of Xbra, which is excluded from the LEM. Patterning of the marginal zone along the animal-vegetal axis derives from interaction of nodal and FGF signals; inhibition of FGF signaling eliminates Xbra expression and expands hematopoietic mesoderm (Kumano and Smith, 2000; Kumano and Smith, 2002). Using in situ hybridization, we observed normal Xbra expression at gastrulation in embryos injected with the Szl morpholino, suggesting that early FGF signaling and the primary animal-vegetal regionalization of the marginal zone are unaffected. Yet, at later stages we observed that the domains of ventral markers such as szl and sCFL are expanded, while lateral markers such as xMyoD and Xlim-1 are shifted dorsally in the posterior region of the embryo. These data are consistent with Szl playing a role in patterning the LEM during and after gastrulation.

The VBI are affected by variations in Szl protein levels that induce only mild morphological defects. Although this might suggest that the two phenotypes (formation of the VBI and allocation of ventral-posterior tissue) are not correlated, it seems unlikely that Szl plays a direct role in differentiation of embryonic blood. In fact, the earliest hematopoietic markers are expanded in embryos treated with the Szl morpholino and, most importantly, Szl knockdown or overexpression had no effect on BMP4-induced erythropoiesis in animal caps (Fig. 4). Although animal cap experiments would not detect activity upstream of BMP4, the current understanding of embryonic hematopoiesis defines BMP4 as the earliest signal in the blood differentiation pathway (Davidson and Zon, 2000). Therefore, we think that the VBI respond to small variations in Szl levels because they are the most ventral structure of the post-gastrula embryo.

**What is the biochemical activity of Szl?**

In an attempt to identify the molecular basis of Szl activity, we started with the reasonable speculation that it affects some extracellular component of the Wnt signaling pathway. Two Wnts are detected in ventral mesoderm at gastrulation: xWnt8, which is expressed in a broad ventral sector overlapping the domain of Szl expression but more extended in the dorsolateral direction; and xWnt11, which is expressed in a ring around the blastopore, overlapping with Szl only in the ventral sector. There are contradictory data on the activity of Szl as an inhibitor of Wnt8: Salic et al. showed that Szl could inhibit induction of Siamois by...
Szl as a negative feedback regulator of ventral mesoderm; unexpected complexity of ventral signaling

The findings described in this paper suggest that szl acts in a negative feedback circuit that limits recruitment of cells to extreme ventral fate. Mechanistically, it is possible that Szl inhibits a graded ventralizing signal, setting a threshold so that ventralmost mesoderm forms only in a restricted region where the signal is strong enough to overcome such inhibition. Alternatively, Szl may act at a longer range than the signal that induces it, setting up a ‘remote inhibition’ system similar to the Spitz-Argos feedback loop in Drosophila eye development (Freeman, 2000). Szl could diffuse from its source, preventing cells at a distance from the ventralizing signal from differentiating into ventralmost mesoderm, while cells expressing Szl would be unaffected because they are already committed. Another possibility is that Szl provides a completely independent specification signal that, by interacting at different levels with ventralizing pathways, eventually results in inhibition of extreme ventral fate. In any case, the molecular nature of the ventralizing signal(s) antagonized by Szl remains to be established.

In conclusion, knockdown experiments have uncovered an unexpected and complex function of Szl, raising important biochemical questions of specificity and action of this and other secreted Frizzled-related proteins. Our data suggest the existence of a self-regulatory circuit controlling the allocation of tissue in the ventral mesoderm, with direct consequences for both blood formation and morphogenesis. The ventral organizing activity involving Szl clearly differs from the Spemann organizer in the locality of its effects. However, it is likely that local organization with feedback control will be manifest in many regions of the embryo, raising interesting questions about how specificity is achieved with a limited number of signaling components.

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