Regulation of midline development by antagonism of *lefty* and *nodal* signaling

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Accepted 7 May; published on WWW 21 June 1999

SUMMARY

The embryonic midline is crucial for the development of embryonic pattern including bilateral symmetry and left-right asymmetry. In zebrafish, *lefty1* (*lft1*) and *lefty2* (*lft2*) have distinct midline expression domains along the anteroposterior axis that overlap with the expression patterns of the *nodal*-related genes *cyclops* and *squint*. Altered expression patterns of *lft1* and *lft2* in zebrafish mutants that affect midline development suggests different upstream pathways regulate each expression domain. Ectopic expression analysis demonstrates that a balance of *lefty* and *cyclops* signaling is required for normal mesendoderm patterning and *goosecoid*, *no tail* and *pitx2* expression. In late somite-stage embryos, *lft1* and *lft2* are expressed asymmetrically in the left diencephalon and left lateral plate respectively, suggesting an additional role in laterality development. A model is proposed by which the vertebrate midline, and thus bilateral symmetry, is established and maintained by antagonistic interactions among co-expressed members of the *lefty* and *nodal* subfamilies of TGF-β signaling molecules.

Key words: *lefty*, *nodal*, *cyclops*, Embryonic midline, TGF-β, Axis formation, Zebrasish

INTRODUCTION

The vertebrate midline is essential for generating dorsoventral and anteroposterior pattern, and regulating left-right asymmetry. Initial embryonic patterning is established by localized maternal signals and inductive cell interactions. These signals establish organizing centers that coordinate the body plan. Spemann and Mangold (1924) identified a population of cells from the dorsal margin of amphibian gastrulae which can organize a second embryonic axis when transplanted into the ventral side of a host embryo. The node in birds (Waddington, 1932) and mice (Beddington, 1994) and the embryonic shield in fish (Oppenheimer, 1936) provide analogous organizing functions. In amphibians and zebrafish, TGF-β signals from the endoderm induce overlying marginal cells to adopt mesodermal fates while *wnt* pathway signaling promotes organizer formation. Mesoderm is then patterned along the dorsal-ventral axis in response to a gradient of BMP signaling (reviewed by Harland and Gerhart, 1997; Thomsen, 1997; Schier and Talbot, 1998). At the onset of gastrulation, zebrafish embryonic shield cells are already specified to develop into specific tissue types including the notochord (Shih and Fraser, 1995; Melby et al., 1996). The notochord influences development of overlying neural floorplate. Together these midline tissues serve as a polarizing center, patterning the anteroposterior axis and establishing dorsoventral polarity in adjacent tissues (reviewed by Placzek, 1995).

Secretn signaling molecules of the *nodal* subfamily of TGF-βs are important in gastrulation, axial development and establishment of laterality in vertebrates (reviewed by Varlet et al., 1997; Ramsdell and Yost, 1998). In zebrafish, *nodal*-related proteins (Erter et al., 1998; Rebagliati et al., 1998a) are encoded by *squint* (Feldman et al., 1998) and *cyclops* (Rebagliati et al., 1998b; Sampath et al., 1998). Loss of gene function results in embryos with deficiencies in midline mesendoderm, ventral neuraxis (Hatta et al., 1991; Yan et al., 1995, Feldman et al., 1998) and laterality defects (Chen et al., 1997).

Recently, a novel subfamily of TGF-β proteins encoded by *lefty-1* and *lefty-2* was identified in mice (Meno et al., 1996, 1997). *lefty* RNA is expressed in the primitive streak during gastrulation and in the midline and left lateral plate mesoderm during somitogenesis, *lefty* and *nodal* expression patterns are strikingly similar. Asymmetric expression of *lefty* and *nodal* are similarly perturbed in mouse mutants with laterality defects (reviewed by Varlet and Robertson, 1997), and a knockout mutation of *lefty-1* causes misexpression of *lefty-2* and *nodal* during somitogenesis (Meno et al., 1998). These observations suggest the regulation and functions of *lefty-1*, *lefty-2* and *nodal* are related.

To better understand the roles of *lefty*-related genes in vertebrate development, we identified two zebrafish *lefty* homologs, *lefty1* and *lefty2* are expressed in distinct domains along the anteroposterior axis and the margin. Together, the spatial and temporal expression patterns of these genes coincide with those of *cyclops* and *squint*. Analysis of *lefty*
expression in mutants for defective midline development suggests individual domains of lefty expression are differentially regulated. Ectopic expression of lefty and cyclops have opposing effects on mesendoderm development and suggests that Lefty suppresses cyclops expression while Cyclops induces lefty expression. Co-injection of lefty and cyclops results in normal mesendoderm formation, suggesting that Lefty and Cyclops are antagonistic. We propose a model in which antagonism between co-expressed members of the TGF-β family of signaling molecules regulates midline mesendoderm formation and establishes bilateral symmetry.

MATERIALS AND METHODS

Cloning zebrafish lefty1 and lefty2 cDNAs

RT-PCR was used to identify zebrafish lefty genes using degenerate oligonucleotides designed from conserved regions of mouse lefty and human endomtrial bleeding associated factor. PCR templates were produced by reverse transcription of zebrafish gastrula (80-95% epiboly) mRNA. RNA isolated by Trizol (BRL) extraction and poly(A) selected with Oligotex spin columns (Qiagen) was reverse transcribed with Superscript (BRL) and amplified using Pfu (Stratagene) in the presence of 2 µM degenerate oligonucleotides. A 270 bp PCR product was cloned into pBlueScript KS- (Stratagene) and sequenced. This PCR fragment was labeled with 32P-dCTP (Amersham) using the Decaprime kit (Ambion) and used to screen a zebrafish gastrula-stage cDNA library cloned in λ phHybrizap (Stratagene). Plaques were lifted to Magnalift membranes (MSI) and hybridized at 42°C overnight in 40% formamide, 50% SSPE, 5x Denhardt's solution, 0.5% SDS, 100 µg/ml yeast RNA. Filters were washed at a final stringency of 0.5x SSPE/0.1% SDS at 50°C. 20 positive clones were subjected to three rounds of purification and excised from λ phage into plasmids using Excist helper phage (Stratagene).

Embryo culture and zebrafish stocks

Zebrafish, Danio rerio, were maintained at 28.5°C on a 14 hour/10 hour light/dark cycle. Embryos were collected from natural spawnsings, cultured and staged by developmental time and morphological criteria (Westerfield, 1995). Wild-type embryos were descendants of outbred stocks obtained from Ekkwell Breeders (Gibsonton, Fl). Zebrafish carrying mutations were obtained from stocks originally produced at the University of Oregon (cyc b229, mt160), MGH/Harvard (oeppn134) or Newcastle (lft12).

Ectopic expression assays

Synthetic RNAs for injection were generated from lft1, lft2 and myc epitope-tagged versions of both genes. Coding sequences from lft1 and lft2 were amplified using PCR with Pfu polymerase (Stratagene) and cloned into pCS2+ and pCS2+MT expression plasmids (Rupp et al., 1994; Turner and Weintraub, 1994).

Capped RNA was synthesized from Lefty expression constructs and from the Cyclops expression construct pCS2+Cyclops (Rebagliati et al., 1998b) using the mMessage mMachine SP6 transcription kit (Ambion). Zebrafish embryos at 1- to 8-cell stages were pressure injected with RNA in 0.1 M KCl containing 24 mg/ml FITC-dextran. Following injection embryos with widespread lineage tracer dye were selected, allowed to develop to desired stages, then photographed or fixed for in situ hybridization and immunohistochemistry.

RNA in situ localization and immunolocalization

For in situ hybridizations, embryos were fixed in 4% paraformaldehyde in sucrose buffer (Westerfield, 1995), rinsed in PBS, dehydrated into absolute methanol and stored at -20°C. Riboprobes were synthesized from linearized DNA templates using T3 or T7 polymerases and digoxigenin labeling mixes (BMB). In situ hybridizations were carried out as described by Stachel et al. (1993).

To identify clones of cells expressing myc epitope-tagged RNAs, embryos were refixed following in situ hybridization and were processed for immunohistochemistry. The myc epitope was detected with 9E10 monoclonal antibody diluted 1/500 (Santa Cruz Biotechnology), HRP-conjugated goat anti-mouse diluted 1/200 (Jackson ImmunoResearch) and 3,3'-diaminobenzidine as development substrate.

Embryos were cleared in 70% glycerol/PBS or benzyl benzoate/benzyl alcohol (2:1), and photographed with a Leica MZ12 microscope. Images were collected on Kodak T160 film, scanned and processed using Adobe Photoshop.

RESULTS

Identification of lefty homologs in zebrafish

To understand the roles of lefty-related genes in development, lefty homologs expressed during zebrafish gastrulation were identified and characterized. Two genes, lefty1 (lft1) and lefty2 (lft2), were identified as members of the TGF-β superfamily of signaling polypeptides (GenBank AF132444, AF132445). Amino acid sequence alignments (Fig. 1) place lft1 and lft2 within the TGF-β subfamily that includes mouse and human lefty. One hallmark of the lefty subfamily is the absence of one of seven cysteines conserved in other TGF-βs. Both Lft1 and Lft2 lack this cysteine (Fig. 1). While Lft1 and Lft2 are 70% identical, there is only 32-35% identity between Lft1 or Lft2 and mouse or human Lefties. lefty genes in each organism are more similar to one another than to their homologs in other species; thus orthology of lefty homologs cannot be established between species by sequence comparison. Nomenclature for the zebrafish genes has been assigned based on conservation of late somite-stage expression patterns when compared to mouse lefty-1 and lefty-2 (Men et al., 1997).

Lft1 and Lft2 are expressed in distinct domains in mesendodermal precursors during gastrulation

Early expression patterns of zebrafish lft1 and lft2 are dynamic, with co-localized expression at some stages and distinct expression domains at others. Initiation of expression at stage 4 (Fig. 2A,B). lft1 is localized to the dorsal blastoderm margin (Fig. 2A), as confirmed by double-labeling for goosecoid (gsc) RNA (Stachel et al., 1993) (not shown). In contrast, lft2 is expressed in patches along the blastoderm margin without bias to the dorsal side (Fig. 2B). By dome stage both genes are expressed around the margin (Fig. 2C,D) in cells that will contribute to mesendodermal tissues of the embryo (Kimmel et al., 1990).

Expression of lft1 in the margin is maintained late in gastrulation, while expression of lft2 is downregulated after 50% epiboly. At shield stage, both genes are expressed in inviolated cells of the dorsal hypoblast (Fig. 2F). At 85-95% epiboly, lft1 and lft2 are expressed in the anterior midline in the polster and prechordal plate (Fig. 2G,H). Posteriorly, expression patterns of lft1 and lft2 diverge. lft1 is absent from the posterior midline, which gives rise to notochord and floorplate. Dorsal forerunner cells (Coope and D’Amico, 1996; Melby et al., 1996), which express high levels of lft1 mRNA, are readily seen as marginal lft1 expression begins to
be downregulated at 90% epiboly (Fig. 2G). In contrast, lft2 is expressed along the axis from polster to margin, but is absent from dorsal forerunner cells (Fig. 2H).

During early somitogenesis, both genes are expressed in the polster and prechordal plate. lft1 is also expressed in Kupffer’s vesicle, a dorsal forerunner cell derivative, while lft2 is localized to presumptive neural floorplate cells (Fig. 2LJ). Expression of lft2 is downregulated after the 3-somite stage (Fig. 2LN), while lft1 expression is maintained in the prechordal plate through 6-8 somites (Fig. 2K). During midsomitogenesis lft1 is expressed in the left habenula of the diencephalon and in the posterior notochord (Fig. 2M). Expression domains of lft1 and lft2 extensively overlap those of the nodal-related genes squint (sqt) and cyclops (cyc) (aka ndr1, ndr2, Rebagliati et al., 1998a).

**lft1 and lft2 are asymmetrically expressed during late somitogenesis**

During late somitogenesis, domains of lefty expression exhibit left-right asymmetry (Fig. 20P). lft1 is expressed strongly in the left habenula and weakly in left lateral plate mesoderm. lft1 is also expressed in anterior and posterior notochord. lft1 expression is extinguished by 24 hours postfertilization (hpf). lft2 is strongly expressed in left lateral plate mesoderm from the 19-somite stage through 30 hpf. Low levels of lft2 RNA are present in the left habenula. The asymmetric lefty expression domains are similar to those of cyc (Rebagliati et al., 1998a; Sampath et al., 1998) and nodal-related genes in other vertebrates (Levin et al., 1995; Lowe et al., 1996; Lustig et al., 1996). Analysis of the roles of lft1 and lft2 in left-right development will be discussed elsewhere (unpublished data).

**lft expression domains are differentially affected in midline mutants**

Domains of lft1 and lft2 expression are divergent, suggesting that distinct regulatory pathways direct expression of each gene. To assess upstream regulatory pathways, lefty expression patterns were assessed in four recessive lethal mutants that affect midline tissue differentiation. Mutations in cyclops, a nodal homolog (Hatta et al., 1991), and one-eyed pinhead (oep) (Schier et al., 1996), a member of the EGF-CFC gene family (reviewed in Salomon et al., 1999), cause defects in prechordal plate mesendoderm and floorplate neurectoderm. These genes encode secreted and membrane-bound signaling molecules, respectively (Rebagliati et al., 1998b; Sampath et al., 1998; Zhang et al., 1998). Mutations in no tail (ntl) (Schulte-Merker et al., 1994) and floating head (flh) (Talbot et al., 1995), which are homologs of the transcription factors Brachyury and Nkx respectively, result in loss of notochord and alterations in floorplate (Halpern et al., 1993, 1995).

flh− embryos show no alteration in lft1 or lft2 expression through early somitogenesis (Fig. 3A,B), indicating that genetic changes in midline cells do not necessarily lead to altered lefty expression. In contrast, domain-specific lefty expression was altered in cyc, oep and ntl mutants. In cyc− embryos, lft1 expression was absent from the polster and prechordal plate but wild-type in dorsal forerunner cells and the margin (Fig. 3C). Prior to shield stage, lft2 expression in the blastoderm margin was unaffected. In late gastrulae, lft2 was expressed only in a few involuting cells at the dorsal margin (Fig. 3D). This suggests that Cyclops is essential for maintenance of lft1 and lft2 expression in the anterior midline and floorplate but not the margin. In contrast, expression of lft1
Fig. 2. lefty expression during early zebrafish development. (A-P) In situ localization of \( lft1 \) and \( lft2 \) transcripts at blastula through late somite stages (A,B, animal pole views; C-N, lateral views, dorsal at right; O,P, dorsoanterior views, anterior at left). \( lft1 \) and \( lft2 \) localize to the blastoderm margin at sphere (A,B) and dome stages (C,D). At shield stage (E,F), and at 90% epiboly (G,H) both genes localize to the dorsal hypoblast; \( lft1 \) is also expressed in dorsal forerunner cells (dfc). 1- to 3-somite embryos express both genes in the polster (po) and prechordal plate (pp, I,J); \( lft1 \) is uniquely expressed in Kupffer’s vesicle (Kv) (I), \( lft2 \) is uniquely expressed in floorplate precursors (fp) (J). At 6-8 somites \( lft1 \) expression is maintained (K) while \( lft2 \) is downregulated (L). 13-15 somite embryos express \( lft1 \) in the anterior notochord and left habenula (arrowhead) (M); \( lft2 \) is not expressed (N). 22-24 somite embryos showing \( lft1 \) in the anterior notochord (an), left habenula (arrowhead) and left lateral plate mesoderm (lpm) (O); \( lft2 \) is expressed in left lateral plate mesoderm (P).

Fig. 3. lefty1 and lefty2 expression domains are disrupted in zebrafish midline mutants. (A-H) In situ localization of \( lft1 \) and \( lft2 \) in mutant embryos at 85-90% epiboly (dorsal views). Embryos from heterozygous \( flh \) parents show wild-type expression of \( lft1 \) (A) and \( lft2 \) (B). cyc mutant embryos do not express \( lft1 \) or \( lft2 \) in the anterior midline (C,D) and \( lft2 \) is only expressed in a few cells near the dorsal margin. In oep mutants \( lft1 \) and \( lft2 \) expression in the prechordal plate is limited to a few cells (E,F); \( lft2 \) expression is also reduced in floorplate precursor cells (F). In ntl mutants \( lft1 \) expression is lost in dorsal forerunner and marginal cells (G), and \( lft2 \) expression in floorplate precursors is expanded laterally (H). (I) lefty expression domains at 85% epiboly. \( lft1 \) is expressed at the margin, and in dorsal forerunner cells. \( lft2 \) is expressed in the posterior midline in floorplate precursors. Both genes are expressed in the anterior midline in the polster and prechordal plate. Expression domains affected in different midline mutants are summarized at right.
Ectopic Lefty expression inhibits mesendoderm development

To investigate downstream responses to Lefty protein, synthetic RNAs were injected into 2- to 8-cell embryos. Injection of 25 pg of RNA encoding Lft1, Lft2 or myc epitope-tagged variants (Lft1-MT or Lft2-MT) produced embryos with identical phenotypes. Injected embryos were indistinguishable from controls until shield stage. In injected embryos, epiboly and dorsal convergence occurred normally during gastrulation but cells failed to involute, resulting in embryos with a dorsal accumulation of cells and deficient axial development (Fig. 4A,B). At 24 hpf, injected embryos lacked anterior mesendodermal derivatives including cephalic mesoderm, anterior somites and notochord, but had some mesoderm in the tail (Fig. 4C,D). Given the apparent absence of axial mesoderm, lefty-injected embryos had surprisingly normal anteroposterior patterning and neural axis development including the presence of developing eyes and otic vesicles.

Fig. 4. Ectopic expression of Lefty1 or Lefty2 results in loss of anterior mesendoderm and ventral neurectoderm. (A-D) Morphological phenotypes of embryos injected with 25 pg Lft2-MT RNA (lateral views). At 95% epiboly, injected embryos accumulate cells on the dorsal side (B, arrowhead) compared to uninjected controls (A). At 24 hpf, injected embryos (D) lack anterior mesendoderm, notochord, and trunk somites and the anteroposterior axis is shortened relative to uninjected controls (C). The neural tube is present and retains some anteroposterior pattern. (E-T) Effect of ectopic Lefty expression on gene expression in 24 hpf (E-J, lateral views) and gastrula-stage embryos (K-P, S,T, 90% epiboly, dorsal view; Q,R, 50% epiboly, animal pole views). Upper panels in each series are uninjected controls, lower panels are embryos injected with 25 pg RNA encoding Lft1-MT (J,L,P,R) or Lft2-MT (F,H,N,T). Embryos were processed by in situ hybridization (E-T), followed by anti-myc immunohistochemistry to identify clones of cells expressing Lft1-MT or Lft2-MT (K-T). At 24 hpf, krx-20 (E,F) is expressed at the normal anteroposterior level in the hindbrain of injected embryos although the bands of expression within rhombomeres 3 and 5 are compressed. shh expression (G,H) is abolished from the ventral brain and floorplate of injected embryos. col2a, expression in the floorplate, notochord and hypochord (I) is reduced to a few cells in injected embryos (J). In gastrulae, ntl expression is reduced in the midline and dorsal margin (K,L) and axial expression is reduced in the midline and abolished in endodermal precursors (arrows, M,N) of injected embryos. tbx6 expression is suppressed in much of the ventrolateral margin (O,P), and pitx2 expression in the margin is abolished (Q,R). The otx2 expression domain is shifted toward the margin (S,T) in injected embryos.
confirming the absence of ventral neural tissue. col2a1 (Yan et al. 1995) is expressed in head mesenchyme, notochord, hypochord and floorplate (Fig. 4I). In lefty-injected embryos, col2a1 is abolished anteriorly and is expressed in only a few cells of unknown fate in the trunk (Fig. 4J). Vascular endoderm that expresses fkh-1 (Liao et al., 1997) is absent in lefty-injected embryos (not shown). These results suggest that, with the exception of neural floorplate, neural ectoderm can develop fairly normal anteroposterior pattern in the absence of underlying mesendoderm.

To address whether neural development observed in 24 hpf embryos might be due to earlier mesodermal induction, expression of mesendodermal and neur ectodermal markers was assessed in late gastrulae. All mesendodermal markers tested including cyc (Rebagliati et al., 1998a,b; Sampath et al., 1998), ntl (Schulte-Merker et al., 1994), gsc (Stachel et al., 1993), axial (Strahle et al., 1993) and tbx6 (Hug et al., 1997) were downregulated in response to ectopic Lefty.

Genes expressed in the anterior midline were more susceptible to downregulation by lefty-injection than those with posterior expression domains. For example, gsc expression in the anterior midline at 80-90% epiboly was abolished in 90% (n=61) of injected embryos (not shown, see Fig. 6B). Genes expressed more posteriorly were less frequently abolished by Lefty (ntl: 42%, n=64; axial: 38%, n=58), but were reduced when clones of Lefty-expressing cells were nearby (Fig. 4K-N). axial expression in endodermal precursors was abolished in all lefty-injected embryos (Fig. 4M,N, arrows). The absence of mesendodermal gene expression from the anterior midline in late gastrulae suggests that neural tube development observed at 24 hpf did not require mesodermal induction earlier in development.

Mesendodermal genes expressed in the margin vary in their sensitivity to ectopic Lefty. ntl was downregulated only when clones of Lefty expression contacted the dorsal margin (Fig. 4K,L). Expression of tbx6 in the ventrolateral margin was inhibited considerable distances from clones expressing Lefty (Fig. 4O,P). In the most extreme case, expression of the paired homeobox gene pitx2 (GenBank AF132446) was abolished from the blastoderm margin regardless of the site of ectopic Lefty expression (Fig. 4Q,R). The distinct responses of ntl, tbx6 and pitx2 to ectopic Lefty suggests these genes are differentially regulated and that misexpression of Lefty does not result simply in loss of all marginal mesendoderm.

Presumptive anterior notor ectoderm marked by otx2 expression (Li et al., 1995) extends across the animal pole to the equator at 80-90% epiboly (Fig. 4S). In lefty-injected embryos, the otx2 expression domain appeared unchanged in dimension but was shifted dorsoventrally to extend almost to the margin (Fig. 4T), suggesting that early mesoderm formation is required for complete axis elongation. That otx2 expression is not expanded suggests ectopic Lefty does not cause cells that lose expression of mesendodermal markers to trans fate to neur ectoderm.

lefty and cyclops affect expression of each other

Two observations suggest lefty and nodal subfamilies of TGF-βs act in a common signaling pathway. First, the combined temporal and spatial expression patterns of lft1 and lft2 are coincident with those of cyc and sqt. Second, morphological phenotypes and alterations of gene expression resulting from lefty RNA injection are very similar to those observed in sqtcyc double-mutant embryos (Feldman et al., 1998).

To test whether these TGF-βs affect expression of one another, we injected RNAs encoding Lft1, Lft2 or Cyc and examined expression of the others. cyc expression was abolished by ectopic Lft1-MT or Lft2-MT in 76% (n=72) of embryos (Fig. 5B,C). Injection of RNAs encoding Lft1-MT or Lft2-MT abolished expression of its paralog in 76% and 71% of embryos, respectively (Fig. 5E, and not shown). Partial downregulation of midline cyc, lft1 and lft2 expression occurred when Lefty-expressing clones did not encompass the midline, as observed for ntl and axial (Fig. 4L,N). In contrast, injection of cyc RNA induced ectopic expression of lft1 and lft2 in all embryos (Fig. 5F, and not shown). At 50% epiboly, sqt expression was induced by ectopic Cyc and suppressed by Lft2-MT (not shown). These results indicate that nodal subfamily members induce expression of themselves and lefty subfamily members. In contrast, lefty subfamily members suppress expression of themselves and nodal subfamily members.

Competitive interaction of Lefty and Cyclops control mesendoderm induction

As shown above, ectopic expression of Lft1 or Lft2 causes downregulation of cyc and other mesendodermal genes. Conversely, ectopic expression of Cyc expands the expression domains of lft1, lft2 and other mesendodermal genes including gsc and lim1 (this study, Rebagliati et al., 1998a; Sampath et al., 1998). These results suggest that lefty and cyclops have opposite effects, with lefty driving differentiation pathways away from mesendoderm and cyclops promoting differentiation of mesendoderm.

To assess whether lefty and cyclops could counteract each other, we injected RNAs encoding Lft2-MT and Cyc singly or in combination into 1- to 4-cell embryos and assessed mesendodermal gene expression by in situ hybridization. Injection of 25 pg lft2-MT RNA abolished gsc expression in 40-50% epiboly embryos (Fig. 6A,B) and caused slight downregulation of ntl expression in the blastoderm margin relative to uninjected embryos (Fig. 6E,F). Injection of cyc RNA had the opposite effect. Injection of 5 pg or 10 pg cyc RNA expanded gsc expression from a 90 degree marginal arc to encompass 25-60% or 50-100% of the blastoderm, respectively (Fig. 6C), and resulted in ectopic ntl expression in 25-60% to 50-100% of the blastoderm, respectively (Fig. 6G).

Co-injection of 25 pg lft2-MT RNA with 5 pg or 10 pg cyc RNA suppressed the phenotypes that result from overexpression of either gene, yielding embryos with wild-type gsc expression (Fig. 6D). Similarly, ectopic ntl expression was reduced in embryos co-injected with 25 pg lft2-MT RNA and 10 pg cyc RNA, and was nearly extinguished in embryos co-injected with 25 pg lft2-MT RNA and 5 pg cyc RNA (Fig. 6H). Wild-type expression patterns of lft1 and sqt were also restored by co-expression of Lft2-MT and Cyc (not shown). Thus, the regulation of several mesendodermal genes can be normalized by co-expression of Lft2 and Cyc. This suggests that lft2 and cyc, which are co-expressed in many tissues, function as mutual antagonists.

DISCUSSION

We have identified two highly related members of the lefty
subfamily of TGF-β signaling molecules that likely play important roles in the generation of embryonic pattern in zebrafish. The lefty genes are expressed early in development in several distinct domains, some of which subdivide the midline along the anteroposterior axis. As revealed by analysis of expression patterns in midline mutants lefty expression domains are regulated by distinct upstream pathways. Ectopic expression of Lft1 and Lft2 indicates both are equally capable of inhibiting mesendodermal gene expression. This inhibition appears to arise from antagonism of signals from the nodal subfamily of TGF-β. Thus a balance of mutual antagonists expressed in the midline likely regulates embryonic mesendoderm formation.

Inhibitory role of lefty in mesoderm induction

Similar functions of Lefty1 and Lefty2 proteins in ectopic expression assays suggests that both genes function in embryonic patterning, by antagonizing mesendoderm induction. Injection of lefty RNA in zebrafish embryos inhibits expression of early dorsal mesoderm markers such as goosecoid and suppresses formation of the embryonic shield, resulting in impaired cellular involution during gastrulation. These results and the localization of lft1 transcripts in the presumptive shield at sphere stage suggest an early zygotic function for Lefty in dorsal mesoderm induction and organizer development. Ectopic Lefty expression also perturbs genes expressed later in the posterior midline, including axial and no tail. These perturbations correlate with a later loss of mesendodermal derivatives including notochord, somites and vascular endoderm. These results, and the observation that the combined expression domains of lft1 and lft2 encompass the midline and margin during gastrulation, suggest an essential role for Lefty signaling in the regulation of mesendoderm formation during early development.

Early dorsoventral polarity is preserved in embryos overexpressing Lefty, indicating that other positional information signals act in parallel to Lefty. Dorsoventral polarity in zebrafish is likely established by the BMP subfamily of TGF-β signaling molecules (Schulte-Minker et al., 1997; Nguyen et al., 1998). Thus, although Lefty signaling may play a role in mesoderm induction, it is not responsible for establishing the dorsal organizer per se.

Although ectopic Lefty expression suppresses mesendodermal differentiation, embryos retain relatively normal anteroposterior patterning of the neuraxis, as indicated by correct positioning of eyes and otic vesicles and molecular markers such as krx-20. The extensive development of neural tissue in embryos lacking significant mesendoderm indicates vertical signals from dorsal mesoderm are not required for anteroposterior patterning of the neuraxis, concurring with the view that planar signals from other organizing centers induce anteroposterior patterning (reviewed by Schier and Talbot, 1998). Ectopic Lefty expression greatly diminishes shh expression and formation of neural floorplate, indicating that development of the dorsoventral axis of the neural tube is dependent on underlying mesoderm as has been reported (reviewed by Tanabe and Jessel, 1996).

Diverse functions of lefty family members may be regulated by distinct expression domains

Mature Lefty proteins are very similar in amino acid sequence, and ectopic expression of either protein has equivalent effects on embryogenesis. We therefore suggest that, if lft1 and lft2 have distinct functions during embryogenesis, this results from divergent expression patterns, not divergent protein functions. For example, lft2 expression in floorplate precursors of late gastrulae might make this tissue refractory to mesoderm-inducing signals, including Cyclops (Rebagliati et al., 1998a; Sampath et al., 1998), emanating from the underlying notochord and allow these cells to progress toward a neural fate. Tissue-specific regulation of lefty expression is also evident in the asymmetric expression of these genes during late somitogenesis. Comparable to lefty-1 and lefty-2 expression in mice (Meno et al., 1996, 1997), lft1 is expressed at high levels in the neural tube while lft2 is expressed at high levels in the lateral plate mesoderm, suggesting they may play different roles in neural or mesodermal patterning. Recent work has shown that distinct regulatory mechanisms also underlie the asymmetric expression of mouse lefty-1 and lefty-2 (Saijoh et al., 1999).

Expression domains of lft1 and lft2 are differentially affected in midline mutants, indicating that individual domains of lefty expression are under control of distinct, tissue-specific regulatory pathways (Fig. 3). Domains of lefty expression and those of cyc and sqt show considerable overlap. Interestingly, alterations in the patterns of cyc expression reported in midline mutants are similar to the alterations of lefty expression seen in this study. cyc expression is normal in flh- embryos (Sampath et al., 1998), fails to be maintained in cyc- (Sampath et al., 1998) and oep- (Schier et al., 1996) late gastrulae, and is widened in the posterior axis of nil- late gastrulae (Rebagliati et al., 1998a). Thus, both cyc and lefty genes are under similar regulatory controls, suggesting coordinated functions of these related signaling molecules.

Antagonism among co-expressed TGF-β family members

Strikingly, the expression domains of the lefty and nodal subfamilies in zebrafish are coincident, and embryos in which Lefty is overexpressed (Fig. 4) are remarkably similar to nodal-deficient cyc:sqt embryos (Feldman et al., 1998). lft1 and cyclops appear to regulate each other’s expression (Fig. 5). Ectopic expression of Lefty suppresses both lft1 and cyclops expression, whereas ectopic expression of Cyclops induces lefty expression. Furthermore, ectopic co-expression of Lefty and Cyclops drives the embryo back toward normal development (Fig. 6). From these results, we propose that Lefty functions during mesoderm induction and midline differentiation by antagonizing Cyclops signaling, and that a balance between these co-expressed mutually antagonistic signals results in a normally proportioned embryo (Fig. 7).

Recently, a zebrafish TGF-β family member, antivin, has been characterized (Thisse and Thisse, 1999). The protein sequences of Antivin and Lefty1 differ by only a single amino acid suggesting they are alleles of the same gene. The reported expression pattern of antivin is similar to the combined patterns of lft1 and lft2 suggesting that the antivin probe recognized both lefty transcripts. Ectopic expression of Antivin greatly reduced mesendoderm development, similar to our results, but did not alter its own expression, in contrast to our results where ectopic Lft1 and Lft2 suppressed one another. Co-injection experiments indicated that Activin can antagonize the effects
Uninjected embryos (E) express gsc expression (D). (E-H) RNA, lft2-MT response to injection of 25 pg RNA and 10 pg 25 pg lft2-MT expanded by injection of 10 pg abolished by injection of 25 pg expression RNA (H) express (G). Embryos co-injected with 25 pg expression is expanded in embryos injected with 5 pg cyc RNA and Rosa, 1994), and its expression does not overlap Lft1-MT or Lft2-MT. (B) W. Bisgrove, J. J. Essner and H. J. Yost

Fig. 5. Ectopic expression of Lefty1, Lefty2 or Cyclops affects the endogenous expression of each other. (A–C) cyc expression at 85% epiboly. In uninjected embryos cyc is expressed in the midline (A). Expression is abolished in embryos injected with 25 pg of RNA encoding either Lft1-MT (B) or Lft2-MT (C). (D–F) lft2 expression at 85% epiboly. In control embryos lft2 is expressed in the midline (D). Expression of lft2 is abolished in embryos injected with 25 pg of RNA encoding Lft1-MT (E) and is expanded in embryos injected with 10 pg of cyc RNA (F). Embryos (dorsal views) were processed by in situ hybridization followed by anti-myc immunohistochemistry to identify clones of cells expressing Lft1-MT or Lft2-MT.

of Antivin (Thisse and Thisse, 1999). However, activin appears to have a restricted maternal role in development (Withbrodt and Rosa, 1994), and its expression does not overlap extensively that of the lefty genes. In contrast, lefty expression domains described here co-localize with nodal (cyc and sqt) expression domains in the margin, embryonic midline, left habenula and the left lateral plate mesoderm. The multiple endogenous co-expression domains throughout embryogenesis suggest that lefty serves to antagonize nodals rather than activin.

Antagonistic interactions among members of the TGF-β family could arise by several mechanisms. lefty and cyclops could function in two separate pathways whose signaling readouts had opposing effects. Alternatively, interaction between lefty and cyclops could be more direct: First, Lefty and Cyclops might compete for a common receptor. A receptor that could function in Lefty/Cyclops signaling in the midline has been identified in zebrafish. The spatial and temporal expression patterns of TARAM-A, a serine/threonine kinase related to TGF-β type I receptor (Renucci et al., 1996), overlap extensively those of lft1, lft2, cyc and sqt. Additionally, ectopic expression of TARAM-A causes expansion only of dorsal mesoderm, suggesting it is activated only by dorsally localized TGF-β signaling molecules. Binding of Lefty might inhibit signal transduction while binding of Cyclops might initiate signal transduction, inducing mesodermal gene expression. Second, Lefty and Cyclops might form an inactive heterodimeric ligand. Dorsoventral axis specification in amphibians occurs via antagonism of TGF-β signaling, however, the antagonists are not TGF-β family members. During gastrulation, organizer cells secrete noggin, chordin and follistatin, which bind and inactivate BMP4 secreted from ventral mesoderm. These interactions establish fates in marginal mesoderm and pattern dorsal ectoderm (reviewed by Sasai and De Robertis, 1997; Thomsen 1997). Third, an equilibrium between Lefty and Cyclops homodimers and the heterodimer might provide a range of signaling functions arising from different activities of each homodimer and the heterodimer. Heterodimeric forms of BMPs 2 through 7 are more potent than the corresponding homodimers at inducing alkaline phosphatase in vitro and inducing ectopic bone formation in vivo (Isreal et al., 1996). Also, BMP4/7 heterodimers can convey signals for ventral mesoderm induction and patterning in Xenopus while the homodimers show no inductive activity (Nishimatsu and Thomsen, 1998).

A model for the establishment of bilaterality

Previous models of embryonic axis formation invoke antagonistic interactions between signaling components; signals and ‘sinks’ are usually proposed to exist on opposite sides of a cellular field or embryo (Lemaire and Yasuo, 1998). While antagonists positioned on opposite sides likely participate in dorsoventral specification (described above), once the midline is established, it is unlikely that signals and sinks on opposite sides could give refined and proportionate development across the midline. In the proposed model, positive and negative signals are emitted from the same source, the embryonic midline, so cells at fixed distances from the midline on the right or left side receive identical ratios of agonistic and antagonistic signals. The ratio between the signals would then define the proportions of the embryo so that bilateral symmetry is established and maintained.

Fig. 6. Cyclops and Lefty2 have opposite and mutually antagonistic effects on mesendodermal gene expression. (A–D) gsc expression at 50% epiboly. Control embryos (A) express gsc in a 90 degree arc at the margin. gsc expression is abolished by injection of 25 pg lft2-MT RNA (B) and is expanded by injection of 10 pg cyc RNA (C). Co-injection of 25 pg lft2-MT RNA and 10 pg cyc RNA results in wild-type gsc expression (D). (E–H) ntl expression at 50% epiboly. Uninjected embryos (E) express ntl throughout the margin. In response to injection of 25 pg lft2-MT RNA, ntl expression is slightly downregulated at the site of ectopic expression (F). ntl expression is expanded in embryos injected with 5 pg cyc RNA (G). Embryos co-injected with 25 pg lft2-MT RNA and 5 pg cyc RNA (H) express ntl in a similar pattern to uninjected embryos. Embryos (animal pole view) were processed by in situ hybridization followed by anti-myc immunohistochemistry to identify clones of cells expressing Lft2-MT.
Fig. 7. Model of the potential roles of lefty and cyclops signaling in establishment of the embryonic midline and bilateral symmetry. Interaction of inhibitory lefty signals and positive cyclops signals regulates mesendoderm induction within the embryonic midline. Signals emanating from the midline (which may include lefty and cyclops) induce floorplate in the overlying neural tube and pattern lateral mesendoderm, thereby establishing bilateral symmetry.

Evolutionary changes in the ratio between the agonist and antagonists emitted from the midline would allow alterations in the proportions of the embryo. This could occur via subtle changes in the regulation of the agonist or antagonist. Furthermore, by altering the ratios of agonist and antagonist in individual midline domains, specific regions of mesendoderm along the axis of the embryo could be expanded or contracted. Throughout this proposed evolutionary process, bilateral symmetry is maintained regardless of how proportions at specific points along the anteroposterior axis are altered.

We thank W. W. Branford, M. L. Condic, C. J. Cretekos, A. L. Parks and A. F. Ramsdell for their thoughtful comments on the manuscript, and J. Zhang for expert technical assistance. D. J. Grunwald and S. A. F. Ramsdell for their thoughtful comments on the manuscript, and J. Zhang for expert technical assistance. D. J. Grunwald and S. A. F. Ramsdell for their thoughtful comments on the manuscript, and J. Zhang for expert technical assistance.

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