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An SP1-like transcription factor Spr2 acts downstream of Fgf signaling to mediate mesoderm induction

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Fgf signaling, mediated in part by the transcription factor Brachyury/Xbra/Ntl, plays important roles in mesoderm formation during the early development of vertebrate embryos. We have identified a zebrafish gene, *spr2,* which encodes a member of the Sp1-like transcription factor family. *spr2* is expressed in both hypoblast and epiblast cells during late blastulation/early gastrulation, and in some mesodermal and neural tissues at later stages. Injection with *spr2* mRNA enhances *ntl* expression and alleviates the inhibitory effect on *ntl* of XFD, a *Xenopus* dominant-negative FGF receptor. In contrast, morpholino-mediated knockdown of Spr2 activity inhibits *ntl* expression and reduces the inductive effect of Fgfs on *ntl*. We also demonstrate that Fgf signaling relays mesoderm induction activity of Nodal signaling and Spr2 is involved in this signal relay process. Furthermore, the correct spatial expression of *spr2* requires Nodal, Fgf and Wnt signals. We suggest that expression of *spr2* is an immediate-early response to mesoderm induction by Fgfs, which in turn regulates the expression of effector genes involved in the development of mesodermal tissues.

Keywords: fibroblast growth factor/mesoderm/SP1-like transcription factor/zebrafish

Introduction

The formation and patterning of the three germ layers of vertebrate embryos are highly complex processes that are regulated by extensive interactions between several inductive signals. The mesoderm is induced in amphibians by signals derived from the endoderm (Nieuwkoop, 1969), and in zebrafish by signals produced both by the yolk syncytial layer and the yolk cell itself (Mizuno *et al*., 1996; Chen and Kimelman, 2000). Molecular and genetic studies have identified VegT, a T-box transcription factor (Zhang *et al*., 1998; Kofron *et al*., 1999), as an early endogenous mesoderm inducer in *Xenopus*, which is maternally produced and vegetally localized. Previous studies in *Xenopus* suggest that VegT activates the expression of nodal-related genes in the endoderm, which in turn induce the formation of mesoderm (Clements *et al*., 1999; Kofron *et al*., 1999; Agius *et al*., 2000). In addition, mice deficient in Nodal fail to form the primitive streak and lack most mesodermal cells (Zhou *et al*., 1993; Conlon *et al*., 1994). Furthermore, in zebrafish, the simultaneous loss-of-function of two nodal genes, *squint* (*sqt*) and *cyclops* (*cyc*), result in embryos that are missing most of the mesodermal tissues (Feldman *et al*., 1998). Thus, Nodal signal is believed to be a universal, well conserved, mesoderm inducer during vertebrate embryogenesis.

Fgf signals are also known to play a key role in the induction of the mesoderm and, like Nodal, its role in mesoderm induction appears to be conserved in vertebrates. Mutant mice lacking Fgf8 are unable to undergo normal gastrulation, leading to loss of mesoderm- and endoderm-derived tissues (Sun *et al*., 1999). In *Xenopus*, the addition of Fgf causes animal cap explants to form mesoderm tissues (Kimelman and Kirschner, 1987; Slack *et al*., 1987), while a dominant-negative Fgf receptor (dnFGFR) can inhibit the formation of the posterior and lateral mesoderm (Amaya *et al*., 1991, 1993). In zebrafish *acerebellar* mutants, which carry a mutation in the *fgf8* locus, a slight reduction of somatic mesoderm is observed (Reifers *et al*., 1998). In addition, an inhibition of Fgf receptor signaling in the zebrafish embryos leads to complete loss of both the trunk and tail (Griffin *et al*., 1995), which also supports a role for Fgf signaling in mesoderm induction.

It has been suggested from work in *Xenopus* that Fgf signals act in a signal relay mechanism to control mesoderm induction, such that the signals enable cells in the marginal zone to be competent for mesoderm induction by TGFβ signals (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). A well-studied transcription factor that is downstream of Fgf signals is *Brachyury*, which was first identified in mouse (Herrmann *et al*., 1990; Wilkinson *et al*., 1990). Mutant mice homologous for the *Brachyury/T* locus have insufficient mesoderm and lack a notochord (Herrmann *et al*., 1990; Wilkinson *et al*., 1990). The zebrafish homolog of mouse *Brachyury* is the loss-of-function mutation *no tail* (*ntl*) which, like the mouse, lacks a notochord and is also missing a tail (Halpern *et al*., 1993; Schulte-Merker *et al*., 1994). The expression of *ntl* is expanded by the overexpression of eFGF and inhibited by the overexpression of a dnFGFR (Griffin *et al*., 1995; Rodaway *et al*., 1999). As in other species, the expression of *Xenopus brachyury* (*Xbra*), a pan-mesodermal marker, is regulated by Fgf signals (Smith *et al*., 1991; Isaacs *et al*., 1994; Latinkic *et al*., 1997). The ectopic expression of *Xbra* leads to the induction of mesoderm in animal cap explants while its loss-of-function results in mesodermal defects (Cunliffe and Smith, 1992; Conlon *et al*., 1996). The mesoderm inductivity of *Xbra* can be overcome by overexpression of dnFGFR (Schulte-Merker and Smith, 1995). It has been suggested that *Brachyury/ntl* is an

immediate mediator of Fgf signaling in mesoderm induction.

We are interested in identifying other downstream components of the FGF signaling cascade that regulate mesoderm induction. SP1 is a zinc finger transcription factor that is ubiquitously expressed and binds to GC-rich promoter elements to activate the transcription of target genes (Kadonaga *et al*., 1987). To date, many SP1-related transcription factors have been identified in a variety of species and these constitute an SP1 family (reviewed in Kaczynski *et al*., 2003). Unlike *SP1*, some SP1-like genes are expressed in certain types of cells during the development of vertebrate embryos and are involved in specific developmental processes. For example, mouse *Sp5* is a recently identified member that is expressed in the primitive streak during gastrulation and later in the notochord, the neural tube and paraxial mesoderm (Harrison *et al*., 2000; Treichel *et al*., 2001). Loss-of-function of *Sp5* in the T/+ genetic background can enhance the T/+ phenotype in mice, suggesting a genetic interaction between *Sp5* and *Brachyury* (Harrison *et al*., 2000). *Xenopus* XSPR-1 and XSPR-2, and zebrafish Bts1, are closely related to Sp5 (Tallafuss *et al*., 2001; Ossipova *et al*., 2002). Both *XSPR-2* and *bts1* are expressed in the mesoderm precursors. These findings suggest that Sp5 and Sp5-related genes may play a role in mesoderm formation during vertebrate embryogenesis.

In this study we have identified zebrafish *spr2*, an ortholog of *Xenopus XSPR2*. Expression of *spr2* occurs in both hypoblast and epiblast cells during late blastulation and early gastrulation, and at later stages in several mesoderm and neural tissues. We demonstrate that *spr2* expression is dependent on Fgf, Nodal and Wnt signals, and it is implicated in mesoderm induction during early development in zebrafish embryos.

Results

Zebrafish spr2 is a member of the SP1 transcription factor family

A new SP1-related sequence was initially identified by whole-mount *in situ* hybridization from a cDNA library as a gene with a restricted expression pattern during early development in zebrafish embryos. An open reading frame of this gene encodes a putative peptide of 357 residues. A BLAST search revealed that the putative peptide had variable degrees of homology with members of the SP1-like transcription factor family. Further BLAST searches of a public database identified seven SP1 family members in the human genome. A phylogenetic analysis of these sequences is shown in Figure 1, which supports a previously published analysis (Kaczynski *et al*., 2003), but with the addition of two extra human family members. Kaczynski *et al*. (2003) reported three main clades of SP1/KLF genes, called clades I, II and III. Clade I comprised the SP1 genes, clade II included TIEG1, and clade 3 held COPEB in the topology confirmed in the tree in Figure 1. Because our new SP1-related sequence from zebrafish was grouped in a phylogenetic analysis with XSPR-2 of frog (Ossipova *et al*., 2002) and a sequence we uncovered in the fugu database, we named this zebrafish gene *spr2*. The previously described SP1-related factor in zebrafish, Bts1 (Tallafuss *et al*. 2001), fell into a second strongly

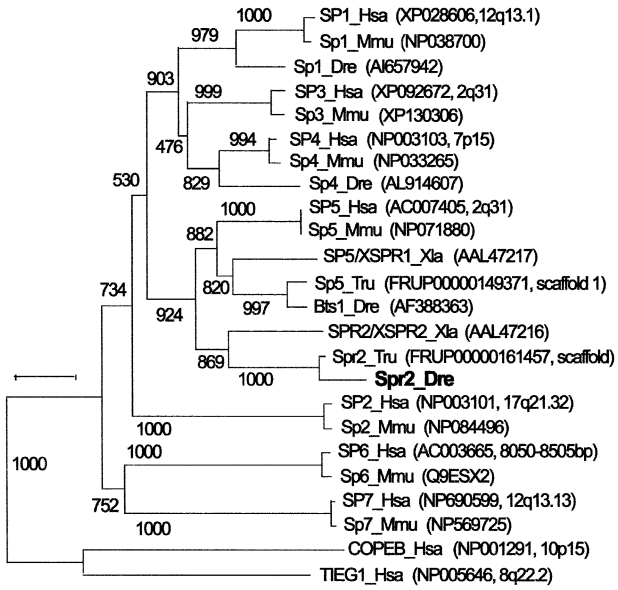

Fig. 1. Phylogenetic analysis of vertebrate SP1-related factors. Sources of the sequences are indicated in parentheses.

supported clade with the frog sequence XSPR-1 (Ossipova *et al*., 2002) and another fugu sequence (Figure 1). These two clades are both related to the previously described *Sp5* gene of mouse (Harrison *et al*., 2000). By searching the human genome sequence database, we found a previously unannotated human sequence on BAC, which is clearly the human ortholog *SP5* as shown by phylogenetic analysis. Because *GAD1* is on the same human BAC clone RP11-570C16 and *GAD1* maps to Hsa2q31 at nucleotide position 171 637 kb, *SP5* should also map just 101 kb away. Supported by chromosomal mapping analysis (data not shown), zebrafish *bts1* may prove to be an ortholog of human *SP5*. The zebrafish Spr2 shares an overall identity in amino acid sequence of 38.5. 56.1, 39.7, 35.8 and 36.6% to XSPR-1, XSPR-2, Bts1, mouse and human SP5, respectively. All of these factors have a conserved btd domain and three zinc finger domains (see Supplementary figure 8 available at *The EMBO Journal* Online), in which they share a sequence identity of over 88.9%. Phylogenetic analysis showed that *Xenopus* has an ortholog of both zebrafish genes *bts1* and *spr2*, thus suggesting that the duplication event that produced these two clades occured before the divergence of the *Xenopus* and teleost lineages, that is, before the divergence of ray-fin fish, which includes teleosts, and lobe-fin fish, which includes tetrapods. Thus, the last common ancestor of frogs and humans had an ortholog of the *Spr2* gene, but the gene does not surface in BLAST searches of the human or mouse genomes, and we assume it is either missing or else so diverged that it does not surface as a member of the SP1 gene family.

spr2 is expressed in mesodermal and ectodermal tissues during embryogenesis

The expression pattern of *spr2* in zebrafish embryos was examined by whole-mount *in situ* hybridization. *spr2* transcripts can initially be detected at 30% epiboly on the future dorsal side of the embryo (Figure 2A and B). The expression domain is about four to eight cells in width and

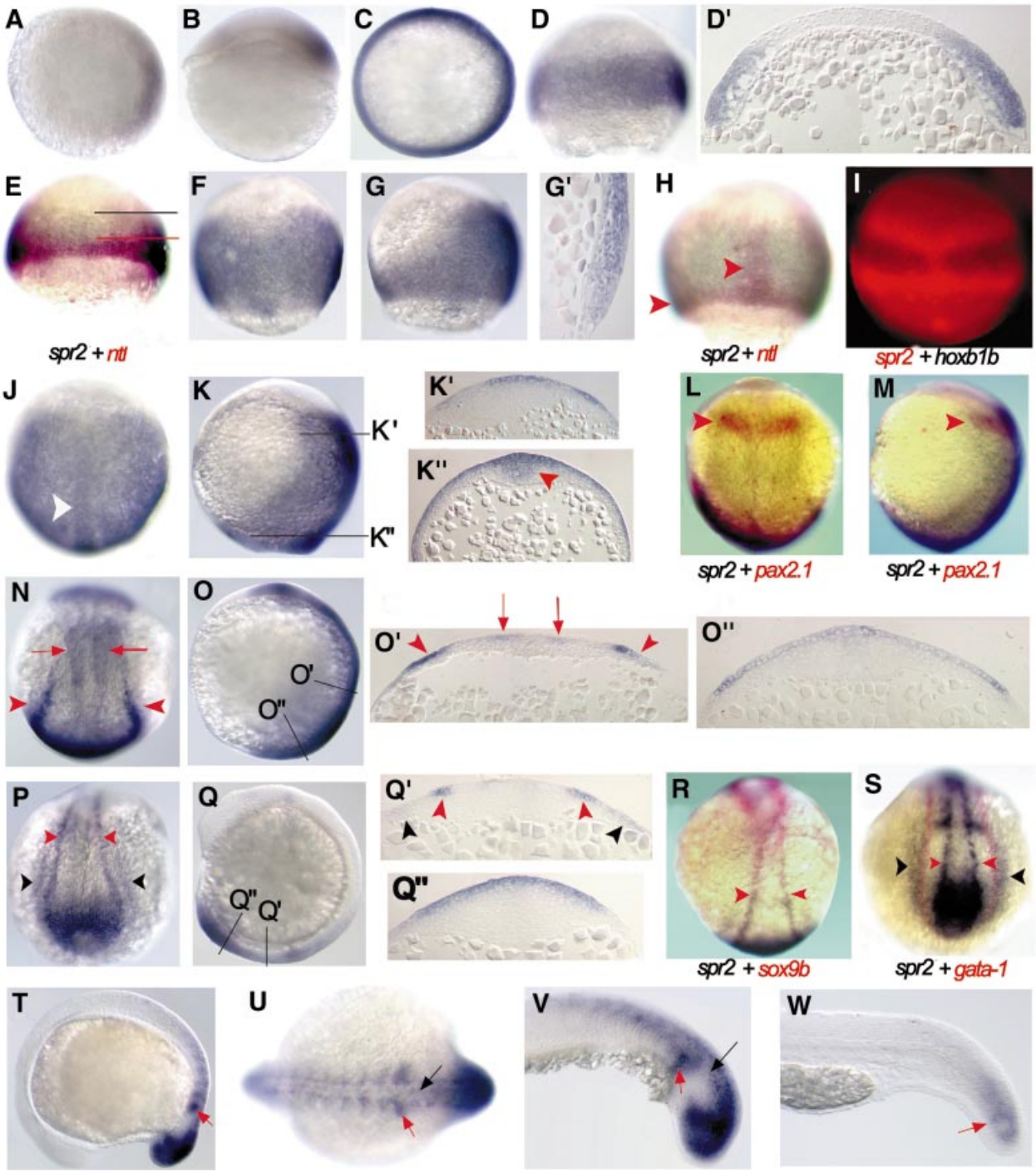

Fig. 2. Expression pattern of *spr2* during the development of zebrafish embryos. Animal-pole (**A**) and lateral (**B**) views of an embryo with dorsal to the right at 30% epiboly stage. Animal-pole (**C**) and lateral (**D**) views of a shield-stage embryo with dorsal to the right. (**D′**) A section in parallel to the animal–vegetal plane of the embryo in (D). Embryos simultaneously labeled for *ntl* (red) and *spr2* (blue) at 50% epiboly (**E**) and 75% epiboly (**H**). The upper limit of *ntl* and *spr2* domains in (E) is indicated by red and black lines, respectively. The red arrowheads in (H) point at the axial and germ ring domains of *ntl*. (**F**, **G** and **G′**) Embryos at 75% epiboly. (F) Dorsal view. (G) Lateral view with dorsal to the right. (G′) The shield area of a section along the anteroposterior axis. (**I**) Fluorescence-microscopic dorsal view of a 60% epiboly-stage embryo simultaneously labeled for *hoxb1b* (blue/black) and *spr2* (red). Dorsal (**J**) and lateral (**K**) views at 95% epiboly. The white arrowhead in (J) points at the midline tissues with a higher level of expression, probably including the neural and the notochord precursors. (**K′** and **K″**) Cross-sections at positions indicated in (K). Expression occurred anteriorly in the outer layer of two to three cell-diameter and posteriorly in the thickening midline tissues (indicated by an arrowhead). Dorsal (**L**) and lateral (**M**) views of a bud-stage embryo simultaneously labeled for *pax2.1* (red) and *spr2* (blue), showing that the anterior border of *spr2* domain matched the *pax2.1* domain (posterior midbrain, indicated by arrowheads). Dorsal (**N**) and lateral (**O**) views of a two-somite stage embryo. (**O′** and **O″**) Cross-sections at positions indicated in (O). The red arrowheads indicate the posterior border of the neuroectoderm [trunk neural crest (TNC) precursors] and red arrows indicate unidentified neural precursors. (**P**–**S**) Emryos at the six-somite stage. (P) Dorsal view of posterior trunk. (Q) Lateral view. (Q′ and Q″) Cross-sections at positions indicated in (Q). (R) Double staining for *sox9b* (red) and *spr2* (blue). Both genes were expressed in the TNC cells. (S) Double staining for *gata-1* (red) and *spr2* (blue). The outermost domain (lateral mesoderm) of *spr2* partially overlapped the *gata-1* domain (hematopoietic progenitors). Red arrowheads in (P–S) point at the TNC and black arrowheads at the lateral mesoderm. Lateral (**T**) and posterodorsal (**U**) views at 14-somite stage. Lateral views of posterior trunk at 18-somite stage (**V**) and 24 h (**W**). The red and black arrows indicate the newly formed somites and neuronal cells.

occupies ~30% of the blastodermal circumference. As epiboly proceeds, the expression domain extends ventrally, so that just before the onset of gastrulation an expression domain 16–20 cells wide encircles the whole blastodermal margin (but again with stronger staining at the dorsal side) (Figure 2C and D). Taking a section through the embryo at this stage reveals that *spr2* is expressed in both hypoblast and epiblast layers (Figure 2D′). Because *ntl* is a mesodermal marker (Schulte-Merker *et al*., 1994), we performed a double *in situ* hybridization with antisense *spr2* and *ntl* probes to determine the tissue types of *spr2*-positive cells. As shown in Figure 2E, the marginal region expressing *spr2* overlaps with the *ntl* domain, suggesting the mesodermal fate of these *spr2*-positive cells. According to the fate map of the early zebrafish gastrula (Kimmel *et al*., 1990) and the location of *spr2* transcripts at later stages, *spr2*-positive cells that are located anterior to the *ntl* domain in the animal pole direction should have a neuroectodermal fate.

During gastrulation the expression of *spr2* further expands towards the dorsal side and along the antero-posterior axis as *spr2*-positive cells proliferate and move dorsally by convergent extension (Figure 2F, G, J and K). At 75% epiboly, expression on the dorsal side extends to an anterior limit presumably marking the midbrain while on the ventral side expression persists only in the blastodermal margin (Figure 2F and G). In contrast, *ntl* expression during the same period becomes restricted to the blastodermal margin and the notochord precursors (Figure 2H). A sagittal section along the dorsal midline discloses *spr2* expression in the hypoblast and epiblast cells (Figure 2G′), suggesting that *spr2*-positive cells may contribute to both mesodermal and ectodermal tissues. Double labeling with antisense *spr2* and *hoxb1b* probes shows that the anterior border of *spr2* expression is several cells ahead of that of *hoxb1b* expression (Figure 2I). Since the anterior border of the *hoxb1b* expression domain marks the boundary between rhombomeres 3 and 4 (Alexandre *et al*., 1996), the most anterior cells that express *spr2* may contribute to the more anterior neural tissues. By the end of epiboly, the expression pattern of *spr2* is changed little from earlier stages (Figure 2J and K). Sections show that the transcripts are present in both outer and deep layers of the posterior midline tissues (Figure 2K″) and in the other areas they are mainly restricted to the outer 2–3 layers of cells (Figure 2K′ and K″). Double labeling with *spr2* and *pax2.1*, a marker for the posterior midbrain (Krauss *et al*., 1991), indicates that the anterior border of *spr2* expression is located in the midbrain (Figure 2L and M).

During the early segmentation period, one of the major tissues expressing *spr2* is the trunk neural crest where *spr2* expression overlaps that of the neural crest marker *sox9b* (Li *et al*., 2002) (Figure 2N–S). A pair of the most lateral bands also covers the expression domain of *gata-1* (Figure 2S), a maker for the blood precursor cells (Detrich *et al*., 1995), suggesting a possible hematopoietic fate for these cells. The other tissues expressing *spr2* during segmentation include the tailbud and the developing somites (Figure 2N–V). Later in development the expression of *spr2* declines such that at 24 h post-fertilization (hpf), it is detected only in the posterior-most region of the embryo (Figure 2W), and at ~36 hpf *spr2* can no longer be detected by *in situ* hybridization.

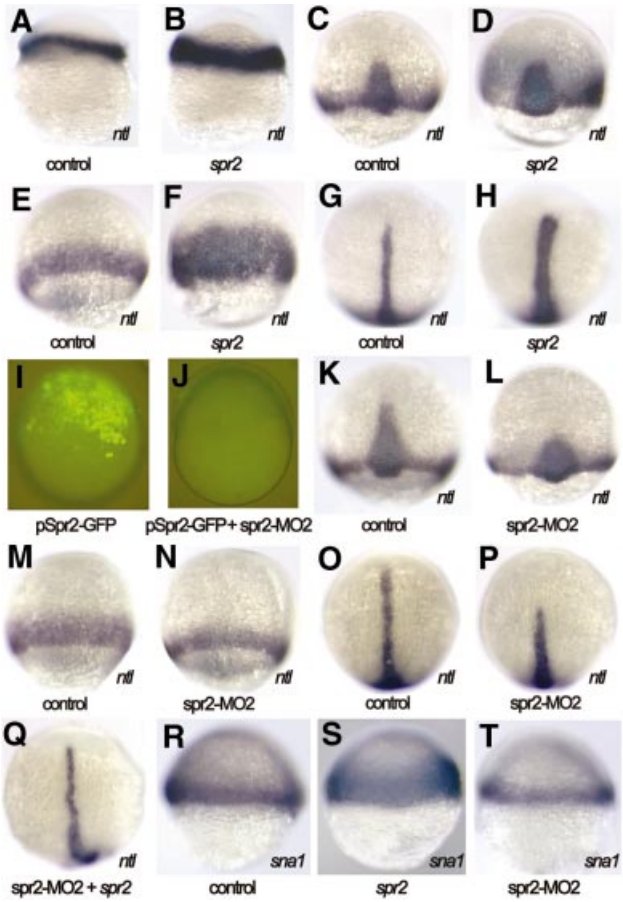

Fig. 3. Spr2 positively regulates expression of *ntl* and *sna1*. (**A**, **C**, **E**, **G**, **K**, **M**, **O** and **R**) Control embryos injected with GFP mRNA or the control morpholino. (**B**, **D**, **F**, **H**–**J**, **L**, **N**, **P**, **Q**, **S** and **T**) Embryos injected with DNA (100 pg), mRNA (100 pg) and/or morpholino (5 ng) as indicated below each picture. (A and B) Lateral views at 40% epiboly, showing enhanced expression of *ntl* in the germ ring (B). (C and D) Dorsal views at 70% epiboly, showing *ntl* expansion in the axial mesoderm (D). (E and F) Ventral views at 70% epiboly, showing *ntl* expansion in the ventral germ ring (F). (G and H) Dorsal views at the bud stage, showing *ntl* expansion in the notochord (H). (I) Lateral view of a live embryo at 60% epiboly, showing green fluorescence expressed by pSpr2-GFP. (J) Co-injection with pSpr2-GFP DNA and spr2-MO2 inhibited Spr2-GFP expression. (K and L) Dorsal views at 75% epiboly. The spr2-MO2 injection caused loss of the anteriormost part of *ntl* expression domain in the axial mesoderm (L). (M and N) Ventral views at 75% epiboly, showing a thinner *ntl* expression domain in the germ ring (N). (O, P and Q) Dorsal views at the bud stage. The morpholino injection resulted in loss of the anterior notochord domain of *ntl* (P), which was rescued by co-injection with *spr2* mRNA (Q). (R–T) Lateral views at 50% epiboly with dorsal to the right. Overexpression of *spr2* expanded *sna1* expression (S) while knocking down with spr2-MO2 led to fewer *sna1*-positive cells (T).

Spr2 positively regulates ntl expression

As shown above, the expression domain of the pan-mesodermal marker *ntl* lies within the *spr2* expression domain during late blastulation and early gastrulation. In order to investigate the possibility that *spr2* might play a role in mesoderm induction by regulating *ntl* expression, we explored the potential effect of Spr2 on *ntl* expression. First we injected synthetic *spr2* mRNA into one-cell stage embryos and examined *ntl* expression at several stages. At the germ ring stage, the expression of *ntl* in the germ ring was enhanced to a certain extent in the injected embryos

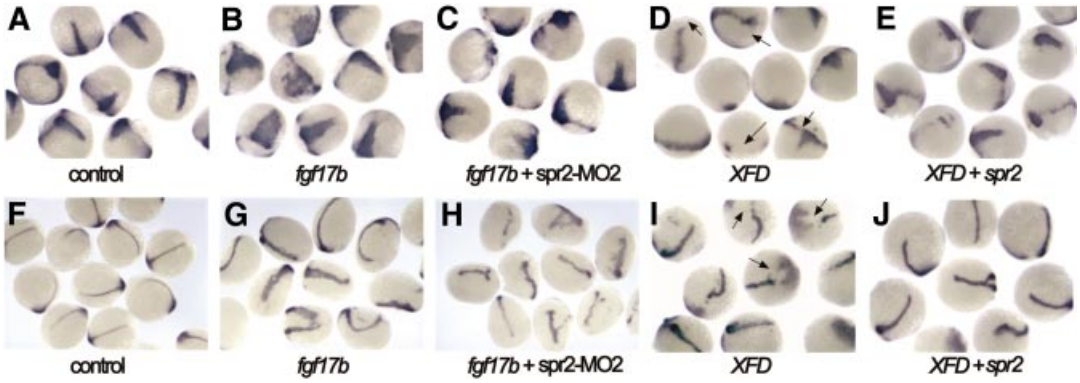

Fig. 4. Spr2 mediates Fgf signaling in mesoderm induction. The embryos were examined for *ntl* expression. (**A**–**E**) 75% epiboly stage. (**F**–**J**) Bud stage. Injection with 10 pg *fgf17b* mRNA caused expansion of *ntl* expression in the axial mesoderm of most of the embryos (B and G), whereas the proportion of the embryos with similarly enhanced *ntl* expression significantly dropped when co-injected with the same amount of *fgf17b* mRNA and 5 ng spr2-MO2 (C and H). Injection with 50 pg *XFD* mRNA led to slightly smaller axial mesoderm domain and interrupted germ ring domain (indicated by arrows) for *ntl* expression (D and I), while the inhibitory effect of XFD was deterred when co-injected with 200 pg *spr2* mRNA (E and J).

(Figure 3B). During gastrulation, phenotypes following *spr2* mRNA injection were more readily recognized because the affected embryos showed a significant increase in *ntl* expression in the axial mesoderm (Figure 3D and H) and in the germ ring (Figure 3F). The inductive effect of *spr2* was clearly dose dependent; when injected with 100 pg *spr2* mRNA, the percentage of embryos that showed expanded *ntl* expression was 43.1% ($n = 181$) at ~70% epiboly, and when injected with 200 pg spr2 mRNA the percentage increased to 65.9% ($n = 41$), respectively. At the bud stage, the two doses affected 40% ($n = 65$) and 51.7% ($n = 116$) of embryos, respectively. The ectopic expression of *ntl* at the animal pole or surrounding regions was not observed in the injected embryos, suggesting that Spr2 is a modulator, rather than an activator, of *ntl* expression.

We then studied the effect of Spr2 on *ntl* expression by blocking the translation of endogenous *spr2* mRNA using morpholino-based knockdown technology (Nasevicius and Ekker, 2000). To test the effectiveness of the morpholinos, fertilized eggs were injected with variable amounts of spr2-MO1 or spr2-MO2 in combination with 100 pg of pSpr2-GFP DNA, an expression construct containing a partial 5′ sequence of *spr2* cDNA fused in-frame to a *GFP* coding sequence. At a dose of 5 ng, the spr2-MO2-injected embryos almost lacked green fluorescence from the GFP fusion protein (Figure 3J), while the spr2-MO1-injected embryos retained visible fluorescence (data not shown), suggesting that spr2-MO2 could more effectively block translation of *spr2* mRNA. Then we examined *ntl* expression, by whole-mount *in situ* hybridization, in the embryos injected with 5 ng of the spr2-MO2. Around the onset of gastrulation, the injected embryos exhibited no obvious changes in *ntl* expression (data not shown). By 75% epiboly, 52.3% ($n = 237$) of the injected embryos appeared to lack the anterior-most axial domain of *ntl* and have a smaller number of *ntl*-positive cells in the germ ring (Figure 3K–N). The effect of spr2-MO2 was easier to identify at the bud stage when *ntl* expression was reduced or abolished in the anterior notochord in 43.2% ($n = 169$) of the injected embryos (Figure 3P). When 100 pg *spr2* mRNA and 5 ng spr2-MO2 were co-injected, however, only 7.9% ($n = 63$) of the embryos showed a loss or significant reduction in *ntl* expression in the anterior notochord at the bud stage, which was far less than the percentage (43.2%) obtained by injecting with spr2-MO2 alone. The successful rescue of *ntl* expression (Figure 3Q) means that the effect of blocking the translation of endogenous *spr2* mRNA is counteracted by the supply of the synthetic *spr2* mRNA. The knockdown results also support the idea that Spr2 is required for maintenance of *ntl* expression.

To further confirm the mesoderm-promoting activity of Spr2, its impact on the expression of *snail1* (*sna1*), which is a lateral mesoderm marker during early gastrulation (Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al*., 1993), was investigated. The overexpression of *spr2* enhanced *sna1* expression in the lateral germ ring at 50% epiboly stage (Figure 3S) while injection with spr2-MO2 caused a reduction in the number of *sna1*-positive cells (Figure 3T), implying a general role in mesoderm induction.

Spr2 is involved in Fgf-mediated mesoderm induction

Because *Xbra/ntl* expression is an immediate-early response to mesoderm induction and *Xbra/Ntl* mediates mesoderm induction activity of Fgfs, we wondered whether Spr2 regulates *ntl* expression by mediating Fgf signaling. To test this possibility, we explored the change in *ntl* expression following co-injection with spr2-MO2 and *fgf* mRNA. We tested two zebrafish Fgf molecules, Fgf8 (Reifers *et al*., 1998) and a new Fgf family member Fgf17b (our unpublished data). At 75% epiboly, all of the embryos injected with 10 pg of *fgf17b* mRNA showed a significant increase in *ntl* expression in the presumptive notochord (Figure 4B). Injection with 50 pg of *fgf8* mRNA also led to the induction of *ntl* expression in 53.7% ($n = 41$) of the embryos at the same stage. When the same doses of *fgf17b* or *fgf8* mRNA were combined with 5 ng spr2-MO2,

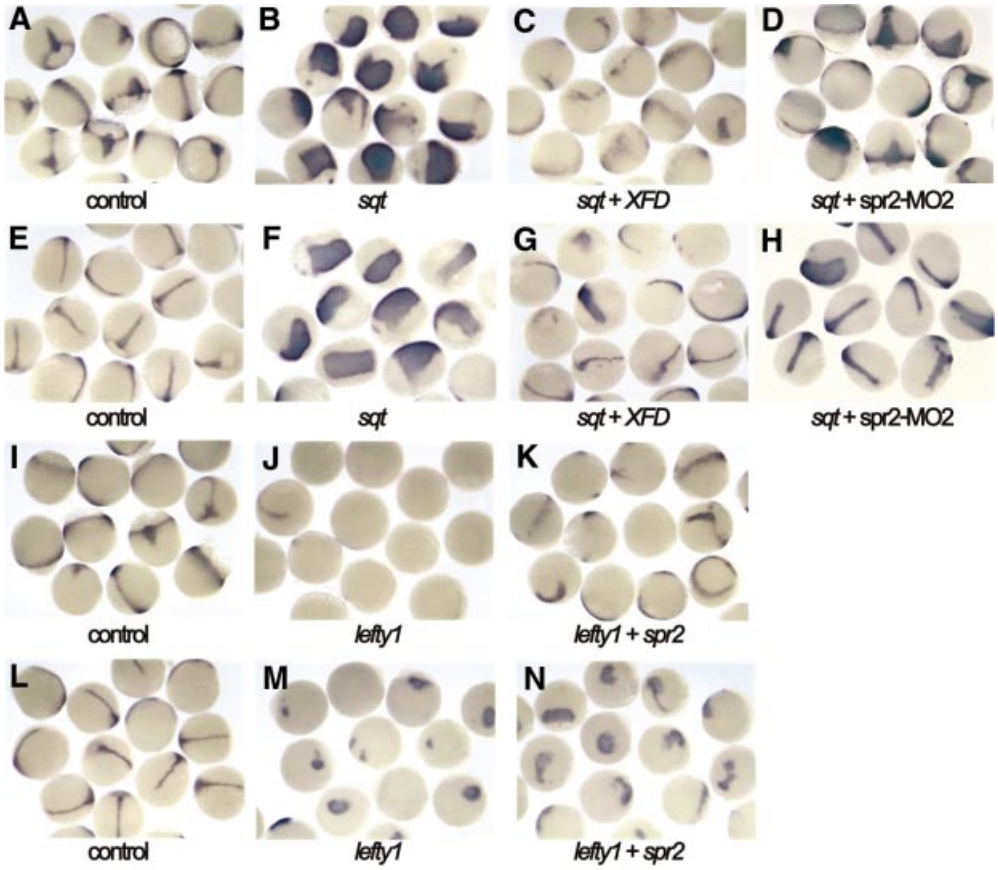

Fig. 5. Fgf signal and Spr2 mediate inductivity of Nodal signal on *ntl*. The embryos were examined for *ntl* expression. (**A**–**D** and **I**–**K**) 75% epiboly stage. (**E**–**H** and **L**–**N**) Bud stage. Almost all of the embryos injected with 0.5 pg *sqt* mRNA alone showed a large increase in *ntl* expression in the axial mesoderm (B and F). Co-injection with 100 pg *XFD* mRNA effectively inhibited the inductivity of *sqt* overexpression (C and G). Co-injection with 5 ng spr2-MO2 also inhibited, although less effectively, the inductive activity of *sqt* overexpression (D and H). Injection with 100 pg *lefty1* mRNA almost abolished *ntl* expression during midgastrulation (J). Co-injection with 50 pg *spr2* mRNA rescued *ntl* expression in some of the embryos (K). At the bud stage, the rescue effect of *spr2* mRNA injection was also obvious (M and N).

only 35.7% (n = 28) or 34.6% (n = 26) of the embryos, respectively, expressed *ntl* at a comparably increased level (Figure 4C). At the bud stage, only 3.1% (n = 65) of the embryos co-injected with 2 pg *fgf17b* mRNA and 5 ng spr2-MO2 exhibited a significant increase in *ntl* expression (Figure 4H), in contrast to 34.8% (n = 46) for the single mRNA injection (Figure 4G). These results demonstrate that knockdown of Spr2 activity inhibits the inductive effect of Fgf on *ntl* expression.

The effect of co-injecting *spr2* mRNA and *XFD* mRNA, which encodes a dominant negative form of a *Xenopus* FGF receptor (Amaya *et al*., 1991), was also investigated. Overexpression of *XFD* alone typically led to a slightly smaller axial mesoderm domain and an interrupted germ ring domain (indicated by arrows in Figure 4D and I) for *ntl* expression during gastrulation. When injected with 50 or 100 pg *XFD* mRNA, the embryos with a reduction in *ntl* expression at the 75% epiboly stage accounted for 75% (n = 40) or 76.7% (n = 43) (Figure 4E), respectively. In contrast, the percentage of the embryos with reduced *ntl* expression, when injected simultaneously with the same two doses of *XFD* mRNA and 200 pg *spr2* mRNA (Figure 4E), was 26.7% (n = 30) and 52.4% (n = 42), respectively. Moreover, the proportion of co-injected embryos with enhanced *ntl* expression at this stage was <15% for the co-injection, compared to ~60% for embryos injected with *spr2* mRNA alone. At the bud stage, the proportion of the co-injected embryos with enhanced *ntl* expression dropped to <5% for the co-injection (Figure 4J). These results suggest that the overexpression of *spr2* complements reduction of Fgf activity in mesoderm induction.

Spr2 mediates mesoderm induction of the Nodal signal

Nodal signaling is a key player in mesoderm induction (Schier and Shen, 2000). As, in *Xenopus,* the mesoderm induction activity of Activin, also a TGFβ ligand, is relayed by Fgfs (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994), so Fgfs may also act as a second signal to relay Nodal signaling in mesoderm induction. To test this hypothesis, we coexpressed ectopic zebrafish *sqt* and *Xenopus XFD*, and examined *ntl* expression in the zebrafish embryos at 75% epiboly and the bud stages. At both these stages, expression of *ntl* was considerably expanded in almost all (n > 32) of the embryos injected with 0.5 pg or 0.25 pg of *sqt* mRNA alone (Figure 5B and F). When 0.5 pg *sqt* mRNA and 100 ng *XFD* mRNA were co-injected, the percentage of embryos with significant induction of *ntl* decreased to 5.1% (n = 39) and 15%

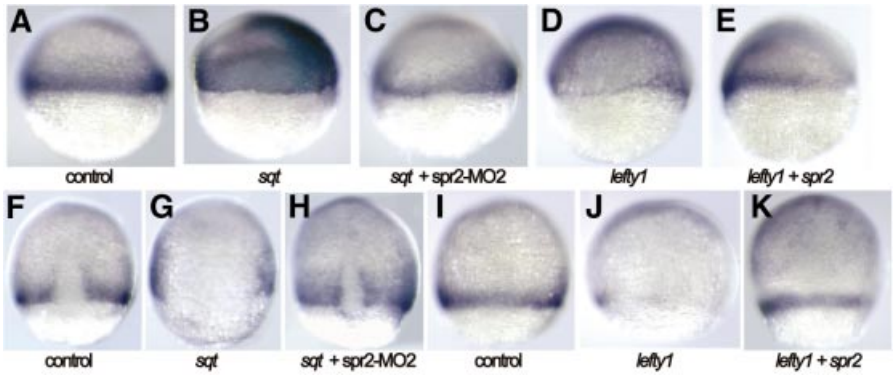

Fig. 6. Spr2 mediates induction of Nodal signal on *sna1*. The embryos were examined for *sna1* expression following injections. (**A**–**E**) Lateral views with dorsal to the right at 50% epiboly. (**F**–**H**) Dorsal views at 80% epiboly. (**I**–**K**) Lateral views at 80% epiboly, with dorsal to the right. Injection doses: *sqt* mRNA, 0.125 pg; *lefty1* mRNA, 100 pg; *spr2* mRNA, 100 pg; spr2-MO2, 5 ng.

($n = 60$) at 75% epiboly and the bud stages (Figure 5C and G), respectively. This suggests that the mesoderm induction activity of Nodal is dependent on Fgf function.

As demonstrated earlier, Spr2 appears to be an effector of Fgf signaling in mesoderm induction. Thus, we speculated that Spr2 might also mediate mesoderm induction activity of Nodal signal. Therefore, we first tested whether it was possible to inhibit Sqt activity by knocking down Spr2. When co-injected with 0.5 pg *sqt* mRNA and 5 ng spr2-MO2, the embryos with *ntl* expansion during gastrulation accounted for ~78% ($n >$ 36), <100% for the *sqt* mRNA injection alone. If the dose of *sqt* mRNA for co-injection was reduced to 0.25 pg, the percentage of the embryos with a comparable level of increase in *ntl* expression further declined to 41.7% ($n = 60$) and 38% ($n = 71$) at 75% epiboly and the bud stages (Figure 5D and H), respectively. This suggests that the inhibition of Spr2 can affect the function of Sqt during mesoderm induction, although this inhibitory effect is not as great as XFD.

Lefty1 is an antagonist of Nodal signaling (Thisse and Thisse, 1999). We injected embryos with 100 pg mRNA of zebrafish *lefty1* and found that 71.8% ($n = 39$) of the embryos had no detectable *ntl* expression and the remaining embryos only showed weak *ntl* expression in the ventral germ ring at 75% epiboly stage (Figure 5J). At the bud stage, only 2 out of 47 injected embryos developed a short and thin notochord domain of *ntl* while the others had a small staining patch in the tailbud (Figure 5M). However, when co-injected with 100 pg *lefty1* and 50 pg *spr2* mRNA, 70.2% ($n = 43$) of the embryos retained *ntl* expression in some domains at 75% epiboly stage (Figure 5K). At the bud stage, 25 out of 44 co-injected embryos (56.8%) expressed *ntl* at a level higher than that observed with the single injection of *lefty1*, 7 of which had a notochord expression domain (Figure 5N). Obviously, the overexpression of *spr2* restored to a certain extent *ntl* expression that was suppressed by the ectopic Lefty1. This again supports the hypothesis that Spr2 is able to mediate mesoderm induction activity of Nodal or related TGFβ signals.

The involvement of Spr2 in mesoderm induction by Nodal signal was confirmed using another mesoderm marker, *sna1*. Compared to dramatic expansion of *sna1* towards the animal pole at 50% epiboly stage after injection with *sqt* mRNA alone (Figure 6B), the expansion was negligible when the embryos were co-injected with *sqt* mRNA and spr2-MO2 (Figure 6C). At 80% epiboly stage, the embryos injected with *sqt* mRNA showed an expanded dorsal gap (Figure 6G), whereas the co-injected embryos had a relatively normal dorsal gap (Figure 6H). The *lefty1* injection led to fewer *sna1*-positive cells in the germ ring at the shield stage (Figure 6D), and the number went back to normal when co-injected with *spr2* mRNA (Figure 6E). More obviously at 80% epiboly, overexpression of *lefty1* almost eliminated *sna1* expression in the lateral and ventral germ ring (Figure 6J), which was recovered by co-injection with *spr2* mRNA (Figure 6K). These results suggest that induction of *sna1* by Nodal signal requires Spr2.

Expression of Spr2 is dependent on Nodal, Fgf and Wnt signals

Since *spr2* is expressed in the mesoderm precursors during early gastrulation and is involved in mesoderm induction by Fgf signals, we asked whether *spr2* was regulated by Nodal and Fgf signals and mediated their activities in a feedback fashion. We analysed *spr2* expression in the zebrafish embryos in which activities of the signals were altered transiently. When injected with 1 pg *sqt* mRNA, *spr2* was induced throughout the blastoderm at 50% epiboly stage (Figure 7B). Injection with 100 pg *lefty1* mRNA completely abolished *spr2* expression on the dorsal side of the blastoderm and also, remarkably, reduced expression on the ventral side (Figure 7C). These data suggest that the Nodal signal is essential for the activation of *spr2* on the dorsal side and is also required for maintenance of *spr2* expression on the ventral side. Likewise, the ectopic expression of *fgf17b* also induced *spr2* expression throughout the blastoderm during early gastrulation (Figure 7E and K). When *XFD* was overexpressed in the embryos, *spr2* expression was almost eliminated in the ventral and ventrolateral domains but retained in the dorsal domain at a lower level (Figure 7F, I and L). Taking a section of an *XFD*-injected embryo at 75% epiboly revealed that on the dorsal side, only the epiblast cells weakly expressed *spr2* whereas the hypoblast cells did not at 75% epiboly (Figure 7N). This suggests that Fgf signaling is absolutely required for *spr2* expression in the hypoblast cells that will give rise to the

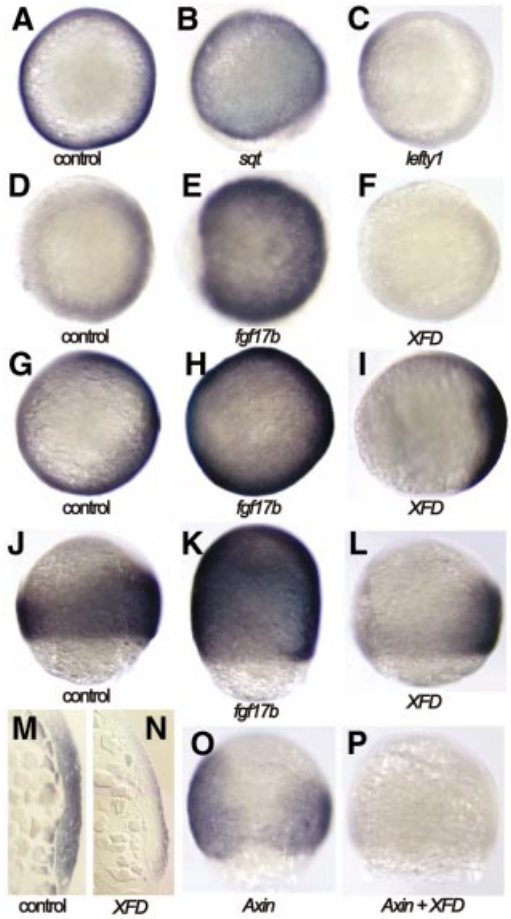

Fig. 7. Dependence of *spr2* expression on Nodal, Fgf and Wnt signals. (**A**–**F**) 50% epiboly stage. (**G**–**L**) 75% epiboly stage. (A–I) Animal-pole views with dorsal to the right. (J–L) Lateral views with dorsal to the right. (O and P) Dorsal views. Injection with 1pg *sqt* or 10 pg of *fgf17b* mRNA caused ectopic expression of *spr2* (B, E, H and K). When injected with 100 pg of *lefty1* mRNA, *spr2* expression was abolished on the dorsal side and reduced in the ventral germ ring (C). When injected with 100 pg *XFD* mRNA, *spr2* expression was eliminated on the ventral side and dramatically decreased on the dorsal side (F, I and L). A section revealed that *spr2* expression on the dorsal of *XFD*-injected embryos persisted in the epiblast layer only (N). Injection with mouse *Axin* mRNA led to suppression of *spr2* expression on the dorsal side (O), while co-injection with *Axin* and *XFD* mRNAs resulted in elimination of *spr2* expression in some of the embryos (P).

mesodermal tissues, while *spr2* expression in the epiblast cells is only partially dependent on Fgf signals. It has been previously demonstrated that in zebrafish the expression of *fgf8* and *fgf17b* in the marginal cells of the late blastulae and early gastrulae is dependent on Nodal signaling (Gritsman *et al*., 1999; our unpublished data). Therefore, Fgf signaling acts during mesoderm formation, at least in most domains, downstream of Nodal signaling to regulate *spr2* expression.

The dorsal epiblast cells of the early gastrula are committed to a neuroectodermal fate. Considering that Wnt signaling contributes to dorsal mesoderm and neural induction (Baker *et al*., 1999; Sokol, 1999; Wilson *et al*., 2001), we speculated that *spr2* expression in the dorsal epiblast cells might be also dependent on Wnt signaling. To test this hypothesis, we injected the zebrafish embryos with an mRNA encoding mouse Axin that is a negative regulator of the canonical Wnt signaling pathway (Kikuchi, 1999). The injected embryos indeed lacked *spr2* transcripts in the dorsal midline and adjacent areas at 75% epiboly (Figure 7O). When both *XFD* and *Axin* were overexpressed, *spr2* expression was almost completely inhibited in some embryos (Figure 7P). This supports the idea that both Fgf and Wnt signaling pathways are essential for the expression of *spr2* in the dorsal neuroectoderm.

Discussion

spr2/XSPR2 and bts1/XSPR1/Sp5 share similar expression patterns

Phylogenetic analysis suggests that *spr2/XSPR2* and *bts1/XSPR1/Sp5* are duplicated from the same ancestor during evolution. Their expression patterns also share a certain degree of similarity. The expression of *spr2* during early gastrulation occurs in the marginal cells, including both epiblast and hypoblast cells, and at later stages in some mesodermal and ectodermal tissues. Like *spr2*, *bts1* is also expressed during early gastrulation in zebrafish (Tallafuss *et al*., 2001). Unlike *spr2*, however, *bts1* expression is mainly restricted to the epiblast cells of the germ ring with its expression in the dorsal hypoblast layer being restricted to very few cells. This suggests that *spr2* and *bts1* may have distinct functions in development of the dorsal mesoderm. The dynamic expression of mouse *Sp5* is also apparent. During early gastrulation *Sp5* is expressed in the primitive streak, including both ectodermal and mesodermal precursors, while in development it is expressed in the midbrain, otic vesicle, the spinal cord, the notochord, somites and even in some endodermal tissues (Harrison *et al*., 2000; Treichel *et al*., 2001). *Xenopus XSPR-2* appears to be predominantly expressed within the presumptive mesoderm during gastrulation, whereas *XSPR-1* expression is restricted to the epithelial and subepithelial layers (Ossipova *et al*., 2002). The expression patterns of these Sp1 family members are indicative of their functions in the formation and patterning of the mesoderm and/or ectoderm.

Spr2 mediates mesoderm induction by Fgf signals

Fgf signaling plays several important roles in mesoderm induction during early development of vertebrate embryos (Kimelman and Kirschner, 1987; Slack *et al*., 1987; Amaya *et al*., 1991, 1993; Griffin *et al*., 1995; Reifers *et al*., 1998). Expression of *Brachyury/Xbra/ntl*, a T-box transcription factor and a specific mesoderm marker, is an immediate response to Fgf induction and is thus also implicated in mesoderm induction. We have demonstrated, mainly using *ntl* as a mesoderm marker, that Spr2 is involved in mesoderm induction via the Fgf signaling pathway. First, the overexpression of Spr2 enhances *ntl* expression, while knockdown of Spr2 activity inhibits *ntl* expression to a certain degree. Second, the overexpression of Spr2 is able to release the inhibition of XFD on *ntl* expression. Third, induction of *ntl* expression by ectopic Fgf is blocked by the simultaneous knockdown of Spr2 activity. However, the extent of increase or decrease in *ntl* expression caused by overexpression or knockdown of *spr2* is not as great as when Fgf or XFD are ectopically

expressed, suggesting that multiple effectors may mediate mesoderm induction via Fgf signaling.

Individual Sp1-like transcription factors function as activators or repressors, depending on which promoter they bind and the coregulators with which they interact (reviewed by Kaczynski *et al*., 2003). We have found that the overexpression of *spr2* enhances *ntl* expression in the germ ring at the onset of gastrulation and in the presumptive notochord during epiboly, which excludes the possibility that Spr2 acts as a repressor on *ntl* promoter. However, the overexpression of *spr2* is unable to induce the ectopic expression of *ntl* in domains where *ntl* is not normally expressed, and when Spr2 is knocked down then *ntl* expression is not completely blocked. This implies that Spr2 is involved in the maintenance rather than activation of *ntl* expression.

Mouse embryos that are homozygous for a targeted mutation in *Sp5* show no obvious phenotype (Harrison *et al*., 2000). Nevertheless, the homozygous mutant mice in a genetic background with a deletion of one *Brachyury* allele have defects in the mesoderm-derived vertebrae, which are not observed in mice with a single mutation. This observation at least suggests that *Sp5* genetically interacts with *Brachyury* to affect development of the mesodermal tissues.

Zebrafish *spr2* is expressed in the epiblast layer during gastrulation and in some neuronal cells at later stages. Our preliminary study has found that *spr2* positively regulates transcription of a posterior neuroectodermal marker *hoxb1b* and might mediate posteriorization of the neuroectoderm during gastrulation via the Fgf signaling pathway (data not shown).

Brachyury/Xbra/ntl may be a direct target of Spr2

Analysis of the mouse *Brachyury* promoter has found binding sites for Sp1 and Sp4, and the deletion of these sites together with the GATA and Pea3 sites reduces reporter gene expression in embryonal carcinoma P19 cells (Yamaguchi *et al*., 1999). In the *Xbra2* promoter a GC box (GCTGGGGGGGGGGGGTG), a potential *cis*-element recognized by Sp1 family members, can be found between –250 and –267. Latinkic *et al*. (1997) have reported that the 381 bp proximal region of the *Xbra2* promoter can elicit responses to Fgf and Activin, whereas the 231 bp promoter, which loses the GC box, fails to respond to Fgf and Activin induction, suggesting requirement of the GC box for such induction. However, further biochemical and molecular studies are needed to confirm the role of Sp1-like factors in transcriptional regulation of *Brachyury/Xbra/ntl*.

Nodal-mediated mesoderm induction is dependent on the action of Fgfs

Nodal proteins have been found to be essential for the development of mesoderm in vertebrates (Schier and Shen, 2000). In this study, we have demonstrated that, like Fgf, the overexpression of *sqt* greatly induces the ectopic expression of *ntl* and this induction can be effectively inhibited by the coexpression of *XFD*, suggesting that action of Nodal signaling depends on the action of Fgf signaling. This is consistent with the findings in *Xenopus* that mesoderm induction by Activin depends on Fgf (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). The fact that induction of *ntl* and *sna1* expression by ectopic Sqt can be reduced by knocking down Spr2 activity, and that the inhibition of *ntl* and *sna1* expression by the nodal-antagonist *lefty1* can be restored by the overexpression of *spr2,* supports the idea that Spr2 mediates mesoderm induction of Nodal signaling by acting downstream of Fgf signaling during early embryogenesis.

Materials and methods

Isolation of spr2 cDNAs

spr2 was first identified from a zebrafish cDNA library as described in Zhao *et al*. (2002). The sequence of *spr2* was deposited in GenBank with an accession number AY338748.

Generation of constructs

The coding sequence of *spr2* was amplified by PCR with a pair of specific primers and cloned into an expression vector pXT7 to generate construct pXT7-spr2 for *in vitro* synthesis of *spr2* mRNA. The coding sequence of *fgf17b*, a new member of Fgf family identified in our laboratory (unpublished results), was similarly cloned to generate pXT7-fgf17b. A recombinant pSpr2-GFP plasmid was constructed by inserting a 787 bp fragment of *spr2*, which contains a 325 bp 5′ UTR and its adjacent coding sequence encoding the first 124 amino acids, in-frame into vector peGFP-N2. This plasmid was used to test the effectiveness of spr2-MO. A GFP coding sequence plus the SV40 polyadenylation signal sequence were cloned into pBluescript KS(–). GFP mRNA was synthesized from the resulting plasmid and was used as an internal injection control.

Morpholino oligonucleotides

Two antisense morpholino oligonucleotides, spr2-MO1 (5′-CCG-CGCTGTTGCTCCTGTTTTTCTG-3′) and spr2-MO2 (5′- CCCCCT-TACACAGCCAGGTGCGTAC-3′), were designed to target *spr2* mRNA and synthesized by Gene Tools, LLC. Co-injection of spr2-MO1 or spr2-MO2 with pSpr2-GFP DNA revealed that spr2-MO2 was much more effective and so this alone was used for all subsequent experiments. Another morpholino oligonucleotide with the sequence 5′- CTG-CTGTAACTACGACCATTTTTGT-3′, which is unrelated in sequence to *spr2* and produces no morphological or molecular changes after injection, was used as a control.

In vitro synthesis of mRNA

Linearized plasmids were used as templates for *in vitro* transcription using an appropriate Cap-Scribe Kit (Roche). The synthesized mRNA was purified using the RNAeasy@ Mini Kit (Qiagen) after treatment with RNase-free DNase and dissolved in nuclease-free water.

Injection

DNA or mRNA was diluted in 0.1 M KCl to an appropriate concentration prior to injection, while the morpholino oligonucleotides were diluted in 1× Danieau's buffer. DNA was injected into the cytoplasm of embryos at the one-cell stage, while the RNAs and morpholinos were injected into the yolk or cytoplasm between the one- and two-cell stages. Injection with GFP mRNA was performed to confirm the effects of overexpression of *spr2* or other genes. The injection dose was an estimated amount received by a single embryo. For co-injection of two mRNAs, they were mixed prior to injection. For co-injection of an mRNA with a morpholino (not RNase-free), an embryo was first injected with the mRNA, followed by a second injection with the morpholino. Data obtained from independent micro-injections were pooled.

Whole-mount in situ hybridization and histological sectioning

Digoxigenin-UTP- or fluorescein-UTP-labeled antisense RNA probes were generated by *in vitro* transcription. Whole-mount *in situ* hybridizations essentially followed the standard protocol with minor modifications. Some of the embryos were sectioned at a thickness of ~10 μm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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