smad2 and smad3 Are Required for Mesendoderm Induction by Transforming Growth Factor-β/Nodal Signals in Zebrafish

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Shunji Jia, Zhen Ren, Xiang Li, Ying Zheng, and Anming Meng

From the Protein Science Laboratory of the Ministry of Education, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

The transforming growth factor-β ligands Nodal, activin, and Vg1 play important roles in mesendoderm induction and patterning during vertebrate embryogenesis. These ligands are believed to transduce the signal through the receptor-activated transcription factors Smad2 and Smad3. However, the roles of smad2/3 genes in development of zebrafish embryos are largely unknown because the presence of multiple smad2/3 genes and their maternal expression have hampered the investigation of their developmental roles. We generated potent and specific dominant-negative forms of zebrafish Smad2, Smad3a, and Smad3b by mutating multiple amino acids. Overexpression of these mutants abolished mesendoderm induction by ectopic Nodal signaling in zebrafish embryos. Expression of dominant-negative smad2/3 abrogated Smad2/3 activities in wild-type embryos and caused various mesendodermal defects similar to those in Nodal-deficient embryos. Smad2/3-deficient cells transplanted into the blastodermal margin of wild-type hosts resulted in more severe mesendodermal defects. Thus, our data reveal that Nodal signaling and mesendoderm induction depend on Smad2/3 and suggest that transforming growth factor-β signals other than Nodal also contribute to Smad2/3 signaling and embryonic patterning.

The formation of the three germ layers is a central event during early embryonic development. Nodal proteins, members of the transforming growth factor-β (TGF-β) superfamily, play crucial roles in mesendoderm induction as well as in dorsoventral and anteroposterior patterning during early development of vertebrate embryos (1–4). Nodal ligands transduce the extracellular signal into intracellular compartments through a mechanism that is common to other TGF-β members. Smad2 and Smad3 are crucial intracellular effectors of Nodal signals (5, 6). Two serine residues in the SXS motif at the C termini of Smad2 and Smad3 are phosphorylated by the activated receptors ALK4 and/or ALK7. Phosphorylated Smad2 and Smad3 associate with the common partner Smad4, and the resulting complex translocates into the nucleus to regulate, in cooperation with other factors, the transcription of target genes.

However, the roles of Smad2 and Smad3 in early development of vertebrate embryos have not been well understood. Genetic studies in mice revealed somewhat controversial results about the function of Smad2 in mesoderm induction. Two groups demonstrated independently that smad2 homozygous mutant mouse embryos fail to undergo gastrulation and lack mesoderm (7, 8), whereas another group reported that the absence of Smad2 causes the entire epiblast to adopt an extraembryonic mesodermal fate (9). Another surprising finding is that smad2-deficient cells with the epiblast of chimeric mouse embryos extensively colonize mesodermal and ectodermal populations (10). Although smad3 knock-out mice develop to term without obvious embryonic defects (11–13), smad2−/−/smad3−/− compound mutant embryos lack anterior axial mesendoderm, implying that smad2 and smad3 may play redundant roles in mesendoderm formation (14). In Xenopus embryos dorsally expressing a dominant-negative mutant (P445H or D450E) of mammalian smad2 or smad3, mesoderm induction still occurs, although the axis is truncated with partial loss of head structures (15), which argues that Smad2/3 activities may not be essential for mesendoderm induction in this species.

The zebrafish genome expresses at least three smad2/3 genes, i.e. smad2, smad3a, and smad3b (16, 17). Their transcripts are abundant in fertilized eggs and throughout blastulation. Even though large-scale mutagenesis screens have been performed in zebrafish (18–20), mutations in smad2/3 genes and their functions have not been reported. Two major impediments hinder the study of the developmental roles of smad2/3 genes by gene knockdown or mutagenesis: (a) the presence of maternal transcripts and proteins that are difficult to remove and (b) the functional redundancy of different smad2/3 genes. In this study, we created dominant-negative mutants of smad2, smad3a, and smad3b, all of which efficiently inhibited TGF-β signaling in vitro. Interference with Smad2/3 activities by overexpressing these mutant forms in zebrafish embryos blocked mesendoderm induction and altered the cell fate of blastodermal marginal cells. Our data

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1 To whom correspondence should be addressed. Tel.: 86-10-6277-2256; Fax: 86-10-6279-4401; E-mail: mengam@mail.tsinghua.edu.cn.

2 The abbreviations used are: TGF-β, transforming growth factor-β; ALK, activin receptor-like kinase; ARE, activin-responsive element; BMP, bone morphogenetic protein; BRE, BMP-responsive element; MO, morpholino oligonucleotide; UTR, untranslated region; hpf, hours post-fertilization; GFP, green fluorescent protein.
indicate that Smad2 and Smad3 play important roles in the mesendoderm by mediating Nodal and non-Nodal signaling during zebrafish embryogenesis.

**EXPERIMENTAL PROCEDURES**

**Fish Embryos**—The Tuebingen and AB strains were used. Embryos were incubated in Holtfreter’s solution at 28.5 °C and staged according to Kimmel et al. (21). Adult oep<sup>−/−</sup> (one-eyed pinhead) fish were obtained using MZoep embryos (which were laid by oep<sup>−/−</sup> females mating with oep<sup>−/−</sup> males) with wild-type oep mRNA. Zoep embryos were generated by crossing oep<sup>+/−</sup> females with oep<sup>−/−</sup> males, and Moep embryos were generated by crossing oep<sup>−/−</sup> females with wild-type males.

**Generation of Dominant-negative Forms of Zebrafish smad2/3**—To generate corresponding dominant-negative alleles, the coding sequences of zebrafish smad2, smad3a, and smad3b were individually amplified by PCR using an upper primer without base substitutes and a lower primer containing base substitutes (underlined below). The primer sequences, which contain an added restriction site, protective bases, and the Kozak sequence (all in lowercase), are as follows: smad2,5′-ggaattcaccATGTCTATTTTACCTTTT-3′ (upper primer) and 5′-ccgctcgagTTAGGGCAACCATGTCAGG-3′ (lower primer); smad3a,5′-ggaattcaccATGTCAATTTTACCTTTT-3′ (upper primer) and 5′-ccgctcgagCTATGCCCGTTAAGGTG-3′ (lower primer); and smad3b,5′-ggaattcaccATGTCAATTTTACCTTTT-3′ (upper primer) and 5′-ccgctcgagTTAGGGCTCACTGGCCCTACTGAGTGAGT-3′ (lower primer). The amplified fragment was digested with EcoRI and XhoI and cloned into the EcoRI/XhoI digested pCMV5-dnsmad3b, respectively. By PCR and subcloned into the pCMV5-HA vector to generate pCMV5-dnsmad3a, ps3a-5′-UTR-GFP, respectively. The DNA of these plasmids was injected alone or co-injected with a corresponding mor- pholin o oligonucleotide.

Whole-mount in Situ Hybridization—Digoxigenin-UTP- or fluorescein-UTP-labeled antisense RNA probes were generated by in vitro transcription. Whole-mount in situ hybridization followed the standard procedure.

**Transplantation**—Embryos, which were used as donors at later stages, were injected between the one- and two-cell stages with 20 ng of dextran tetramethylrhodamine (Molecular Probes) alone or in combination with 600 pg of dominant-negative smad3b mRNA. Donor and host embryos were decorticated by treatment in Holtfreter’s solution containing 6 mg/ml Pronase MS before transplantation. Approximately 20–50 cells were sucked from the animal pole of a donor at the oblong-to-sphere stages and transplanted into the blastodermal margin of wild-type host embryos at the same stage. Locations of transplanted cells in the hosts were determined by fluorescence stereomicroscopy at the shield stage. Distribution of transplanted cells in the hosts was observed and photographed at 24–26 h post-fertilization (hpf) using a Leica MZ16F fluorescence stereomicroscope.

**RESULTS**

Embryos Injected with smad2/3 Morpholino Oligonucleotides Develop without Observable Defects—Zebrafish smad2, smad3a, and smad3b transcripts are present in one-cell embryos, suggesting that these genes are maternally expressed (16, 17). To study their functions during early development of zebrafish embryos, we first tried to use antisense morpholino oligonucleotides (25) to block translation of each smad2/3 gene. We examined the efficacy of these morpholino oligonucleotides by co-injection with a corresponding gene’s 5′-UTR-GFP expression construct and found that smad2-MO1,
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A

ps2-5'UTR-GFP +
ps3a-5'UTR-GFP +
ps3b-5'UTR-GFP +
s2-cMO
s2a-cMO
s2b-cMO
s3a-MO1
s3b-MO1
s3-MO2

B

C

D

E

F

FIGURE 1. Knockdown of smad2, smad3a, or smad3b fails to block mesodermal development. A, the effectiveness of morpholino oligonucleotides was tested using the corresponding 5'-UTR-GFP expression constructs. 90 ng of the 5'-UTR-GFP plasmid DNA was injected alone or with 5 ng of a morpholino oligonucleotide at the one-cell stage, and a group of embryos was photographed during late gastrulation. B–F, wild-type (wt) embryos were injected with morpholino oligonucleotides at the one-cell stage and observed at 24 hpf. For co-injections, different morpholino oligonucleotides were mixed prior to injection. s2, smad2; s3a, smad3a; s3b, smad3b.

smad3a-MO1, smad3b-MO1, and smad3b-MO2 were effective (Fig. 1A). Embryos injected with smad2-MO1, smad3a-MO1, smad3b-MO1, or smad3b-MO2 alone displayed different degrees of growth retardation and neural degeneration (Fig. 1, B–E). (Images for smad3b-MO1 injection are not shown.) When co-injected with smad2-MO1, smad3a-MO1, and smad3b-MO2, embryos showed more severe growth retardation (Fig. 1F). We suspected that these morpholino oligonucleotides-induced phenotypes might be caused by off-targeting via p53 activation (23). Then, we co-injected the smad2/3 morpholino oligonucleotides with a p53 morpholino oligonucleotide. The co-injected embryos showed normal morphology without any apparent mesodermal defects (Fig. 1, C–F). We assume that the inability of smad2/3 knockdown to block mesoderm induction and to cause any developmental defects could be due to the presence of maternal Smad2/3a/3b proteins as well as redundant functions of zygotic smad2/3 products.

Generation of Dominant-negative Alleles of Zebrafish smad2, smad3a, and smad3b—We then developed an alternative approach using dominant-negative forms of Smad2/3 as used previously in Xenopus (15). Previous studies have demonstrated that mammalian Smad2 mutants carrying an S465A and/or S467A mutation within the SXS motif at their C terminus act as dominant-negative forms to block TGF-β signaling by stably binding to the TGF-β type I receptor and by preventing translocation of Smad2/Smad4 complexes into the nucleus (26, 27). The Smad2(P445H) mutant or its equivalent Smad3 mutant also functions as a dominant-negative allele to inhibit TGF-β signaling by preventing recruitment of Smad4 from the cytoplasm into the nucleus to form a transcription activation complex (15). By placing three mutant amino acids corresponding to P445H/S465A/S467A of mammalian Smad2 into a single molecule, we created dominant-negative forms of zebrafish Smad2, Smad3a, and Smad3b (Fig. 2A). Their effects on TGF-β signaling transduction were tested in mammalian Hep3B cells using the (ARE)3-luciferase reporter system, which uses three copies of the ARE in the Mix.2 promoter (28). The expression of this reporter was stimulated by expression of the constitutively active TGF-β receptor ALK4, but this effect was efficiently inhibited by cotransfection of dominant-negative smad2, smad3a, or smad3b (Fig. 2, B–D). Reporter expression was also induced by transfection of zebrafish smad2, smad3a, or smad3b, which was attenuated by cotransfection of dominant-negative smad3b (Fig. 2, E–G) or the other two mutants (data not shown). These results indicate that all three Smad2/3 mutant forms are able to inhibit TGF-β signaling.

BMP signals, which are mediated by Smad1/5/8, also play important roles in early patterning of vertebrate embryos, but their roles differ from those of Smad2/3-mediated TGF-β signals (29, 30). To test whether the Smad2/3 mutant forms would also interfere with BMP signal transduction, we used the BRE-luciferase reporter system, which has BREs in the promoter (31). Induction of BRE-luciferase expression by the constitutively active BMP receptor ALK1 was not disturbed by cotransfection of dominant-negative smad3b (e.g. Fig. 2H) or other mutant alleles (data not shown). These results indicate that the inhibitory effects of the dominant-negative Smad2/3 mutants are restricted to Smad2/3-mediated TGF-β signaling.

Mesoderm Induction by Nodal Signals Absolutely Requires Smad2/3 Activities—Nodal signals play a pivotal role in mesendoderm induction (3). Overexpression of sqt or the constitutively active form (tar*) of acvr1b/taram-a induces ectopic expression of mesoderm and endoderm markers (32, 33). We investigated whether our dominant-negative mutants could block the mesendoderm induction activity of sqt or tar* in vivo. When injected with either 0.5 pg of sqt or 5 pg of tar* mRNA, >90% (n > 30) of the embryos displayed dramatic induction of the expression of the organizer-specific marker goosecoid (gsc) and of the mesoderm marker no tail (ntl) (Fig. 3, A–P). In addition, embryos injected with 5 pg of tar* mRNA also showed induction of the endoderm marker sox32 (Fig. 3, Q–W). When the same amount of sqt or tar* mRNA was co-injected with 600 pg of dominant-negative smad2 or smad3b mRNA, however, none of the embryos exhibited induction of gsc, ntl, or sox32 expression. Compared with wild-type

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embryos, the co-injected embryos had reduced expression of those markers, as did the embryos injected with dominant-negative smad2 or smad3b mRNA alone. Thus, Smad2/3 activities are essential for mesendoderm induction by Nodal signals in zebrafish embryos.

Interference with Smad2/3 Activities Inhibits Development of Mesendodermal Tissues in Wild-type Embryos—Next, we investigated the roles of Smad2/3 in the development of zebrafish embryos by injecting mRNAs encoding dominant-negative Smad2/3 into one-cell stage embryos. Injection with an mRNA encoding any of the dominant-negative Smad2/3 mutant forms (Smad2, Smad3a, and Smad3b) or with a mixture of three mRNAs caused similar embryonic defects at 24 hpf (Fig. 4, A–C). The affected embryos could be morphologically classified into two groups. Embryos in the first group showed closer or fused eyes and a slightly curved notochord (Fig. 4A), resembling ZOep mutants (34, 35). The majority of the affected embryos from single injections could be placed in this group (Fig. 4B). Embryos in the second group lacked the head mesoderm, the notochord, anterior somites, and the endoderm, resembling cyc/sqt (36) and MZOep mutants (37). A much higher proportion of embryos co-injected with dominant-negative smad2, smad3a, and smad3b mRNAs, each at a dose of 600 pg, belonged to the second group, suggesting that these mutant forms have the same effect. As in ZOep or MZOep embryos, the expression of the axial mesoderm/ventral neural tube marker sonic hedgehog (shh) was partially or completely lost (Fig. 4C and data not shown). Furthermore, some of the embryos in both groups also had smaller heads. The zebrafish oep locus encodes a membrane-anchoring protein (35), which acts as the coreceptor for Nodal (3, 38). ZOep mutants have maternal oep mRNA but are unable to zygotically express oep mRNA, resulting in an insufficient amount of Nodal signals during early embryogenesis (37). MZOep mutants lack maternal as well as zygotic wild-type oep mRNA and thus are deficient in Nodal signaling (37). The similarity between dominant-negative smad2/3-induced and MZOep phenotypes suggests that Smad2/3 activities are required for maternal and zygotic Nodal signaling in mesendoderm induction.

To further test the specificity of dominant-negative Smad2/3 in vivo, dominant-negative smad3b mRNA was co-injected with smad3a, smad3b, or smad2 to see if dominant-negative smad3b-induced defects could be rescued. As demonstrated previously (16, 17, 39, 40), overexpression of smad3a and smad3b caused embryonic dorsalization, which was characterized by a smaller trunk and tail at 24 hpf, whereas smad2 overexpression had no visible effect on embryonic development. As shown in Fig. 4D, none of the co-injected embryos showed MZOep-like phenotypes, and the ratio of ZOep-like embryos dramatically dropped. On the other hand, the ratio of dorsalized embryos induced by smad3b or smad3a overexpression decreased when co-injected with dominant-negative smad3b. These effects were confirmed by examination of shh expression (Fig. 4E). Taken together, these data suggest that dominant-negative smad3b (and dominant-negative smad2/3a) specifically inhibits Smad2/3 functions in vivo.

To confirm and extend the effect of dominant-negative Smad2/3 on mesendoderm induction, we examined a set of specific markers by whole-mount in situ hybridization following injection with 600 pg of dominant-negative smad3b mRNA. As shown in Fig. 5 (A–B), the expression of the shield-specific markers gsc and floating head (flh) was reduced or eliminated in the injected embryos at the shield stage. The injected embryos at the bud stage were missing
the shield-derived anterior axial mesodermal (polster) that was labeled by *hgg1* expression (Fig. 5, C and C'). *ntl* is expressed at the shield stage throughout the germ ring, in which cells give rise to mesodermal and endodermal tissues at later stages (Fig. 5D), and its expression in the dorsal germ ring was apparently eliminated in dominant-negative *smad3b*-injected embryos (Fig. 5D'). Accordingly, the expression of the endoderm markers *sox17* and *sox32/casanova* was inhibited during mid-gastrulation in the injected embryos (Fig. 5, E–F'). Dominant-negative *smad3b*-induced type II (MZoep-like) embryos did not express *shh* in the ventral brain and anterior notochord at 26 hpf (Fig. 5, G and G'), and they did not express *myoD* in the head mesoderm at 48 hpf (H and H'). Furthermore, the ventral mesoderm marker *vent* showed reduction at the shield stage (Fig. 5, I and I'), and the ventral marker *gata1*, which labels hematopoietic progenitors, was also reduced at 24 hpf (I' and I''), suggesting that development of ventral mesodermal tissues also requires Smad2/3 activities. We conclude that mesendodermal development absolutely requires Smad2/3 functions. In contrast, the ventral ectoderm marker *gata2*, which is activated by ventral BMP signals, showed expansion (data not shown),
implying that Smad2/3 activities play a role in antagonizing the epidermis-inducing activity of BMP signaling.

Smad2/3-abrogated Cells in the Blastodermal Margin Differentiate Predominantly into Neural Tissues Instead of Mesendodermal Tissues—Previous studies have demonstrated that a deficiency in Nodal signaling in mouse embryos results in excessive embryonic ectoderm at the expense of mesoderm (41, 42) and in zebrafish leads to transformation of dorsal mesoderm progenitors into neural fates (36, 43, 44). We wondered whether the interference with Smad2/3 functions similarly leads to change in the mesodermal fate of cells in the blastodermal margin of zebrafish embryos. To address this question, we transplanted labeled cells from the animal pole area of a donor embryo at the oblong-dome stages into the blastodermal margin of a wild-type host embryo. Donor embryos were either wild-type or injected with 600 pg of dominant-negative smad3b mRNA. We found that, at 24 hpf, donor wild-type cells gave rise predominately to mesendodermal cells, including primarily pharyngeal endoderm, notochord, muscle, and blood cells as well as some ectodermal cells (Fig. 6, E–G). For example, 40.9 and 81.8% (n = 22) of host embryos had labeled wild-type cells in the notochord and muscle, respectively. The transplanted wild-type cells also differentiated into neurons and epidermal cells in 45.5% of the host embryos, which were located mainly in the head region, with fewer in the trunk region. In contrast, dominant-negative smad3b-injected cells contributed to the neural tube in 75% (n = 40) of the host embryos, with a large number of injected cells in the head or trunk region or throughout the neural tube (Fig. 6, H–J). We found only 1 of 40 embryos that had two dominant-negative smad3b-injected cells integrated into the notochord. In addition, 32.5% of the host embryos had labeled muscle cells, most of which appeared in the posterior trunk. Taken together, these data suggest that when Smad2/3 activities are inhibited, blastodermal marginal cells preferentially commit to neural fate instead of mesendodermal fate. Thus, Smad2/3 activities are essential for mediating mesendoderm-inducing signals such as Nodal signals.

Interference with Smad2/3 Activities in Zoep and Moep Mutants Leads to Loss of More Mesodermal Tissues—Zoep mutants are missing the anterior axial mesoderm, endoderm, and floor plate, but have a notochord and paraxial mesoderm (34). It is likely that, in Zoep mutants, Smad2/3 could be activated by Nodal signaling in the presence of maternal Oep and that activated Smad2 and Smad3 are sufficient for formation of the trunk mesoderm. We thus expected that overexpression of dominant-negative smad2/3 deteriorates defects in Zoep mutant embryos. We tested this hypothesis by injecting 600 pg of dominant-negative smad3b mRNA into one-cell progeny of oep+/− females and oep−/− males. Of the un.injected embryos used in this experiment, about half (47.4%, n = 95) were Zoep mutants, and the remaining were wild type. At 24 hpf after injection, 48% (n = 177) of the embryos had $M_{\text{Zoep}}$-like phenotypes; 32.2% showed Zoep-like phenotypes; and 19.8% appeared normal morphologically (Fig.

FIGURE 4. Overexpression of dominant-negative smad2/3 produces phenotypes resembling Nodal-deficient mutants in zebrafish. A, shown is the morphology of representative 24-hpf live embryos injected with dominant-negative smad2/3 mRNAs. B, shown are the ratios of embryos with different morphological phenotypes. The doses for dominant-negative (dn) smad2, smad3a, and smad3b were 550, 700, and 600 pg, respectively. Mixed RNAs means a mixture of dominant-negative smad2, smad3a, and smad3b mRNAs, each at a dose of 600 pg. C, shown are the ratios of embryos based on shh expression. The injected embryos were examined after morphological observation for shh expression at 24 hpf by whole-mount in situ hybridization. D and E, dominant-negative smad3 (600 pg)-induced phenotypes were rescued by overexpression of smad2 (350 pg), smad3a (50 pg), or smad3b (50 pg), as justified by morphological observation (D) and shh expression examination (E). Note that whereas smad2 overexpression had no effect, smad3a or smad3b overexpression led to dorsalization (not shown). The number of observed embryos is indicated in parentheses. WT, wild type.
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FIGURE 5. Interference with Smad2/3 activities in zebrafish embryos inhibits expression of mesendoderm markers. A–F and G–J, uninjected embryos. A'–F' and G'–J', embryos injected with 600 pg of dominant-negative (dn) smad3b mRNA. All of the examined mesendoderm markers showed eliminated or reduced expression. These markers included the organizer-specific markers gsc and flh at the shield stage, the prechordal plate mesoderm marker hgg1 at the bud stage, the pan-mesoderm marker ntl at the shield stage, the endoderm markers sox17 and sox32 at the 75% epiboly stage, the axial mesoderm and ventral brain marker shh at 24 hpf, the head muscle marker myoD at 48 hpf, the ventral mesoderm marker vent at the shield stage, and the hematopoietic marker gata1 at 24 hpf. Dorsal views are shown for gsc, flh, sox17, and sox32; animal pole views are shown for ntl and vent; and lateral views are shown for the others.

FIGURE 6. Smad2/3-deficient cells in the blastodermal margin preferentially differentiate into neural tissues. Cells from the labeled donor embryo were transplanted into different locations of the blastodermal margin of wild-type host embryos at the oblong-sphere stages. The locations of donor cells were observed at the shield stage. The donor-derived progenitors (red) were examined at 24–26 hpf by fluorescence microscopy. A–D, examples of locations of transplanted cells in host embryos at the shield stage. Embryos were viewed from the animal pole with the shield on the right. The locations of transplanted cells were as follows: dorsal (A), dorsolateral (B), lateral (C), and ventral (D). E–G, differentiation of transplanted wild-type cells in 1-day embryos (anterior to the left). E, transplanted cells differentiated into the head endoderm (white arrowhead), ventral brain (white arrow), notochord (yellow arrows), and neurons in the trunk (yellow arrowheads). F, transplanted cells differentiated mainly into muscle (green arrows) and notochord cells (yellow arrows). G, transplanted cells were located primarily in posterior somites and neural tube. H–J, differentiation of transplanted dominant-negative smad3b-injected cells in 1-day embryos (anterior to the left). The dominant-negative smad3b-injected cells contributed preferentially to neural tissues (white arrows). Note that not all labeled cells are indicated. For the same host embryo, the fluorescent image was superimposed on the bright-field image.

7, A–C). To confirm the effect, the injected embryos were examined for shh expression by whole-mount in situ hybridization. We noted that 30.8% (n = 52) of the injected embryos were missing shh expression in the ventral brain and in most of the notochord, a phenotype typical of MZoep mutants; 38.5% of the embryos lacked the ventral brain domain of shh expression, as did Zoep mutants; and the other had a normal shh expression pattern (Fig. 7C). Thus, compared with dominant-negative smad3b injection into wild-type embryos, the ratio of MZoep-like embryos markedly increased when Zoep embryos were injected with dominant-negative smad3b. We suggest that the amount of activated Smad2/3 in Zoep embryos is less and that interference with dominant-negative smad3b transforms Zoep embryos into MZoep embryos more efficiently.

We took another approach to test the importance of Smad2/3 activity in mesendoderm induction. By crossing oep<sup>−/−</sup> females with wild-type (oep<sup>+</sup>/+) males, we obtained a genetically homogeneous Moeop population. Although these embryos lack maternally supplied oep mRNA or its protein product, they do not have detectable abnormalities (37). When injected with 600 pg of dominant-negative smad3b mRNA, 43.5% (n = 69) of the embryos showed MZoep-like morphology, with partial loss of the head at 24 hpf; 39.1% of the embryos were Zoep-like; and only 17.4% of the embryos looked normal (Fig. 7, D and E). These phenotypes were confirmed by shh expression (Fig. 7F). Apparently, interference with Smad2/3 activities by dominant-negative smad3b induced a higher ratio of MZoep- and Zoep-like embryos from Moeop embryos than from wild-type embryos, suggesting that Moeop embryos have less amounts of Smad2/3 activities.

Smad2/3 Proteins May Mediate Non-Nodal Signaling in Mesoderm Induction—To test whether Smad2/3 might have functions in addition to Oep-dependent TGF-β/Nodal signals, we investigated whether overexpression of dominant-negative smad3b in MZoep embryos worsens their anomalies. We obtained MZoep embryos by mating oep<sup>−/−</sup> females with oep<sup>−/−</sup> males. The dominant-negative smad3b-injected MZoep embryos had a shortened trunk with 8.5 somites on average (n = 40), whereas un.injected MZoep embryos from the same batch had 16.4 somites (n = 30) (Fig. 7, G and H). This phenomenon was also observed in MZoep mutant embryos treated with the small compound SB431542 (39), which specifically inhibits ALK4/5/7-mediated TGF-β signaling. Our results support the idea that Oep-independent non-Nodal signals mediated by Smad2/3 play a role in mesoderm induction, particularly in posterior mesoderm formation.
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**FIGURE 7. Overexpression of dominant-negative smad2/3 deteriorates abnormalities in Oep-deficient embryos.** A–C, dominant-negative (dn) smad3b mRNA (600 pg) was injected into one-cell stage Zoep mutant embryos, which were produced by crossing oep<sup>−/−</sup> females with oep<sup>−/−</sup> males. A, live embryos at 24 hpf, representing three types. B, ratios of different types of embryos based on morphology. C, ratios of different types of embryos based on shh expression. Compared with injection into wild-type (WT) embryos, more Zoep-like embryos were seen. Embryos that did not have recognizable structures or deform before observation were not included for statistics. D–F, dominant-negative smad3b mRNA (600 pg) was injected into one-cell stage MZoep embryos, which were produced by crossing oep<sup>−/−</sup> females with wild-type (oep<sup>+/+</sup>) males and usually do not develop visible defects. The injection resulted in a high ratio of Zoep- and MZoep-like embryos, suggesting an important role of maternal Smad2/3. G and H, dominant-negative smad3b mRNA (600 pg) was injected into one-cell stage MZoep embryos, which were produced by crossing oep<sup>−/−</sup> females and oep<sup>−/−</sup> males. G, morphology of live embryos at 24 hpf. H, number of somites in uninjected and injected MZoep embryos. The dominant-negative smad3b-injected MZoep embryos had fewer numbers of somites, suggesting that Smad2 and Smad3 mediate Oep-independent signaling in mesendoderm induction.

**DISCUSSION**

In this study, we generated potent and specific dominant-negative alleles of zebrafish smad2, smad3a, and smad3b. Using these mutants, we demonstrated that Smad2/3 activities are required for mesendoderm induction and that Nodal signals exert their biological effects during embryonic development depending on Smad2/3 activities.

It appears that injection with dominant-negative smad3b mRNA gives rise to more MZoep-like embryos than injection with dominant-negative smad2 or smad3a mRNA (Fig. 4B). However, dominant-negative smad3b overexpression in mammalian cells also inhibits TGF-β signaling more efficiently than dominant-negative smad2 and smad3a (Fig. 2). The reason for the greater potency of dominant-negative Smad3b is not known. We are unable to speculate the relative importance of different smad2/3 genes for embryonic development in zebrafish. Nevertheless, the facts that smad2, smad3a, and smad3b mRNAs are all present in large amounts during early stages of embryonic development (16, 17) and that overexpression of their wild-type mRNAs can rescue mesendodermal defects induced by dominant-negative smad3b make us believe that these smad2/3 genes play redundant functions in zebrafish embryos.

Like zebrafish sqt/cyc double mutants (36), MZoep embryos are able to develop posterior mesodermal tissues (37). These observations suggest that other inducing signals are required for posterior mesoderm formation. We found that interference with Smad2/3 activities in MZoep embryos could further reduce the number of somites as observed at 24 hpf. It is likely that signals for posterior mesoderm induction, which may be other signals rather than Nodal signals, require Smad2/3 activities for their biological functions. One candidate could be derriere, a member of the TGF-β superfamily. In Xenopus, derriere plays a crucial role in mesendodermal patterning and development of posterior regions (45). However, the zebrafish ortholog of Xenopus derriere has yet to be identified. Although BMP and zygotic Wnt signals have been found to play important roles in the development of ventral/tail mesodermal tissues (46, 47), we could exclude the possibility that Smad2 and Smad3 mediate such functions of these signals because dominant-negative smad2/3 failed to block BMP signaling (see above) or β-catenin-induced bozozok expression (data not shown). The fact that interference with Smad2/3 activities or smad3b overexpression in MZoep embryos is still able to influence neural induction and patterning also supports the idea that Smad2/3 activities mediate non-Nodal signals in neural development.

The dominant-negative forms of smad2/3, which harbor three mutant residues, specifically and efficiently inhibit
Smad2/3 activities. We also made mutant forms of zebrafish Smad3b by mutating the two serine residues within the SXS motif at its C terminus or by substituting Pro401 with histidine only. These two mutant forms were much less effective in blocking mesendoderm induction of zebrafish embryos (data not shown). Dominant-negative Smad2/3 with three mutant residues may be used to investigate the tissue- or organ-specific roles of Smad2/3 activities through inducible expression systems, such as the heat shock-inducible (48) or GAL4/UAS-inducible (49) system.

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