The zebrafish sensory posterior lateral line (pLL) has become an attractive model for studying collective cell migration and cell morphogenesis. Recent studies have indicated that chemokine, Wnt/β-catenin, Fgf, and Delta-Notch signaling pathways participate in regulating pLL development. However, it remains unclear whether TGFβ signaling pathway is involved in pLL development. Here we report a critical role of TGFβ1 in regulating morphogenesis of the pLL primordium (pLLP). The tgfβ1a gene is abundantly expressed in the lateral line primordium. Knockdown or knockout of tgfβ1a leads to a reduction of neuromast number, an increase of inter-neuromast distance, and a reduced number of hair cells. The aberrant morphogenesis in embryos depleted of tgfβ1a correlates with the reduced expression of atoh1a, deltaA, and n-cadherin/cdh2, which are known important regulators of the pLLP morphogenesis. Like tgfβ1a depletion, knockdown of smad5 that expresses in the pLLP, affects pLLP development whereas overexpression of a constitutive active Smad5 isoform rescues the defects in embryos depleted of tgfβ1a, indicating that Smad5 mediates tgfβ1a function in pLLP development. Therefore, TGFβ/Smad5 signaling plays an important role in the zebrafish lateral line formation.

Keywords: zebrafish, lateral line, neuromast, TGFβ1a, Smad5

Introduction

The lateral line, a mechanosensory system existing in fish and some amphibian, is involved in several behaviors including water movement detection, prey detection, predator avoidance, and so on (Coombs and Van Netten, 2005). The zebrafish lateral line system is composed of two major branches, the anterior lateral line (aLL) and posterior lateral line (pLL). The pLL primordium (pLLP) arises from a group of cells just posterior to the otic placode and begins to migrate caudally along the horizontal myoseptum at ~20 h postfertilization (hpf) (Metcalfe et al., 1985; Glysen and Dambly-Chaudiere, 2007; Aman and Piotrowski, 2011; Valdivia et al., 2011). Within the pLLP, cells in the trailing zone are organized into center-oriented epithelial rosettes with hair cell progenitors at the center (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Aman and Piotrowski, 2009). During pLLP migration, the rosettes are sequentially deposited as proneuromasts at regular intervals, ultimately generating five or six primary neuromasts along the trunk and two or three terminal neuromasts at the tip of the tail (Gompel et al., 2001).

The pLL formation is a coordinated process of cell migration, deposition, and maturation (Raible and Kruse, 2000; Gompel et al., 2001; Aman and Piotrowski, 2011; Chitnis et al., 2012). This process is regulated by cooperation of distinct signaling pathways, including chemokine, Wnt/β-catenin, Fgf, and Delta-Notch signaling pathways (Aman and Piotrowski, 2009, 2011; Chitnis et al., 2012). For example, chemokine signaling pathway is important in regulating the pLLP migration. Knockdown of its ligand cxcl12a or its receptor cxcr4b or cxcr7b leads to strong migration defects (David et al., 2002; Li et al., 2004; Haas and Gilmour, 2006; Dambly-Chaudiere et al., 2007; Valentin et al., 2007; Dona et al., 2013; Venkiteswaran et al., 2013). In addition, Wnt/β-catenin and Fgf signaling feedback system is essential for pLLP morphogenesis (Aman and Piotrowski, 2008, 2009, 2011; Chitnis et al., 2012). Loss of lef1 function reduces cell proliferation and results in closer neuromast deposition and premature termination of the pLLP (Gamba et al., 2010; McGraw et al., 2011; Valdivia et al., 2011; Matsuda et al., 2013). Fgf3/10 signals localized in the leading zone of the pLLP are necessary for primordium migration, proneuromast formation and deposition (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Breau et al., 2012). Another important aspect of the pLLP morphogenesis is neuromast maturation, during which cells within a proneuromast are epithelialized to become a rosette. It has been demonstrated that Fgf and DeltaA ligands are essential to induce and restrict the expression of...
atoh1a in the central cells of the rosette, which are then specified as hair cell progenitors (Itoh and Chitnis, 2001; Chitnis et al., 2012). The maturation of epithelial rosettes contributes to the proper pLLP morphogenesis and neuromast deposition (Nechipour and Raible, 2008; Matsuda and Chitnis, 2010; Matsuda et al., 2013).

Transforming growth factor-β (TGFβ) superfamily comprises various TGFβ, bone morphogenetic protein (BMP), Nodal, Activin, and other related protein ligands. Among these TGFβ ligands, Nodal and BMPs have been demonstrated to play important roles in germ layer formation and patterning in vertebrate embryos (Tian and Meng, 2006; Langdon and Mullins, 2011; Jia et al., 2012; Liu et al., 2013; Xu et al., 2014; Xue et al., 2014). There are three different TGFβ ligands, TGFβ1, TGFβ2, and TGFβ3 in mammals. These mature TGFβ ligands usually bind to and activate the receptors TβRII and TβRI (ALK5), and the activated ALK5 further phosphorylates the intracellular effectors Smad2 and Smad3 (Derynck and Zhang, 2003; Shi and Massague, 2003; Massagué, 2012). However, TGFβ ligands can also transduce the signal through ALK1, ALK2, or ALK3 to the effectors Smad1 and Smad5 in certain cell types (Miettinen et al., 1994; Oh et al., 2000; Daly et al., 2008; Finnson et al., 2008; Park et al., 2008; Massagué, 2012). Therefore, TGFβ signaling plays various roles in development and carcinogenesis through distinct mechanisms (Padua and Massagué, 2008; Akhurst and Hata, 2012; Massagué, 2012; Pickup et al., 2013). Nevertheless, it is unclear whether TGFβ ligands are implicated in the formation of the lateral line system.

In this study, we disclose a novel function of TGFβ signaling in regulating the pLLP morphogenesis. By knocking down tgfβ1a expression with its specific morpholinos or generating tgfβ1a(45)145 mutant with TALENs approach, we show that loss of TGFβ1a signaling results in the disrupted lateral line system with decreased neuromast number and increased inter-neuromast distance. TGFβ1a positively regulates the expression of n-cadherin/cdh2, deltaA, and atoh1a in pLLP and affects pLLP migration, maturation, and deposition. Mechanistically, function of TGFβ1a in pLLP development is mediated by its downstream effector Smad5.

Results

**tgfβ1a is expressed in zebrafish lateral line system**

We have been interested in the developmental functions of different TGFβ ligands in the zebrafish embryo. According to ZFIN (http://zfin.org), four tgfβ genes, tgfβ1a, tgfβ1b, tgfβ2, and tgfβ3, have been annotated. tgfβ1a is the only one that expresses in the lateral line system (Figure 1A and B, and Supplementary Figure S1A), while the other three are undetectable in the primordium of pLL (Supplementary Figure S1B–D). We then focused on tgfβ1a. A previous study indicated that tgfβ1a transcripts are present in the immature oocytes (Kohli et al., 2003). We found that tgfβ1a transcripts were undetectable before early somitogenesis by in situ hybridization (Figure 1A), and therefore, tgfβ1a may not function during early embryonic development. At the 10-somite stage, tgfβ1a was expressed in the telencephalon and otic placodes. Its transcripts were detected in the pLLP and enriched in the central cells around 24 hpf and in all neuromasts of the lateral line system by 48 hpf (Figure 1A and B). The expression of tgfβ1a in the pLLP suggests a potential role in lateral line development.

**tgfβ1a knockdown reduces neuromast numbers and increases inter-neuromast distance**

In order to investigate the function of tgfβ1a in pLL formation, two morpholinos, named tgfβ1a-MO1 and tgfβ1a-MO2, were designed to target different regions surrounding the translation start site of tgfβ1a mRNA. The effectiveness of these two morpholinos was confirmed by their ability to block the expression of the corresponding 5’UTR-GFP reporters (Figure 1C). Next, we investigated tgfβ1a function with Tg(−8.0cldnb:lynEGFP) transgenic line, in which GFP is expressed in the lateral line primordium and neuromasts as previously reported (Haas and Gilmour, 2006) (Figure 1D and E). In std-MO injected control embryos, the pLLP migrated and deposited an average of 5.58 neuromasts (n = 31) at regular intervals along the horizontal myoseptum, and stopped at the tip of the tail with two or three terminal neuromasts at 2 day postfertilization (dpf). In the Tg(−8.0cldnb:lynEGFP) transgenic embryos injected with 5 ng tgfβ1a-MO1 or 0.5 ng tgfβ1a-MO2 alone, the number of deposited pLL neuromasts was reduced to 3.42 and 3.23 on average, respectively, and the distance between neuromasts was increased obviously at 2 dpf. Like control embryos, tgfβ1a morphants had two or three terminal neuromasts, suggesting that the pLLP is able to migrate to the tail tip eventually in the absence of TGFβ1a. To exclude the general off-target effect of morpholinos due to p53 activation (Robu et al., 2007), we co-injected p53-MO with tgfβ1a-MO2. Co-injected embryos still had fewer neuromasts than control embryos (Figure 1D and E), implying that the defects may not be caused by p53-induced apoptosis. To further substantiate the specific function of tgfβ1a in pLL formation, we examined the rescuing effect of tgfβ1a overexpression in morphants. Although tgfβ1a overexpression by injecting a high dose (500 pg) of tgfβ1a mRNA at the one-cell stage resulted in embryonic dorsoventral patterning defects (data not shown), injecting a low dose (2.5 pg) of tgfβ1a mRNA neither caused any morphological defects (data not shown) nor induced more neuromasts (Figure 1D). When embryos were co-injected with 2.5 pg tgfβ1a mRNA and 0.5 ng tgfβ1a-MO2, the trunk neuromast number was recovered to 4.49 (n = 59) and the inter-neuromast distance also appeared normal (Figure 1D and E), indicating a specific role of tgfβ1a in pLL development.

We further defined the positions of neuromasts relative to the somite positions along the trunk at 2 dpf. As illustrated in Figure 1F, tgfβ1a knockdown caused a posterior shift in neuromast distribution compared with the control, which could be rescued by co-injection of tgfβ1a mRNA. Taken together, these data suggest that tgfβ1a is implicated in the primary lateral line system formation.

**tgfβ1a knockdown impairs the pLLP migration and deposition**

To understand how the neuromast number is reduced with an increase of the inter-neuromast distance in tgfβ1a morphants, we investigated pLLP migration and neuromast deposition at different stages, neuromast number was significantly reduced (Figure 2A, B, C, and D). The results were in agreement with the co-injection experiments by co-injecting tgfβ1a mRNA and a low dose of tgfβ1a-MO2 (Figure 2E and F). To investigate the mechanism of tgfβ1a knockdown impairs the pLLP migration and deposition, we investigated the effect of tgfβ1a on pLLP migration and deposition in vivo. To this end, we injected tgfβ1a-MO at the one-cell stage and collected embryos at 40 hpf (Figure 2G). We found that tgfβ1a-MO injected embryos had fewer neuromasts than control embryos. Furthermore, the pLLP deposition was delayed and affected at 40 hpf (Figure 2G). To further investigate whether tgfβ1a knockdown impairs the pLLP migration and deposition is mediated by Smad5, we injected Smad5-MO at the one-cell stage and observed the neuromast number and the location of the pLLP at hpf (Figure 2G). We found that Smad5-MO injected embryos had more neuromasts than control embryos. Furthermore, the pLLP migration and deposition were significantly delayed and affected at 40 hpf (Figure 2G). Together, these results suggest that tgfβ1a knockdown impairs the pLLP migration and deposition is mediated by Smad5.

** tgβ1a knockdown impairs the pLLP migration and deposition**

To understand how the neuromast number is reduced with an increase of the inter-neuromast distance in tgfβ1a morphants, we investigated pLLP migration and neuromast deposition at different stages, neuromast number was significantly reduced (Figure 2A, B, C, and D). The results were in agreement with the co-injection experiments by co-injecting tgfβ1a mRNA and a low dose of tgfβ1a-MO2 (Figure 2E and F). To investigate the mechanism of tgfβ1a knockdown impairs the pLLP migration and deposition, we investigated the effect of tgfβ1a on pLLP migration and deposition in vivo. To this end, we injected tgfβ1a-MO at the one-cell stage and collected embryos at 40 hpf (Figure 2G). We found that tgfβ1a-MO injected embryos had fewer neuromasts than control embryos. Furthermore, the pLLP deposition was delayed and affected at 40 hpf (Figure 2G). To further investigate whether tgfβ1a knockdown impairs the pLLP migration and deposition is mediated by Smad5, we injected Smad5-MO at the one-cell stage and observed the neuromast number and the location of the pLLP at hpf (Figure 2G). We found that Smad5-MO injected embryos had more neuromasts than control embryos. Furthermore, the pLLP migration and deposition were significantly delayed and affected at 40 hpf (Figure 2G). Together, these results suggest that tgfβ1a knockdown impairs the pLLP migration and deposition is mediated by Smad5.
time points. First, after in situ hybridization for eya1 expression in injected embryos, we quantified the migration rate of the pLLP as the ratio of its migrated distance (MD, the distance from the caudal edge of the otic vesicle to the caudal edge of the pLLP) to the whole trunk distance (WD, the distance from the caudal edge of the otic vesicle to the tail terminus) (Figure 2A). The pLLP migration rate in tgfβ1a morphants at all four examined stages was significantly lower than that in the control embryos (Figure 2B). This observation suggests that the pLLP migrates slower in the morphants and the increased inter-neuromast distance is not caused by a faster migration of the pLLP. Then, we performed a time-lapse examination, by confocal microscopy, of the

Figure 1 tgfβ1a is required for zebrafish lateral line development. (A and B) Detection of tgfβ1a transcripts by whole-mount in situ hybridization at indicated stages. Images shown are lateral views. otp, otic placode; tb, tail bud; te, telencephalon; nm, neuromast (indicated by arrows). The pLLP is outlined by dotted lines at a high magnification in B. (C) Effectiveness of tgfβ1a-MO1 and tgfβ1a-MO2. The reporter tgfβ1a-5’UTR-GFP DNA plasmid DNA was co-injected with 5 ng std-MO, tgfβ1a-MO1, or tgfβ1a-MO2 at one-cell stage and the injected embryos were observed for GFP expression at late gastrulation stages. (D) Effect of tgfβ1a knockdown on lateral line formation. Tg(-8.0cldnb:lynEGFP) transgenic embryos at the one-cell stage were injected with indicated reagents and examined at 2 dpf under a dissection fluorescence microscopy for GFP-positive neuromasts. Red arrows indicate trunk neuromasts; yellow chevrons indicate terminal neuromasts. The representative embryos are laterally viewed with anterior to the left. (E) The average trunk neuromast number. n, the number of observed embryos. ***P < 0.001. (F) Illustration of the axial positions of L1–L6 neuromasts relative to somite positions in embryos injected with indicated reagents. Scale bar, 200 μm for the whole embryo in A and D; 40 μm for the pLLP in B; 500 μm in C.
pLLP migration in *Tg(-8.0cldnb:lynEGFP)* during earlier migration stages. As shown in Figure 2C and Supplementary Movie S1, the observation of primordium migration in control embryos began at 23 hpf (0 min), and the first proneuromast L1 was deposited successfully from a group of concerted slowing down cells at the trailing edge of the primordium within 180 min, which was consistent with others’ observations (Gompel et al., 2001; Haas and Gilmour, 2006; Nechiporuk and Raible, 2008). Then we focused on the primordium migration and deposition in *tgfβ1a* morphants. Results revealed that not only the migration rate was much slower, but the first rosette (r1) was also not deposited properly to form proneuromast L1 by 300 min in *tgfβ1a* morphants (Figure 2C and Supplementary Movie S2). It seemed that the cells of the rosette r1 in the morphants were slowing down relative to the other rosettes at 180 min and almost separating from the other cells in pLLP at 210 min, but they integrated to the primordium again to continue migrating at 270 min and failed to be deposited until 300 min. From the above observations, we conclude that *tgfβ1a* activity is required for normal migration and deposition of the pLLP, as well as the neuromast formation.

*tgfβ1a* may promote pLLP cell cohesion by positively regulating *n-cadherin/cdh2* expression

It has been shown that chemokine signaling promotes pLLP migration, which largely depends on the expression of its ligand *cxcl12a* along the presumptive migratory path and the polarized expression of its receptors *cxcr4b* in the leading zone and *cxcr7b* in the trailing zone (Haas and Gilmour, 2006; Dambly-Chaudiere et al., 2007; Valentin et al., 2007; Dona et al., 2013; Venkiteswaran et al., 2013). Because *tgfβ1a-MO2* injection leads to primordium migration defects, we set to verify whether it acts through chemokine signaling pathway or not. By *in situ* hybridization, we found that the expression patterns of *cxcl12a*, *cxcr4b*, and *cxcr7b* in *tgfβ1a* morphants were similar to those in control embryos (Figure 3A). Thus, we speculated that the *tgfβ1a* in pLLP may not function to regulate chemokine gene expression.
Since several cadherin proteins including E-cadherin/Cdh1, N-cadherin/Cdh2, R-cadherin/Cdh4, and Cdhl6 may contribute to cohesive interactions between cells within the pLLP (Liu et al., 2003; Kerstetter et al., 2004; Wilson et al., 2007), we wondered whether their expression is altered in tgfβ1a morphants. Whole-mount in situ hybridization results showed that cdh1, cdh4, and cdh6 expression in the pLLP were almost unchanged (Figure 3B), while cdh2 expression was remarkably reduced in

Figure 3 Knockdown of tgfβ1a downregulates cdh2 but not chemokine genes. (A) The expression patterns of cxc12a, cxcr4b, and cxcr7b in tgfβ1a morphants. Wild-type embryos were injected with 0.5 ng std-MO or tgfβ1a-MO2 at the one-cell stage and fixed at 28 hpf for in situ hybridization. Embryos were orientated with anterior to the left. Note that these markers appeared unaffected in tgfβ1a morphants. (B) The expression patterns of cdh1, cdh4, and cdh6 were unaltered in the pLLP of tgfβ1a morphants. Embryos were harvested at 28 hpf for in situ hybridization and laterally viewed with anterior to the left. Note that the expression patterns of cdh1, cdh4, and cdh6 in the pLLP (indicated by arrowheads) of tgfβ1a morphants were comparable to those in control embryos. (C) cdh2 expression was positively regulated by tgfβ1a. Wild-type embryos were injected with 0.5 ng std-MO or tgfβ1a-MO2, alone or together with 2.5 pg tgfβ1a mRNA, at the one-cell stage and fixed at 28 hpf for examining cdh2 expression by in situ hybridization. Embryos were laterally viewed with anterior to the left. The ratio of embryos with exhibited pattern is indicated. (D and E) Knockdown of cdh2 led to the pLL developmental defects. Tg(-8.0cldnb:lynEGFP) transgenic embryos were injected with 5 ng std-MO or cdh2-MO at the one-cell stage and examined by fluorescence microscopy at 2 dpf. Arrows indicate pLL neuromasts. The average neuromast numbers are shown in E with examined embryo numbers indicated below. ***P < 0.001. Scale bar, 200 μm for the whole embryo in A, B, and D; 40 μm for the pLLP in A and C.
the pLLP of tgfβ1a morphants at 28 hpf (Figure 3C). Furthermore, the reduction of cdh2 expression in tgfβ1a morphants could be partially rescued by 2.5 pg tgfβ1a mRNA co-injection (Figure 3C). Like tgfβ1a knockdown, cdh2 knockdown also led to the lateral line defects (Figure 3D and E, Kerstetter et al., 2004). Thus, we propose that tgfβ1a may regulate pLLP migration in part by maintaining cdh2 expression and then controlling cell–cell adhesion.

**tgfβ1a may control proneuromast maturation and deposition by regulating deltaA expression**

The migrating pLLP consists of 3–4 proneuromasts at various stages of maturation. The patterning of these proneuromasts is regulated by at least two important signaling feedback centers, involving Wnt/β-catenin–Fgf system for leading zone–trailing zone patterning and Fgf–Delta system for the hair cell–support cell patterning (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Gamba et al., 2010; Matsuda and Chitnis, 2010; McGraw et al., 2011; Valdivia et al., 2011). The relative enrichment of tgfβ1a transcripts in the small groups of cells located at the center of forming proneuromasts (Figure 1B) suggests a role in the patterning process of the primordium. To investigate the possibility of tgfβ1a in regulating primordium patterning, we looked at several molecular markers with specific expression domain in the pLLP, including cxcr4b, cxcr7b, lef1, axin2a, fgf3, fgf10a, and pea3 (Matsuda and Chitnis, 2010; McGraw et al., 2011). In situ hybridization indicated that the expression of cxcr7b and pea3 in the trailing zone and cxcr4b, lef1, axin2a, fgf3, and fgf10a in the leading zone appeared unaltered in tgfβ1a morphants at 28 hpf (Figures 3A and 4A), suggesting that tgfβ1a does not take part in the leading zone–trailing zone patterning of the primordium.

Each proneuromast forms a center-oriented epithelial rosette with central cells committed to the hair cell fate (Lecaudey et al., 2008; Matsuda and Chitnis, 2010). It has been demonstrated that Fgf signaling from the leading zone is crucial to induce deltaA expression in the central cell, and deltaA restricts atoh1a expression in the center cell for hair cell specification (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Matsuda and Chitnis, 2010). Once atoh1a expression is stabilized, it drives expression of fgf10a and establishes a second atoh1a-dependent Fgf signaling system.

**Figure 4 tgfβ1a knockdown inhibits hair cell maturation.** (A) The expression patterns of lef1, axin2a, fgf3, fgf10a, and pea3 in the pLLP. Embryos were injected with 0.5 ng std-MO or tgfβ1a-MO2 at the one-cell stage and collected at 28 hpf for in situ hybridization. Note that fgf10a expression was decreased in some hair cell regions (indicated by arrows) of the pLLP trailing zone but remained almost unchanged in the leading zone in tgfβ1a morphants while the other markers were not significantly changed. (B and C) The change of hair cell numbers. Hair cells in neuromasts (L1–L6) of injected embryos at 3 dpf were labeled by immunostaining using anti-Myosin antibody. All the trunk neuromasts of each embryo were counted. The average hair cell numbers per neuromast are shown in C. n, number of examined embryos. ***P < 0.001. (D) atoh1a expression was decreased in proneuromast central cells (indicated by arrows) of tgfβ1a morphants at 28 hpf. (E and F) tgfβ1a regulation of deltaA expression in proneuromast central cells (indicated by arrows) at 28 hpf. Note that tgfβ1a knockdown caused a reduction of deltaA expression, which was rescued by co-injection of 2.5 pg tgfβ1a mRNA. The average numbers of deltaA-positive clusters are shown in F. n, the number of examined embryos. ***P < 0.001. The ratios of embryos with representative pattern are indicated at the right corner of each picture. Scale bar, 40 μm in A, D, and E; 5 μm in B.
exhibited a decrease of pLL neuromast number and an increase with control embryos, embryos injected with tgfβ1a, and atoh1a expression at 26 hpf (Figure 5F). Taken together, these results support the idea that Smad5 could mediate the function of TGFβ1a in pLL development.

tgfβ1a mutants have an abnormal lateral line

To further validate the function of tgfβ1a in pLL development, we knocked out the tgfβ1a gene by transcription activator-like effector nucleases (TALENs) technology (Huang et al., 2011; Bedell et al., 2012; Zu et al., 2013). By targeting exon 1 of the tgfβ1a gene (Supplementary Figure S3A), we obtained three kinds of deletion mutations (td1 with 1 bp deletion, td5 with 5 bp deletion, and td8 with 8 bp deletion), all of which led to a shift of the open reading frame with a premature stop codon (Supplementary Figure S3B and C). An initial observation indicated that both tgfβ1aΔ5/Δ5 and tgfβ1aΔ6/Δ6 homozygous embryos, but not tgfβ1aΔ3/Δ3 homozygotes, showed identical defects in the posterior lateral line (data not shown). We then focused on tgfβ1aΔ5 mutation in subsequent analyses. Heterozygous (tgfβ1aΔ5+/−) and homozygous (tgfβ1aΔ5/Δ5) embryos of this line appeared to develop normally without obvious morphological defects during embryogenesis (Supplementary Figure 3D and E). However, ~25% of tgfβ1aΔ5/Δ5 larva could grow up to adulthood and the others died, implying that tgfβ1a may be required for later development or homeostasis to certain degrees. F2 embryos derived from tgfβ1aΔ5/Δ5 heterozygous intercrossing were classified, based on deposited pLL neuromasts labeled with the fluorescent dye FM 1-43FX at 2 dpf, into three groups, i.e. 3NMs with three or less neuromasts, 4NMs with four neuromasts, and 5NMs with five or more neuromasts (Figure 6A). After neuromasts were counted, embryos were individually genotyped by PCR (see examples in Figure 6B). Results showed that the average number of neuromasts in wild-type (tgfβ1a+/−), heterozygotes (tgfβ1aΔ5/Δ5), and homozygotes (tgfβ1aΔ5/Δ5) was 5.03 (n = 31), 4.87 (n = 62), and 3.04 (n = 27), respectively. All homozygotes had only three or four neuromasts with an expanded inter-neuromast distance (Table 1). Thus, deficiency of tgfβ1a led to a reduction of neuromast numbers and an increase of inter-neuromast distance, which were similar to the defects observed in tgfβ1a morphants.

We then tested whether overexpression of tgfβ1a could rescue the neuromast number in tgfβ1aΔ5/Δ5 F2 population, by judging the average neuromast number per embryo. Injection with an increasing amount of ectopic tgfβ1a mRNA in the range of 0.5–2.5 pg per embryo resulted in increasingly more efficient recovery of neuromast number in tgfβ1aΔ5/Δ5 F2 embryos (Figure 6C). Importantly, the decreased trunk neuromast number in tgfβ1aΔ5/Δ5 mutants could be partially rescued by overexpression of casmsd5 at 2 dpf, which was in sharp contrast to the inefficiency of casmsd1 overexpression (Figure 6D and E). These results further support the notion that TGFB1a regulates posterior lateral line formation through smad5.

tgfβ1a function in pLLP morphogenesis is mediated by Smad5

Previous reports have shown that TGFB proteins regulate cell functions primarily through ALK5 to Smad2 and Smad3 (Derynick and Zhang, 2003; Shi and Massague, 2003; Feng and Derynick, 2005; Massagué, 2012; Pickup et al., 2013), and in a few cell types through ALK1/ALK2/ALK3 to Smad1/Smad5 (Oh et al., 2000; Derynick and Zhang, 2003; Daly et al., 2008; Massagué, 2012). To determine which downstream effectors mediate tgfβ1a signaling during pLLP morphogenesis, we tested which smad upon overexpression could rescue the reduction of neuromast number in tgfβ1a morphants using mRNAs coding for constitutively active Smads (Casmads). Results indicated that casmsd5 but not casmsd1 or casmsd2 mRNA injection led to a recovery of pLL neuromast number and inter-neuromast distance in tgfβ1a morphants at 2 dpf (Figure 5A and B). The in situ hybridization showed that smad5 transcripts, but not smad1 or smad8, appeared to be enriched in the pLLP at 28 hpf (Figure 5C). The expression of smad5 in the migrating primordium of the pLL persisted at 36 hpf but not in the deposited neuromasts (Supplementary Figure S4C). Therefore, we speculated that TGFB1a signaling may be mainly mediated by Smad5 in the pLLP.

Previous studies showed that smad5 mutants exhibit obvious dorsoventral patterning defects (Hild et al., 1999; Kramer et al., 2002). Consistent with previous reports (Lele et al., 2001; McReynolds et al., 2007), injection with high dose (10 ng) of smad5-MO could phenocopy smad5 mutants in our hands (Supplementary Figure S2). In order to bypass early embryonic patterning defects, we used a low dose (2.5 ng) of smad5-MO to investigate its function in pLLP morphogenesis at late stage. Compared with control embryos, embryos injected with 2.5 ng smad5-MO exhibited a decrease of pLL neuromast number and an increase of inter-neuromast distance at 2 dpf (Figure 5D and E) as those injected with tgfβ1a-MO (Figure 1D and E). These pLL defects caused by smad5 knockdown could not be rescued by co-injection of p53-MO (Figure 5D and E), indicating a specific effect. Furthermore, similar to tgfβ1a knockdown (Figures 3C, 4D and E), smad5 knockdown caused a reduction of cdh2, deltaA, and atoh1a expression at 26 hpf (Figure 5F). Together, these results support the idea that Smad5 could mediate the function of TGFβ1a in pLL development.

(Matsuda and Chitnis, 2010). We observed that tgfβ10a expression was missing in some hair cell clusters of the pLLP traling zone, though its expression remained unchanged in the leading zone in tgfβ3a morphants (Figure 4A), suggesting a role in hair cell specification. To further study tgfβ1a function in hair cell formation, we first examined hair cells within trunk neuromasts at 3 dpf by labeling the hair cells with anti-Myosin6 antibody. Compared with an average of 8.16 hair cells per neuromast in control embryos (n = 15), tgfβ1a morphants (n = 17) had only 4.98 hair cells per neuromast (Figure 4B and C), which suggests a role of tgfβ1a in hair cell formation. Next, we investigated the expression of tgfβ1a in control embryos (Figure 4D and E). To verify the specific function of tgfβ1a, we co-injected tgfβ1a mRNA with tgfβ1a-MO and examined deltaA expression. Results disclosed that deltaA expression could be rescued efficiently at 28 hpf (Figure 4E and F). These data together indicate that tgfβ1a can regulate deltaA expression to regulate pLLP hair cell formation and then promote pLLP maturation.
We also examined hair cells in the trunk neuromasts. Immunostaining with anti-Myosin 6 antibody detected fewer hair cells within each neuromast in tgfβ1a<sup>ds/ds</sup> mutants (Supplementary Figure S5). The expression of cdh2, deltaA, and atoh1a in the pLLP was reduced in tgfβ1a<sup>ds/ds</sup> mutants (Figure 7A). The amount of Cdh2 protein in the apical constriction of rosettes, as examined by immunostaining, was obviously reduced (Figure 7C). In addition, the immunodetection revealed the missing of the tight junction protein ZO-1, which labels the center of rosettes in the pLLP, in the less-organized leading region in tgfβ1a<sup>ds/ds</sup> mutants (Figure 7D). Importantly, overexpression of tgfβ1a or casmad5 mRNA in tgfβ1a<sup>ds/ds</sup> mutants could largely restore the expression of cdh2, deltaA, and atoh1a in the pLLP (Figure 7A and B), although tgfβ1a or casmad5 overexpression in wild-type embryos did not obviously alter these gene expression patterns (Figures 3C and 4E, and Supplementary Figure S6). These data further support the view that tgfβ1a regulates cell adhesion and hair cell formation during pLLP morphogenesis.
The reduced pLLP neuromast number in tgfβ1<sup>atd</sup> mutants may not be ascribed to reduced cell proliferation and boosted apoptosis. It is well known that TGFβ1 signaling is involved in both negative and positive regulations of cell proliferation and apoptotic processes (Massague et al., 2000). We asked whether the reduced neuromast number in the pLLP could be due to a reduced cell proliferation or an excessive apoptosis. To test these possibilities, we...
Figure 7 **tgfb1a<sup>dss/dss</sup>** mutants exhibit pLLP defects resembling *tgfb1a* morphants. (A and B) *cdh2*, *deltaA*, and *atoh1a* expression patterns in the pLLP at 28 hpf. The pLLP is outlined by black dotted lines. The *deltaA* and *atoh1a* expression clusters within proneuromasts are indicated by red arrows. The ratios of embryos with representative pattern are indicated. Note that the reduction of these markers in pLLP could be largely restored by overexpression of *tgfb1a* or *casmad5* mRNA. The average numbers of *deltaA* - and *atoh1a*-positive clusters are shown in B. *n*, the number of examined embryos. ***P < 0.005. (C) *Cdh2* protein level was reduced in the pLLP of *tgfb1a<sup>dss/dss</sup>* mutants. (D) The tight junction protein ZO-1 was missing in rosettes in the leading region of the *tgfb1a<sup>dss/dss</sup>* primordia. Wild-type (WT) and *tgfb1a<sup>dss/dss</sup>* mutant embryos in C and D were immunostained at 28 hpf using anti-*Cd2* and anti-ZO-1 antibodies, respectively. The ZO-1 signal is indicated by white arrows. The embryos were laterally viewed with anterior to the left. The ratios of embryos with representative pattern are indicated. The genotype of each embryo was confirmed after *in situ* hybridization or immunostaining. Scale bar, 40 μm in A, C, and D. (E) Proposed signaling network involving TGFβ1a in pLL development. Our findings are indicated by red arrows and interactions reported previously are indicated by black arrows or symbols (see Discussion for details).
first performed BrdU incorporation assay to investigate cell proliferation activity. Results indicated that the number of BrdU-positive cells in the pLLP of *tgfb1aΔA5/ΔDs* mutants was comparable to that in wild-type embryos at 25 and 31 hpf (Supplementary Figure S7A and B), suggesting that cell proliferation in the pLLP of mutants is not impaired. We then detected apoptotic cells by carrying out TUNEL assay. Generally, the number of apoptotic cells in the pLLP was extremely low in either wild-type or *atd* mutants, and the intracellular effectors Smad2 and Smad3 (Derynck and Zhang, 2003; Shi and Massague, 2003; Massagué, 2012). However, the total number of cells within the pLLP in mutants was comparable to that in wild-type embryos (Supplementary Figure S7C and D), which implies that enhanced apoptosis in *tgfb1aΔA5/ΔDs* mutants may not contribute to the reduction of neuromast numbers.

Discussion

We have demonstrated in this study for the first time that *tgfb1a* is required for migration of the primordium, maturation of proneur-masts, and deposition of neuromasts during posterior lateral line development in the zebrafish embryo. As summarized in Figure 7E, TGFβ1a regulates primordium migration and neuromast deposition by promoting *cdh2* expression but not by affecting chemokine signaling related genes; it regulates neuromast maturation by maintaining the expression of the Fgf ligand gene *fgf10a*, the Delta ligand gene *deltaA*, and the bHLH transcription factor *atoh1a* in the trailing zone of the primordium. It seems that TGFβ1a does not play a role in the trailing zone—leading zone patterning of the primordium.

Zebrafish *tgfb1a* is highly expressed in ovarian follicles at stage I through stage III and becomes decreased at stage IV (Kohli et al., 2003). It is found to play an inhibitory role in oocyte maturation (Kohli et al., 2003, 2005). We found that *tgfb1a* transcripts are undetectable in zebrafish embryos before early segmentation and zygotic expression of *tgfb1a* appears to occur around 10-somite stage predominantly in the olfactory and otic placodes (Figure 1A and B). *tgfb1aΔA5/ΔDs* mutants or *tgfb1a* morphants do not exhibit morphological abnormalities during early development. Therefore, *tgfb1a* may not play a role in early embryonic development, and the pLL developmental defects due to deficiency of TGFβ1a may not be secondary to defects in other tissues occurring at earlier developmental stages. The continuous expression of *tgfb1a* in the pLLP and neuromasts could support a specific role in pLL development.

TGFβ1 signaling is well known to enhance tumorigenic cell migration, invasion, survival, and epithelial-mesenchymal transition (EMT), which are associated with the upregulation of N-cadherin/Cdh2, a key regulator of collective cell migration (Derynck et al., 2001; Massague, 2008; Shih and Yamada, 2012). However, the function of *cdh2* in zebrafish lateral line formation is still controversial. Recently, Revenu et al. (2014) reported that Cdh2 protein is enriched in apical adherens junctions in the pLLP, but the *cdh2* mutant *parachute* appears normal in pLLP migration and neuromasts formation. It is explained by the redundancy of cadherins in the pLLP. However, another study has shown that knockdown of *cdh2* could severely disrupt the zebrafish lateral line system with reduced neuromast number both in the aLL and pLL, which is further confirmed by analyzing another *cdh2* mutant *glass onion* (Kerstetter et al., 2004). Compared with *parachute*, the mutation in *glass onion* occurs at a very important and well-conserved residue (Malicki et al., 2003). This might be the reason why *glass onion* mutant exhibits much stronger lateral line defects than *parachute*. In this study, we demonstrate that deficiency of TGFβ1a in the pLLP leads to remarkable elimination of *cdh2* expression, indicating an important role of TGFβ1a in regulation of cadherins in the pLLP for the first time.

TGFβ1 plays important roles in cell migration, proliferation, and apoptosis, as well as in development and tissue homeostasis. In most cases, TGFβ1 is found to be mediated by the type I receptor and the intracellular effectors Smad2 and/or Smad3 (Derynck and Zhang, 2003; Shi and Massague, 2003; Massagué, 2012). However, TGFβ1 signaling could be mediated by ALK2 in mammary epithelial cells (Miettinen et al., 1994) and by ALK1 in endothelial cells through the intracellular effectors Smad1/5 (Oh et al., 2000; Park et al., 2008). Furthermore, TGFβ1 can form large complexes with TβRII, ALK5, and ALK2 or ALK3 to activate Smad2/3 and Smad1/5 together (Dalý et al., 2008), or with TβRII, ALK1, ALK5, Endoglin, and Betaglycan in human chondrocytes to activate Smad1/5 signaling but to inhibit Smad3 signaling (Finnson et al., 2008). In this study, we propose that TGFβ1a transduces the signal in the zebrafish lateral line system to Smad5 rather than to Smad2/3, which represents another case of Smad2/3-independent TGFβ1 signaling in a novel system. Our proposal is supported by three lines of evidence. First, overexpression of constitutively active smad5, but not smad1 or smad2, is able to rescue lateral line defects in *tgfb1a* morphants or mutants (Figures 5A and B, 6D and E, and 7A and B). Second, *smad5* is highly expressed in the pLLP, while *smad1* and *smad8* expression is not enriched there (Figure 5C and Supplementary Figure S4). Third, knockdown of *smad5* by using a lower dose (2.5 ng) of *smad5*-MO phenocopies *tgfb1a* morphants and mutants (Figure 5D–F). Unfortunately, we were unable to detect p-Smad5 in the pLLP using a p-Smad1/5/8 antibody that recognizes zebra-fish p-Smad1/5/8 around gastrula stages (Liu et al., 2013; Xue et al., 2014). Detection of p-Smad5 in the pLLP may need further improving immunostaining technique or using a better antibody. Besides, we do not know at present which type II receptor and/or co-receptors are required for TGFβ1a signaling in the lateral line cells. To address this issue, it is necessary to investigate the expression patterns of various TGFβ and Activin-like kinase receptors and co-receptors (e.g. Endoglin) and identify those expressing in the lateral line cells.

Smad5 or Smad5/Smad4 complex may directly regulate target gene transcription by physically binding to the responsive regulatory DNA elements. We demonstrate that Smad5 positively regulates *cdh2*, *deltaA*, and *atoh1a* expression in the lateral line primordium (Figure 5F). However, we were unable to investigate whether this regulation is direct or indirect because of difficulties to sort out sufficient number of pLLP cells for chromatin immunoprecipitation. Dissection of promoters/enhancers of these genes through transgenic approach may help address the issue.
Another interesting issue is whether the tgfβ1a functions in other systems of zebrafish embryos are mediated by Smad5. Since both tgfβ1a and smad5 are also expressed in the otic placodes (Figures 1A, 5C, and Supplementary Figure S4), we investigated the effect of tgfβ1a deficiency on otic hair cells. Compared with wild-type embryos, tgfβ1a/mesoderm mutants at 2 dpf and 3 dpf did not show a significant change of the hair cell number in the inner ear (Supplementary Figure S8). This phenomenon might be ascribed to redundant functions of other signals in the formation of hair cells in the inner ear. Lack of detectable defects in other systems in tgfβ1a/mesoderm mutants disallows us to investigate the functional specificity of the TGFβ1a/Smad5 pathway.

Although tgfβ1a and smad5 are co-expressed in the pLLP, smad5 expression is not regulated by tgfβ1a, because smad5 mRNA levels in the pLLP were not obviously altered in tgfβ1a/mesoderm mutants (Supplementary Figure S4). It remains elusive which upstream signals, either non-TGFβ signals or other TGFβ superfamily members, regulate smad5 expression in the pLLP.

Materials and methods
Zebrafish strains
Zebrafish wild-type embryos from Tuebingen strain and Tg(-8.0cldb:nynEGFP) (Haas and Gilmour, 2006) transgenic embryos were used. Embryos were incubated in Holtfreter’s solution at 28.5°C and staged as previously described (Kimmel et al., 1995).

Constructs
For in vitro mRNA synthesis, tgfβ1a full coding sequence was amplified with the forward primer 5′-ATGAGGTGTTGGTGTTTG-3′ and the reverse primer 5′-TTAAGTCAGTGAATGTTCCCT-3′, and then subcloned into the pX7 vector that has 5′ and 3′ UTRs from Xenopus β-globin gene. tgfβ1a-5′UTR-GFP plasmid was generated by inserting a part of 5′UTR and coding sequence of tgfβ1a into pEGFP-N3 (Clontech) with the forward primer 5′-CCAGGTCTCAGTATCCCTTCTTCTTCT-3′ and the reverse primer 5′-GGGTGTAATACACCCAGTCCATTATCGGCTGTG-3′.

The probe for detecting tgfβ1a, tgfβ1b, tgfβ2, or tgfβ3 mRNA was amplified with the primers (as follows) and inserted into EZ-T vector (Genstar, #T618-10):
tgfβ1a: 5′-CCAGAGAGCGGAGCAGATTC-3′ (forward) and 5′-GTC AAGGATTGCAGTACACA-3′ (reverse); tgfβ1b: 5′-CAGAGAAAC AGAAGTAGA-3′ (forward) and 5′-CGAGCATAGGCAAGCAG-3′ (reverse); tgfβ2: 5′-ATGAAATCTTGACTCGTACG-3′ (forward) and 5′-CTAGGTCAGTTGCAGA-3′ (reverse); tgfβ3: 5′-ATGATTTGGG CAAAAGCTATT-3′ (forward) and 5′-TACGCTGACATTGCGAG-3′ (reverse).

mRNAs, morpholinos, and microinjection
The mRNAs were generated with corresponding linearized plasmids by in vitro transcription using mMessage mMachine kit (Ambion) and purified using RNeasy Mini Kit (Qiagen). Morpholinos were synthesized by Gene Tools, LLC. The target sites of tgfβ1a MOs are located in the 5′UTR of tgfβ1a. The sequences of antisense morpholinos are as follows: tgfβ1a-MO1, 5′-TACGACAAACTGCGTACCCAG AAGCAA-3′; tgfβ1a-MO2, 5′-AAGGAAAAACGAAATGGAAAGAT-3′; standard-MO, 5′-CCTCTACCTCGTACATTTAAT-3′; p53-MO, 5′-GACCTCTCTCTGCAACTACATG-3′; chd2-MO, 5′-TCTGATATGAAACCGGATAGAGTT-3′ (Lele et al., 2002); smad5-MO, 5′-AACAGA CTGACATTGCGTGATAG-3′ (Lele et al., 2001). About 1–1.5 nl mRNA or morpholino solution was injected into the yolk of each embryo between one-cell and two-cell stages with the typical MPPI-2 quantitative injection equipment (Applied Scientific Instrumentation Co.). We used the sequential injection when two different reagents needed to be co-injected. The injection dose was the amount of the mRNA or morpholino received by a single embryo.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was carried out as previously described (Xiong et al., 2006). Digoxigenin-UTP-labeled antisense RNA probes were synthesized in vitro using a linearized plasmid or PCR product as template. The templates of tgfβ1a, tgfβ1b, tgfβ2,eya1,cxcl12a,cxcr4b,cxcr7b,axin2a,fgf3,pea3,deltaa, and cdh1 were linearized from corresponding plasmids, and those for lef1,fgf10a,atorh1a,cdh2,cdh4,cdh6,smad1,smad5, and smad8 were amplified by PCR with the following primers:
lef1: 5′-ATGCCCGAGTTGTGCTG-3′ (forward) and 5′-TAATAC GACTCATACTAGGGATGCTAGGTTTCTCATATA-3′ (reverse); fgf10a: 5′-GGAGGAGGGTGAAGCATGTTTTC-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse); atorh1a: 5′-TTCCAGGGAAATATCCAG-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse); cdh2: 5′-AAGGAGGAGGATCTGTTCTTCCCTAAGAGTGTCC-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse); cdh4: 5′-GTCTGATATGAAACCGGATAGAGTT-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse); cdh6: 5′-CGGAGGTGCTTGCTATGCTTTTCCCTAAGAGTGTCC-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse); smad1: 5′-TACGACTCAAGGGCCGCTCAGTACAGTTACA-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (forward) and 5′-TACGAGCTTACCTGCAAGGATTACACCA-3′ (reverse); smad5: 5′-GTCTGATATGAAACCGGATAGAGTT-3′ (forward) and 5′-GTCTGATATGAAACCGGATAGAGTT-3′ (reverse); smad8: 5′-AAGGAGGAGGATCTGTTCTTCCCTAAGAGTGTCC-3′ (forward) and 5′-TAATACGCTACTATAGGGCCGCTCAGTACAGTTACA-3′ (reverse).

Embryos after in situ hybridization were immersed in glycercol and photographed using the Ds-Ri1 CCD camera under a Nikon SMZ1500 stereoscope. The migration distance was calculated by Image J software.

BrdU labeling and TUNEL assay
BrdU incorporation assay was performed as previously described (Plaster et al., 2006). Zebrafish embryos at 23 hpf stage were incubated with BrdU (10 μM) on ice for half hour and fixed with 4% paraformaldehyde (PFA) at different stages. To detect the apoptotic cells in lateral line primordium, TUNEL assay was performed with ApopTag® Red In Situ Apoptosis Detection Kit (Millipore, S7165).

Whole-mount immunofluorescence
Zebrafish whole-mount immunofluorescence was performed as previously described (Han et al., 2011; Zhang et al., 2012). The following antibodies were used: rabbit anti-Mysinos6 (a gift from...
Dr Dong Liu, rabbit anti-ZO-1 (61–7300, Invitrogen), rabbit anti-Cdh2 (GTK125885, GeneTex), and DyLight 488-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch). Immunostained embryos were imaged under Nikon A1RMPSi lasers scanning confocal microscope.

Time-lapse imaging
Tg(-8.0clelnb:lynEGFP) transgenic embryos at 23 hpf were immobilized in 1% low melting point agarose and 0.02% Tricaine (Sigma), and imaged with two-photon laser excitation on a Olympus FV1000MPE with 40× objective. The Z-projections of the confocal stacks were generated using ImageJ software.

Hair cells labeling
Embryos at 2 or 3 dpf were incubated with FM 1-43FX (Invitrogen cat. F-35355, 1:1000 diluted) for 30 sec. Then, the embryos were immediately rinsed for several times and observed under Zeiss Imager M2 using an AxioCam MRm camera.

Generation of tfgb1a<sup>plantid;hspa</sup> mutant lines with TALENs
The TALENs target site, consisting of the left TALENs binding site, the spacer, and the right TALENs binding site, was predicted with the online software (Cermak et al., 2011) (http://boglab.xpl2.iastate.edu/TALEN/help.php). The corresponding expression vectors were constructed following the protocol from Dr Bo Zhang’s lab (Huang et al., 2011). The screens of founder fish (F<sub>0</sub>) and F<sub>1</sub> embryos carrying mutated genomic DNA were performed as previously described (Zu et al., 2013). For genotyping, genomic DNA was isolated from the tail of individual fish or from the whole embryo after FM 1-43FX staining; the specific region of the tfgb1a locus was amplified with the forward primer 5′-CTCGCTGGACAGTGTGGTCGT-3′ and the reverse primer 5′-TCACATCTGGGGGTTATATAACAC-3′; the amplified DNA fragment was purified for sequencing or subjected to enzymatic digestion with HphI; the digested DNA was resolved on an agarose gel.

Statistical analysis
Student’s t-tests (two-tailed, unequal variance) were used to determine P-values of all measurements in this study. For pLPP migration distance and whole length of each embryo, data were averaged from three times of measurements by Image J software and expressed as mean plus s.d. *P < 0.05 and ***P < 0.005 indicated the significance levels.

Supplementary material
Supplementary material is available at Journal of Molecular Cell Biology online.

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References


