INTRODUCTION

Tongue formation is a relatively recent evolutionary adaptation of craniofacial musculoskeleton, appearing to be coincident with terrestrial amphibian species (Iwasaki, 2002; Noden and Francis-West, 2006). The mammalian tongue is composed of numerous tissues, including mesoderm-derived skeletal muscle, cranial neural crest (CNC)-derived supportive connective tissue and a stratified, squamous, non-keratinized epithelium. Studies using chick and mouse models suggest that the myogenic precursors of tongue muscles are hybrids because they originate from somatic hypaxial somites (2-5) and complete their development in the craniofacial region (Noden, 1983; Huang et al., 1999). As these myogenic precursors first enter the craniofacial region (the first branchial arch), they immediately establish intimate contact with the CNC cells. This close association between the two cell types continues throughout the entire course of tongue morphogenesis, suggesting that tissue-tissue interaction may play an important role in regulating cell fate determination. To date, there is no definitive analysis comparing the regulatory mechanisms of tongue muscle development with those of trunk or cranial muscle formation. Thus, further studies are required to elucidate the functional significance of signaling molecules in regulating tongue formation.

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A TGFβ-Smad4-Fgf6 signaling cascade controls myogenic differentiation and myoblast fusion during tongue development

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SUMMARY

The tongue is a muscular organ and plays a crucial role in speech, deglutition and taste. Despite the important physiological functions of the tongue, little is known about the regulatory mechanisms of tongue muscle development. TGFβ family members play important roles in regulating myogenesis, but the functional significance of Smad-dependent TGFβ signaling in regulating tongue skeletal muscle development remains unclear. In this study, we have investigated Smad4-mediated TGFβ signaling in the development of occipital somite-derived myogenic progenitors during tongue morphogenesis through tissue-specific inactivation of Smad4 (using Myf5-Cre;Smad4fl/fl mice). During the initiation of tongue development, cranial neural crest (CNC) cells occupy the tongue buds before myogenic progenitors migrate into the tongue primordium, suggesting that CNC cells play an instructive role in guiding tongue muscle development. Moreover, ablation of Smad4 results in defects in myogenic terminal differentiation and myoblast fusion. Despite compromised muscle differentiation, tendon formation appears unaffected in CNC cells. This intimate relationship suggests that CNC cells play an instructive role in guiding tongue muscle development. Furthermore, ablation of Smad4 results in a significant reduction in expression of several members of the FGF family, including Fgf6 and Fgfr4. Exogenous Fgf6 partially rescues the tongue myoblast fusion defect of Myf5-Cre;Smad4fl/fl mice. Taken together, our study demonstrates that a TGFβ-Smad4-Fgf6 signaling cascade plays a crucial role in myogenic cell fate determination and lineage progression during tongue morphogenesis.

KEY WORDS: Smad4, TGFβ signaling, Tongue development, Myogenesis, Myogenic differentiation, Myoblast fusion, Fgf6, Mouse

TGFβ family members play important roles in regulating myogenesis during skeletal muscle development (Kollias and McDermott, 2008). Specifically, TGFβ signaling controls the proliferation and fusion of myoblasts (Olson et al., 1986). Myogenic cells exposed to truncated TGFβ type II receptor show inhibition of terminal differentiation (Filvaroff et al., 1994). A recent study shows that TGFβ signaling is specifically required in CNC-derived fibroblasts and controls myogenic cell proliferation through tissue-tissue interactions during tongue morphogenesis (Hosokawa et al., 2010).

Smad4 occupies the central position of the canonical TGFβ signaling pathway in regulating organogenesis. Our preliminary studies have demonstrated that Smad4 is expressed in both myogenic progenitors and CNC-derived cells in the tongue primordium. However, mice that lack Smad4 die before the initiation of tongue formation, making it impossible to investigate the role of Smad4-mediated TGFβ signaling in regulating tongue development (Sirard et al., 1998; Ko et al., 2007). To test the hypothesis that Smad4-mediated TGFβ signaling controls the development of myogenic progenitors during tongue morphogenesis, we generated tissue-specific Smad4 gene ablation in mesoderm-derived myogenic progenitors (Myf5-Cre;Smad4fl/fl mice). We provide the first evidence that CNC cells are the sole population within the tongue buds that initially form and that myogenic progenitors subsequently migrate into the tongue primordium and establish contact with CNC cells. This intimate relationship suggests that CNC cells play an instructive role in guiding tongue muscle development. Furthermore, there is a cell-autonomous requirement for Smad4-mediated TGFβ signaling during myogenic differentiation and myoblast fusion. Our study demonstrates that a TGFβ/FGF signaling cascade is specifically required during tongue myogenesis.
MATERIALS AND METHODS

**Mice**

The Myf5-Cre, Wnt1-Cre (Chai et al., 2000), ROSA26 reporter (R26R) (Soriano, 1999) and conditional Smad4 (Dpc4) allele (Yang et al., 2002) have been described previously. Genotyping was previously described (Chai et al., 2000).

**Histological analysis and scanning electron microscopy**

For Hematoxylin and Eosin (H&E) staining, samples were processed and stained according to standard procedures. Bromodeoxyuridine (BrdU; Sigma) injections were performed as reported previously (Hosokawa et al., 2010) and detected using the BrdU Staining Kit (Invitrogen). Apoptosis was detected by TUNEL assay using the In Situ Cell Death Detection Kit (Fluorescein; Roche). Samples for SEM analysis were processed and viewed as previously described (Xu et al., 2006).

**β-Galactosidase activity assays**

E10.5 embryos were harvested and stained for β-galactosidase (β-gal) activity according to standard procedures (Chai et al., 2000). For detection of β-gal activity in tissue sections, samples were processed and stained as previously described (Chai et al., 2000).

**In situ hybridization**

In situ hybridizations were performed following standard procedures (Xu et al., 2005). Digoxigenin-labeled antisense probes were generated from mouse cDNA clones that were kindly provided by several laboratories: myogenin (Achim Gossler, Institute for Molecular Biology, Medizinische Hochschule Hannover, Germany); scleraxis (Eric N. Olson, University of Texas Southwestern Medical Center, USA); Fgf6 and Fgf4 (Pascal Maire, Institute Cochin, France).

**Immunostaining**

Immunostaining was performed using primary antibodies against myosin heavy chain (MHC; DSHB); Pax3 (DSHB); MyoD1, desmin, Ki67 and phospho-Smad3 (Abcam); and phospho-Smad1/5/8 (Cell Signaling). Alexa Fluor 488 and 568 (Molecular Probes) were used for detection. Slides were mounted with Vectashield Mounting Medium (VECTOR) and imaged by fluorescence microscopy.

**Western blot analysis**

Tongue primordia were collected from E13.5 embryos, treated with 2.4 U/ml Dispase I (Roche) on ice for 1 hour, then the tongue mesenchyme was used for protein extraction. Protein samples were analyzed by SDS-PAGE using NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen). After protein transfer to a Millipore Immobilon-P membrane, polyclonal antibodies against Fgf6, Fgfr4 and Smad4 (Santa Cruz) were used for protein transfer to a Millipore Immobilon-P membrane, polyclonal antibodies against Fgf6, Fgfr4 and Smad4 (Santa Cruz). Immunostaining was performed using primary antibodies against myosin heavy chain (MHC; DSHB); Pax3 (DSHB); MyoD1, desmin, Ki67 and phospho-Smad3 (Abcam); and phospho-Smad1/5/8 (Cell Signaling). Alexa Fluor 488 and 568 (Molecular Probes) were used for detection. Slides were mounted with Vectashield Mounting Medium (VECTOR) and imaged by fluorescence microscopy.

**Gene expression analysis**

RNA was isolated from tongue primordia at various stages and converted to cDNAs using RNeasy Mini and QuantiTect Reverse Transcription Kits (Qiagen), respectively. Real-time PCR was conducted using 2×SYBR-green PCR master mix on the iCycler (Bio-Rad). Gene-specific primer sequences were obtained from the Primer Bank (Wang et al., 2012). Values were normalized against Gapdh using the 2ΔΔCt method (Livak and Schmittgen, 2001). The global gene expression analysis was performed as previously described (Iwata et al., 2012).

**Isolated myofibers and measurements**

Single myofibers were isolated and cultured from the tongue muscles of E18.5 embryos as previously described (Kuang et al., 2007). After 24 hours of culture, individual fibers were processed for immunostaining (Kuang et al., 2006). For quantification of length, myofibers were prepared from three sets of E18.5 Myf5-Cre; Smad4flox/− mice that were at least 10 fibers were scored per sample. All measurements and counting were performed with ImageJ 1.45k software. Results were assessed for statistical significance using Student’s t-test.

**RESULTS**

CNC-derived cells are the first to arrive during the initial development of tongue buds

CNC-derived cells and myogenic cells are closely associated during tongue morphogenesis (Hosokawa et al., 2010); however, the question remains of whether CNC-derived or myogenic cells initiate tongue development. To address this, we examined the initial development of tongue in Wnt1-Cre; R26R and Myf5-Cre; R26R mice. At E10.5, two swellings emerge on the floor of both sides of the first branchial arch, called the tongue buds (also referred as lateral lingual swellings; Fig. 1A). Significantly, all cells...
in the two tongue buds were CNC derived at E10.5 (Fig. 1C). In Myf5-Cre;R26R mice, X-gal stains β-galactosidase (the protein product of lacZ) in myogenic cells and no lacZ-positive cells were detected within the tongue buds at E10.5 (Fig. 1E). At E11.5, both tongue buds merged and formed the tongue primordium (Fig. 1B). Myogenic cells were first detectable in the tongue primordium of Wnt1-Cre;R26R (Fig. 1D; lacZ-negative cells) and Myf5-Cre;R26R mice (Fig. 1F; lacZ-positive cells) at E11.5, indicating that myogenic precursors have started invading the tongue primordium. After these myogenic precursors enter the craniofacial region, they are circumscribed by CNC-derived cells. This close association between the two cell types continues throughout tongue morphogenesis. Our data suggest that CNC-derived cells form the initial tongue buds and may guide tongue morphogenesis.

Ablation of Smad4 in myogenic cells results in microglossia and fewer muscle fibers in the tongue
To test the hypothesis that Smad4-mediated TGFβ signaling plays a cell-autonomous role in controlling the fate of myogenic cells during tongue development, we generated Myf5-Cre;Smad4flox/flox conditional knockout mice. After FDG staining and FACS (Fig. 2A,B), we found that ablation of Smad4 in myogenic cells is specific and efficient (Fig. 2C). Myf5-Cre;Smad4flox/flox mice die at birth and show microglossia (Fig. 2D-I). Histological analysis revealed that the muscle fibers in the tongue were disorganized and present in low density in Myf5-Cre;Smad4flox/flox mice compared with the well-organized muscle fibers in control mice (Fig. 2J,K). To evaluate the status of myogenic differentiation, we analyzed expression of MHC, a marker for fully differentiated myoblasts. We detected a significant decrease in the number of MHC-positive muscle fibers in newborn Myf5-Cre;Smad4flox/flox mice, accompanied by a moderate increase in connective tissue (Fig. 2L-N). We also observed numerous nuclei located in the center of the muscle fibers in Myf5-Cre;Smad4flox/flox mice, instead of their normal location at the periphery in control mice (Fig. 2L,M). However, the total number of tongue mesenchymal cell nuclei in Myf5-Cre;Smad4flox/flox mice is comparable with that of control (Fig. 2O), indicating that more connective tissue may be present per field owing to the lack of intervening myofibrils. Our results suggest that there is a cell-autonomous requirement for Smad4-mediated TGFβ signaling in myogenic cells during tongue morphogenesis.

Loss of Smad4 in myogenic cells does not affect myogenic progenitor cell migration, proliferation or apoptosis
In contrast to the other skeletal muscles in the craniofacial region that are derived from cranial paraxial mesoderm, the myogenic progenitor cells in the tongue migrate from the occipital somites (Noden, 1983; Noden and Francis-West, 2006). To determine whether loss of Smad4 in myogenic cells of the tongue affects myogenic progenitor cell migration, we performed Western blot analysis of Smad4 expression in myogenic cells of the tongue during tongue development, we generated Myf5-Cre;Smad4flox/flox conditional knockout mice. After FDG staining and FACS (Fig. 2A,B), we found that ablation of Smad4 in myogenic cells is specific and efficient (Fig. 2C). Myf5-Cre;Smad4flox/flox mice die at birth and show microglossia (Fig. 2D-I). Histological analysis revealed that the muscle fibers in the tongue were disorganized and present in low density in Myf5-Cre;Smad4flox/flox mice compared with the well-organized muscle fibers in control mice (Fig. 2J,K). To evaluate the status of myogenic differentiation, we analyzed expression of MHC, a marker for fully differentiated myoblasts. We detected a significant decrease in the number of MHC-positive muscle fibers in newborn Myf5-Cre;Smad4flox/flox mice, accompanied by a moderate increase in connective tissue (Fig. 2L-N). We also observed numerous nuclei located in the center of the muscle fibers in Myf5-Cre;Smad4flox/flox mice, instead of their normal location at the periphery in control mice (Fig. 2L,M). However, the total number of tongue mesenchymal cell nuclei in Myf5-Cre;Smad4flox/flox mice is comparable with that of control (Fig. 2O), indicating that more connective tissue may be present per field owing to the lack of intervening myofibrils. Our results suggest that there is a cell-autonomous requirement for Smad4-mediated TGFβ signaling in myogenic cells during tongue morphogenesis.

**Fig. 2.** Myf5-Cre;Smad4flox/flox mice exhibit microglossia and a reduction of muscle fibers. (A) Schematic diagram of fluorescence activated cell sorting (FACS) approach based on fluorescein di-β-D-galactopyranoside (FDG) staining. (B) FACS plots of FDG-positive (myogenic cells, R5) and -negative cells (CNC-derived cells, R2) from preparations. (C) Western blot analysis of Smad4 expression in tongue myogenic cells [FDG (+)] and CNC-derived cells [FDG (–)] from control and Myf5-Cre;Smad4flox/flox;R26R (C.K.O.) mice. (D-M) Macroscopic appearance (D,E), Hematoxilin and Eosin staining (F-K) and MHC immunofluorescence (L,M) of tongues from Myf5-Cre;Smad4flox/+ control (D,F,H,J,L) and Myf5-Cre;Smad4flox/E,G,I,K,M) newborn mice. (F-K) Black arrows indicate disorganized muscle fibers in Myf5-Cre;Smad4flox/+ mice (G,K). Boxed areas in H and I are shown magnified in J and K. (L,M) MHC immunofluorescence (MHC, green; DAPI, blue) shows MHC-positive muscle fibers. Numerous nuclei (white arrows) are located in the center of the muscle fibers in Myf5-Cre;Smad4flox/+ mice (M). (N,O) Quantitation of the MHC-positive muscle fiber number (N) and total tongue mesenchymal cell nuclei number (O) from L and M. Five randomly selected non-overlapping samples were used from each experimental group. Graphs show average ± s.d. *P<0.05; n=5. Scale bars: 1.5 mm in D,E; 500 µm in F-I; 50 µm in J-M.
immunofluorescence in the tongue primordium (Fig. 3E,F), and the Pax3-positive cells were detectable by the migrating progenitor cells in the hypoglossal cord express Pax3 the number of progenitor cells that arrived in the tongue staining in sections (C,D) of E10.5 protein (Myod1) acts as a myoblast determination gene, expressed the expression level of cyclin D1, a cell cycle progression marker, in myoblast fusion and differentiation (Li et al., 1994). The results showed striking changes in the relative proportion of myoblasts and multinucleated myotubes obtained from control and Myf5-Cre;Smad4flox/flox mice (Fig. 6E-L). Statistical analyses revealed that the myoblast fusion index and myotube length were significantly reduced in Myf5-Cre;Smad4flox/flox samples (Fig. 6M,N); however, the number of MyoD1-positive cells in Myf5-Cre;Smad4flox/flox samples was comparable with that of control at each time point (Fig. 6O). These results indicate that the myoblasts from tongue mesenchyme of Myf5-Cre;Smad4flox/flox mice experience a fusion defect during differentiation rather than decreased proliferation.

Smad4-mediated TGFβ signaling controls myogenic cell differentiation and myoblast fusion in the tongue

To define the progression of tongue myogenesis in Myf5-Cre;Smad4flox/flox mice more precisely, we analyzed the expression of myogenic regulatory factors (MRFs). Myoblast determination protein (Myod1) acts as a myoblast determination gene, expressed by undifferentiated proliferating myoblasts (Berkes and Tapscott, 2005). We detected MyoD1-positive cells by immunofluorescence analysis in the tongue of both control and Myf5-Cre;Smad4flox/flox mice at E13.5 (Fig. 5A-B’), and the number of MyoD1-positive cells in Myf5-Cre;Smad4flox/flox mice was comparable with that of control (Fig. 5C). We also evaluated the relative expression level of MyoD1 by real-time PCR and detected no significant difference between control and Myf5-Cre;Smad4flox/flox mice at E12.5 and E13.5 (Fig. 5D), indicating that the determination of myoblasts was unaffected in Myf5-Cre;Smad4flox/flox mice. Myogenin is a myogenic differentiation determinant, essential for the terminal differentiation of committed myoblasts (Braun and Gautel, 2011). At E13.5, myogenin was strongly expressed in differentiating myoblasts of the intrinsic tongue muscles, extrinsic tongue muscles, such as the genioglossus and geniohyoid, and the other craniofacial muscles in control mice (Fig. 5E,F’). In Myf5-Cre;Smad4flox/flox mice, myogenin expression was significantly reduced in the intrinsic and extrinsic tongue muscles and the other craniofacial muscles (Fig. 5F,F’), indicating that the terminal differentiation of myoblasts was compromised. The reduced myogenin expression in the tongue of Myf5-Cre;Smad4flox/flox mice was confirmed by real-time PCR at E13.5 and E14.5 (Fig. 5G).

This defective differentiation could result from inefficient fusion of myoblasts and myotubes. Therefore, we performed single muscle fiber isolation and culture of tongue muscle from E18.5 control and Myf5-Cre;Smad4flox/flox mice. We observed that the length of muscle fibers was significantly decreased in Myf5-Cre;Smad4flox/flox mice (Fig. 6A-C). Moreover, the number of nuclei contained in each muscle fiber was significantly reduced in Myf5-Cre;Smad4flox/flox mice (Fig. 6A,B,D). To quantify the effect on myoblast fusion, we performed primary tongue mesenchymal cell culture from E13.5 control and Myf5-Cre;Smad4flox/flox mice. The fusion of myoblasts was visualized using antibodies against Myod1 and desmin at various time points of culture. Desmin, a muscle-specific intermediate filament protein, is linked to proper myoblast fusion and differentiation (Li et al., 1994). The results showed striking changes in the relative proportion of myoblasts and multinucleated myotubes obtained from control and Myf5-Cre;Smad4flox/flox mice (Fig. 6E-L). Statistical analyses revealed that the myoblast fusion index and myotube length were significantly reduced in Myf5-Cre;Smad4flox/flox samples (Fig. 6M,N); however, the number of MyoD1-positive cells in Myf5-Cre;Smad4flox/flox samples was comparable with that of control at each time point (Fig. 6O). These results indicate that the myoblasts from tongue mesenchyme of Myf5-Cre;Smad4flox/flox mice experience a fusion defect during differentiation rather than decreased proliferation.

We also examined the expression levels of several genes involved in fusion during myogenesis: caveolin 3, β1-integrin and prostacyclin (Galbiati et al., 1999; Schwander et al., 2003; Bondesen et al., 2007). Results from real-time PCR analysis showed that these fusion-related genes were significantly downregulated in the tongues of Myf5-Cre;Smad4flox/flox mice at E13.5 and E14.5 (Fig. 6P-R), consistent with a fusion defect in tongue myoblasts of Myf5-Cre;Smad4flox/flox mice. Moreover, the expression level of cyclin D1, a cell cycle progression marker, in tongues of Myf5-Cre;Smad4flox/flox mice was comparable with that of control mice at the same stages (Fig. 6S), indicating that the compromised myoblast fusion in the tongues of Myf5-Cre;Smad4flox/flox mice is not the consequence of reduced myoblast number or decreased proliferation. In order to analyze whether the observed phenotype in Myf5-Cre;Smad4flox/flox mice is due to loss

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of either TGFβ or BMP signaling in myogenic cells, we examined the expression pattern of phospho-Smad1/5/8, the downstream effectors of BMP, and phospho-Smad3, the downstream effector of TGFβ. We found that both were strongly expressed in myogenic cells in the tongue of E13.5 control mice (supplementary material Fig. S2A-D), indicating that both TGFβ and BMP signaling pathways were activated and may regulate tongue myogenesis. Moreover, at the newborn stage, Myf5-Cre;Tgfbr2fl/fl mice exhibit microglossia, but the tongue of Myf5-Cre;Bmpr1afl/fl mice is indistinguishable from control (supplementary material Fig. S2E-P). It remains a possibility that other BMP receptors are expressed in tongue myogenic cells and regulate tongue myogenesis. Nevertheless, our results suggest that Smad4-mediated TGFβ signaling is required for myoblast fusion and myotube formation.

CNC-derived tendon formation is independent of Smad4-mediated TGFβ signaling in myogenic cells during tongue morphogenesis

Muscles and tendons interact during fetal myogenesis (Edom-Vovard and Duprez, 2004). In the trunk and limb region, the differentiation and maintenance of tendon cells depends on their interaction with well-differentiated muscle cells (Schweitzer et al., 2010). We have previously shown that tissue-tissue interaction is crucial during tongue morphogenesis (Hosokawa et al., 2010). In Myf5-Cre;Smad4fl/fl mice, compromised myogenic cell differentiation might result in a defect in tendon cell differentiation via tissue-tissue interaction. To test this hypothesis, we analyzed the expression of a tendon marker, scleraxis, a bHLH transcription factor expressed in the mature tendons of limbs and trunk as well as their progenitors (Schweitzer et al., 2001). Scleraxis was expressed in the central septum of the intrinsic tongue muscles and in tendons of the genioglossus in control mice at E13.5, E14.5 and E15.5 (Fig. 7A-C). Although scleraxis expression was diminished in the intrinsic muscles of the tongue in E13.5 Myf5-Cre;Smad4fl/fl mice (Fig. 7D), probably owing to delayed development, the intensity and pattern of scleraxis expression in the tendons of the intrinsic tongue muscle and genioglossus were indistinguishable in control and Myf5-Cre;Smad4fl/fl mice at subsequent stages (Fig. 7E,F). To evaluate the differentiation of CNC-derived cells further, we analyzed the relative expression level of scleraxis and type I collagen. Type I collagen, the main component of connective tissue, is expressed in CNC-derived central septum and dense lamina propria during tongue morphogenesis (Hosokawa et al., 2010). Real-time PCR results showed that expression of scleraxis and type I collagen was significantly downregulated in the tongues of Myf5-Cre;Smad4fl/fl mice at E13.5 (Fig. 7G); however, no significant difference was detectable between control and Myf5-Cre;Smad4fl/fl mice at E14.5 and E15.5 (Fig. 7H,I). Therefore, we conclude that the differentiation and maintenance of CNC-derived tendon cells are independent of Smad4-mediated TGFβ signaling in myogenic cells during tongue morphogenesis.

FGF signaling functions downstream of Smad4 in regulating tongue myogenic cell differentiation

To elucidate the molecular mechanism of Smad4-mediated TGFβ signaling during tongue myogenesis, we performed microarray analysis to compare gene expression profiles of the tongue in control and Myf5-Cre;Smad4fl/fl mice at E13.5. We detected significant reductions in expression of several members of the FGF family, including Fgf4, Fgf5, Fgf6, Fgf7 and Fgf8. (All data are available at the NCBI GEO repository: www.ncbi.nlm.nih.gov/geo/ under Accession Number GSE35357; supplementary material Tables S1, S2.) Although numerous FGFs are expressed in developing skeletal muscle (Hébert et al., 1990), only Fgf6 and one
of its receptors, Fgfr4, exhibit a restricted expression profile predominantly in the myogenic lineage in developing and regenerating skeletal muscle (deLapeyrière et al., 1993; Han and Martin, 1993). Using in situ hybridization, we found that the expression pattern of Fgf6 exhibits dynamic changes from E12.5 to E16.5 (supplementary material Fig. S3). Fgf6 expression was restricted to myogenic cells and developing myotubes of the transverse intrinsic muscle of the tongue in control mice at E13.5 and E14.5 (Fig. 8A,E). Fgfr4 transcripts were expressed more broadly than Fgf6 transcripts at the same stage; Fgfr4 was expressed in myogenic cells and developing myotubes of the transverse, longitudinal, and vertical intrinsic tongue muscles and extrinsic muscles, such as genioglossus, in control mice at E13.5 and E14.5 (Fig. 8C,G). In Myf5-Cre;Smad4flox/flox mice, the expression level of Fgf6 and Fgfr4 was significantly reduced at E13.5 and E14.5 (Fig. 8B,D,F,H,I). Thus, our data suggest that there is a cell-autonomous requirement for Smad4-mediated TGFβ signaling to regulate Fgf6 and Fgfr4 expression directly or indirectly in myogenic cells during tongue myogenic differentiation.

**Partial rescue of tongue myoblast fusion in Myf5-Cre;Smad4flox/flox mice using exogenous Fgf6**

To test the hypothesis that Fgf6 acts downstream of Smad4-mediated TGFβ signaling to control myogenic differentiation and myoblast fusion, we performed rescue experiments using primary tongue cell culture from E13.5 embryos. The myoblasts from the control sample proliferated, differentiated, increased in cell length and fused with each other to form multinucleated myotubes (Fig. 9A). Addition of exogenous Fgf6 had no effect on control samples (Fig. 9B,E,F). In Myf5-Cre;Smad4flox/flox samples, there was a significant reduction in the myotube length and myoblast fusion index after 3 days culture (Fig. 9C,E,F). We found that the addition of exogenous Fgf6 resulted in an increase in the myotube length of Myf5-Cre;Smad4flox/flox samples (Fig. 9D). Statistical analyses revealed that myoblast fusion and myotube length increased in Myf5-Cre;Smad4flox/flox cell cultures treated with Fgf6, but were not completely restored to the control level (Fig. 9E,F). Furthermore, we analyzed the changes in the expression level of several myogenic differentiation and myoblast fusion-related genes after exogenous Fgf6 treatment. Results from real-time PCR analysis showed that the levels of the Fgf6 receptor Fgfr4, the myogenic differentiation determinant myogenin, and myoblast fusion-related genes caveolin 3, β1-integrin and prostacyclin were all significantly increased in the Myf5-Cre;Smad4flox/flox samples after exogenous Fgf6 treatment for 3 days (Fig. 9I-M). By contrast, cyclin D1 and Myod1 expression were not changed after treatment (Fig. 9G,H), suggesting that the addition of Fgf6 has no effect on proliferation in Myf5-Cre;Smad4flox/flox samples. Although exogenous Fgf6 treatment significantly increased the expression levels of these myogenic differentiation and myoblast fusion-related genes in Myf5-Cre;Smad4flox/flox samples, the expression level of these genes was not completely restored to the control level (Fig. 9I-M). Taken together, these results indicate that addition of exogenous Fgf6 in Myf5-Cre;Smad4flox/flox primary tongue cell culture partially rescues myoblast fusion, and we conclude that TGFβ-Smad4-Fgf6 signaling cascade plays an important role in regulating myogenic differentiation and myoblast fusion during tongue myogenesis (Fig. 9N,O).

**DISCUSSION**

Skeletal muscle development, growth and regeneration are governed by the precise regulation of signaling networks. In this study, we demonstrate that there is a cell-autonomous requirement for Smad4-mediated TGFβ signaling during tongue myogenic differentiation and myoblast fusion. Furthermore, we show that a TGFβ-Smad4-Fgf6 signaling cascade plays a crucial role in tongue skeletal muscle development.

**Smad4 is required for myogenic differentiation and myoblast fusion**

Previous in vitro and in vivo studies have led to the conclusion that TGFβ signaling is a potent repressor of differentiation for skeletal muscle (Biressi et al., 2007; Drooguet al., 2010).
Myostatin (GDF8), a member of the TGFβ superfamily, is a negative regulator of skeletal muscle development. Myostatin-null mice or mice in which the myostatin has been disrupted show enhanced skeletal muscle growth (Kambadur et al., 1997; McPherron et al., 1997). By contrast, a recent study reveals that over-expression of Bmp4 at the tips of chick limb skeletal muscles increases the number of fetal muscle progenitors and satellite cells, indicating that TGFβ superfamily members may also promote skeletal muscle development (Wang et al., 2010). Consistent with this, our study clearly shows that inactivation of Smad4 in tongue myogenic cells results in defects in myogenic differentiation and myoblast fusion, suggesting a positive role for TGFβ signaling in regulating tongue myogenesis. One possible explanation of the seemingly opposite functions of TGFβ superfamily members in myogenesis is that members of the TGFβ superfamily might regulate differential downstream target genes to control myogenesis.

A transcriptional regulatory network of the myogenic regulatory factor (MRF) family governs the determination and terminal differentiation of muscle cells during skeletal muscle formation. MyoD1 is essential for progenitor cell commitment to the myogenic lineage, whereas myogenin plays a crucial role in
mediated TGFα signaling is crucial for myoblast terminal differentiation during tongue myogenesis. Moreover, in Myf5-Cre;Smad4flox/flox mice, we detected compromised myogenin transcript expression not only in tongue myogenic cells, but also in head muscles, including masseter and extraocular muscles. Although a recent study shows that distinct regulatory cascades regulate extraocular and branchiomeric muscle progenitor cell fates (Sambasivan et al., 2009), our results suggest that Smad4-mediated TGFα signaling is universally required by skeletal muscle progenitor cells in the craniofacial region to induce myogenin expression, which allows lineage progression and promotes myoblast terminal differentiation.

Myoblast fusion is a key cellular process that shapes the formation and repair of muscle. In vitro data suggest that myoblast fusion can be further partitioned into two phases. First, individual myoblasts undergo fusion with one another to generate nascent myotubes, which retain few nuclei. In the second phase of fusion, additional differentiated myoblasts incorporate into the forming myotube, leading to the further maturation of the nascent myofiber during which the myofiber increases in size and begins to express contractile proteins (Rochlin et al., 2010). Following the fusion of myoblasts into multinucleated myofibers, myonuclei move to a peripheral position and spread along the length of the myofiber. We found that, in Myf5-Cre;Smad4flox/flox mice, the myoblast fusion defect in the tongue muscles leads to an atrophic phenotype, with both reduced myotube length and reduced average myonuclei number per myotube. Moreover, in Myf5-Cre;Smad4flox/flox mice tongue muscle, numerous nuclei are located in the center of the muscle fibers, instead of at the periphery. Improperly positioned nuclei are a hallmark of numerous muscle diseases in human, including centronuclear myopathy (Romero, 2010). Individuals with this disease show severe muscle weakness and low muscle tone. Thus, the central location of the myonuclei in tongue muscle of Myf5-Cre;Smad4flox/flox mice suggests that muscle contractile function may be compromised.

**CNC-derived tendon formation is independent of Smad4-mediated muscle development in the tongue**

Muscle and tendon interactions during myogenesis are crucial for their development (Schweitzer et al., 2010). Although the major molecular regulators of tendon induction and differentiation may be shared throughout the vertebrate body, the cellular dynamics and muscle-tendon interactions directing these processes may vary in different sections of the body. In the trunk and limb regions, myogenic cells, tendon cells and their surrounding tissue are derived from mesoderm. The induction of axial tendon progenitors in the trunk depends on signals from the myotome. In limb buds, the induction of tendon progenitors is independent of muscle; however, signals from the muscles are essential for tendon differentiation at subsequent stages (Kardon, 1998; Eloy-Trinquet et al., 2009). The tendons of branchiomeric muscles are derived from the CNC, whereas branchiomeric muscles differentiate from the mesodermal core of the branchial arches (Trainor et al., 1994). As in the limb, the induction of tendon progenitors of branchiomeric muscles does not depend on muscle. For example, in Tbx1−/− null mice, the branchiomeric muscles fail to form or are severely reduced in size. Although the induction of tendons of branchiomeric muscles is normal, tendon cell differentiation fails in the Tbx1−/− mutants by E15.5, demonstrating that tendon differentiation depends on an interaction with branchiomeric muscle (Grifone et al., 2008; Grenier et al., 2009). The tongue is unique because its myogenic progenitor cells migrate from occipital somites and its tendons arise from CNC cells. Moreover, the anatomical site of the tongue, located between the head and trunk, suggests that the regulatory mechanism of tendon differentiation in the tongue is independent of Smad4-mediated signals from the muscle. Because Myf5-Cre;Smad4flox/flox mice are the first mesoderm-specific conditional knockout model for the study of tongue muscle development, it remains to be seen whether the muscle-independent tendon development is specific for Myf5-Cre;Smad4flox/flox mice or a universal mechanism during tongue morphogenesis. Nevertheless, muscle-independent tendon formation and repair of muscle is not affected, suggesting that early myoblast determination does not rely on Smad4-mediated TGFα signaling, but Smad4-mediated TGFβ signaling is crucial for myoblast terminal differentiation during tongue myogenesis. Moreover, in Myf5-Cre;Smad4flox/flox mice, we detected compromised myogenin transcript expression not only in tongue myogenic cells, but also in head muscles, including masseter and extraocular muscles. Although a recent study shows that distinct regulatory cascades regulate extraocular and branchiomeric muscle progenitor cell fates (Sambasivan et al., 2009), our results suggest that Smad4-mediated TGFα signaling is universally required by skeletal muscle progenitor cells in the craniofacial region to induce myogenin expression, which allows lineage progression and promotes myoblast terminal differentiation.

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development in the tongue suggests that muscle-tendon interactions in the tongue may be different from that of the trunk, limb and branchiomeric muscles.

**Smad4 is upstream of FGF signaling in regulating myogenic differentiation and myoblast fusion during tongue development**

Among the FGF family members, Fgf6 exhibits a restricted expression profile predominantly in the myogenic lineage in adult and developing skeletal muscle (deLapeyrière et al., 1993; Han and Martin, 1993), suggesting that it may be a component of signaling events associated with somite formation (Grass et al., 1996) and the regeneration process of adult muscle (Zhao and Hoffman, 2004). Fgf6 induces a transduction signal, preferentially via Fgfr1 and Fgfr4 (Zhang et al., 2006). In vitro analysis indicates that both Fgf6 and Fgfr4 are uniquely expressed by myofibers and satellite cells, whereas Fgfr1 is ubiquitously expressed by myogenic and nonmyogenic cells (Kästner et al., 2000). Moreover, during muscle regeneration, Fgf6 and Fgfr4 proteins are strongly expressed in differentiating myoblasts and newly formed myotubes, suggesting that Fgfr4 is probably the key receptor for Fgf6 during muscle regeneration (Zhao and Hoffman, 2004).

The expression patterns of Fgf6 and Fgfr4 transcripts are not completely overlapping in the tongues of E12.5 to E16.5 mouse embryos. Fgfr4 transcripts are more widespread than Fgf6 transcripts. The Fgfr6 expression pattern shows dynamic changes during developmental stages. These dynamic changes may reflect the state of maturation of the muscle fibers and/or their future muscle fibers type. Alternatively, it is conceivable that Fgf6 might be secreted from the differentiating myoblasts and newly formed myotubes, and function via the more widespread Fgfr4 to regulate tongue myogenesis. However, based on the mRNA expression...
Significantly, we have demonstrated that expression of Fgf6 and Fgf4 mRNA and protein was dramatically downregulated following the loss of Smad4 in vivo, suggesting that both Fgf6 and Fgf4 can be directly or indirectly regulated by Smad4-mediated TGFβ signaling during tongue myogenic differentiation and myoblast fusion.

Fgf6 is involved in the control of both phases of skeletal muscle myogenesis, proliferation and differentiation, depending on concentration and alternative receptor use (Pizette et al., 1996;Israeli et al., 2004). In vitro studies using muscle cell lines or primary satellite cells show that a low concentration of exogenous Fgf6 (5 ng/ml) increases the expression of a subset of myogenic differentiation markers and triggers myogenic differentiation. By contrast, a high concentration of Fgf6 (25 ng/ml) promotes opposing effects and stimulates myoblast proliferation (Pizette et al., 1996; Kästner et al., 2000). In our study, we show that exogenous Fgf6 (5 ng/ml) partially rescues the compromised tongue myoblast fusion of Myf5-Cre;Smad4<sup>fl/fl</sup> mice in vitro. One possible explanation for the partial rescue is that Fgf6 may require Fgfr4, or additional members of the FGF family, to regulate myogenic differentiation. Another possibility is that some transcription factors may also mediate TGFβ signaling to control myogenic differentiation and myoblast fusion during tongue development. Taken together, our study provides the first in vivo evidence that TGFβ relies on Smad4 to regulate Fgf6 and Fgfr4 expression during tongue myogenesis. The discovery of a genetic hierarchy involving TGFβ and FGF and the elucidation of its role in cell fate determination will greatly enhance our understanding of the molecular and cellular mechanisms involved in normal and abnormal tongue development. Information from this study may provide future therapeutic strategies to prevent and rescue tongue defects, and facilitate tongue regeneration following surgical resection.

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The authors declare no competing financial interests.

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