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

Dong Han[1,2], Hu Zhao[1], Carolina Parada[1], Joseph G. Hacia[3], Pablo Bringas, Jr[1] and Yang Chai[1,*]

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;Smad4$^{flox/flox}$* 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 the tongue of *Myf5-Cre;Smad4$^{flox/flox}$* mice, suggesting that the differentiation and maintenance of CNC-derived tendon cells are independent of Smad4-mediated signaling in myogenic cells in the tongue. Furthermore, loss 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;Smad4$^{flox/flox}$* 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 myogenesis.

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

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.

[1]Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA 90033, USA. [2]Department of Prosthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China. [3]Department of Biochemistry and Molecular Biology, Broad Center for Regenerative Medicine and Stem Cell Research, University of Southern California, Los Angeles, CA 90089, USA.

*Author for correspondence (ychai@usc.edu)

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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 TGF 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;Smad4$^{flox/flox}$* 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* (Tallquist et al., 2000), *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 carried out using PCR on tail tip or yolk sac DNA.

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 *Fgfr4* (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 western blot analysis. Bovine serum albumin served as a negative control and was not recognized by any of the antibodies tested.

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 $2^{\Delta\Delta Ct}$ 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;Smad4*$^{flox/+}$ control and *Myf5-Cre;Smad4*$^{flox/flox}$ mouse embryos, and at least 10 fibers were scored per sample. All measurements and counting were performed with Image-J 1.45k software. Results were assessed for statistical significance using Student's *t*-test.

Tongue mesenchymal cell culture

Tongue primordium was harvested from E13.5 embryos, treated with 2.4 U/ml Dispase I (Roche Applied Science) for 30 minutes at 37°C, then tongue epithelium was removed and tongue mesenchymal cells were isolated and cultured as previously described (Biressi et al., 2007). Where indicated, human recombinant Fgf6 (5 ng/ml; R&D) was added to the medium 24 hours after plating and re-added every 24 hours, followed by cell culture for 3 days. Each day, half of the medium was replaced with fresh medium. To quantify the level of myoblast fusion, we determined the myoblast fusion index as the percentage of myogenic cell nuclei present in myotubes compared with the total number of nuclei present in the observed field. Ten fields (40×) from each genotype were used for quantification of myotube length and myoblast fusion index.

FDG (fluorescein di-β-D-galactopyranoside) staining and fluorescence activated cell sorting (FACS)

At E14.5, tongue mesenchymal cells were isolated as described above. FDG staining and FACS analysis were performed as reported previously (Hosokawa et al., 2010).

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

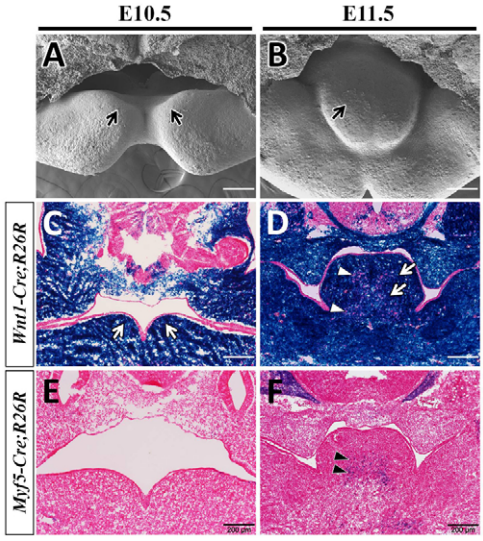

Fig. 1. The relationship between cranial neural crest and myogenic cells in the tongue buds and tongue primordium. (**A**,**B**) Scanning electron microscope images of the tongue buds (black arrows) at E10.5 (A) and tongue primordium (black arrow) at E11.5 (B) in C57BL/6J mouse embryos. (**C-F**) *lacZ* expression assayed by X-gal staining (blue) in sections from *Wnt1-Cre;R26R* (C,D) and *Myf5-Cre;R26R* (E,F) mice. White arrows indicate CNC-derived *lacZ*-positive cells in the tongue buds at E10.5 (C). CNC-derived *lacZ*-positive cells (white arrows) circumscribe the *lacZ*-negative cells (white arrowheads) at E11.5 (D). Myogenic *lacZ*-positive cells are not detectable in tongue buds at E10.5 (E), but a few *lacZ*-positive cells are detectable (black arrowheads) in the center of the tongue primordium at E11.5 (F). Scale bars: 200 μm.

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;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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 β-galactosidase staining on whole-mount embryos and in tissue sections of E10.5 *Myf5-Cre;Smad4$^{flox/+}$;R26R* control and *Myf5-Cre;Smad4$^{flox/flox}$;R26R* mice. Myogenic progenitors migrated from the occipital somites and started to invade the first branchial arch through the hypoglossal cord at E10.5 in control mice (Fig. 3A,C). Based on the presence of *lacZ* in sections, we measured the distance of myogenic progenitor migration and found there

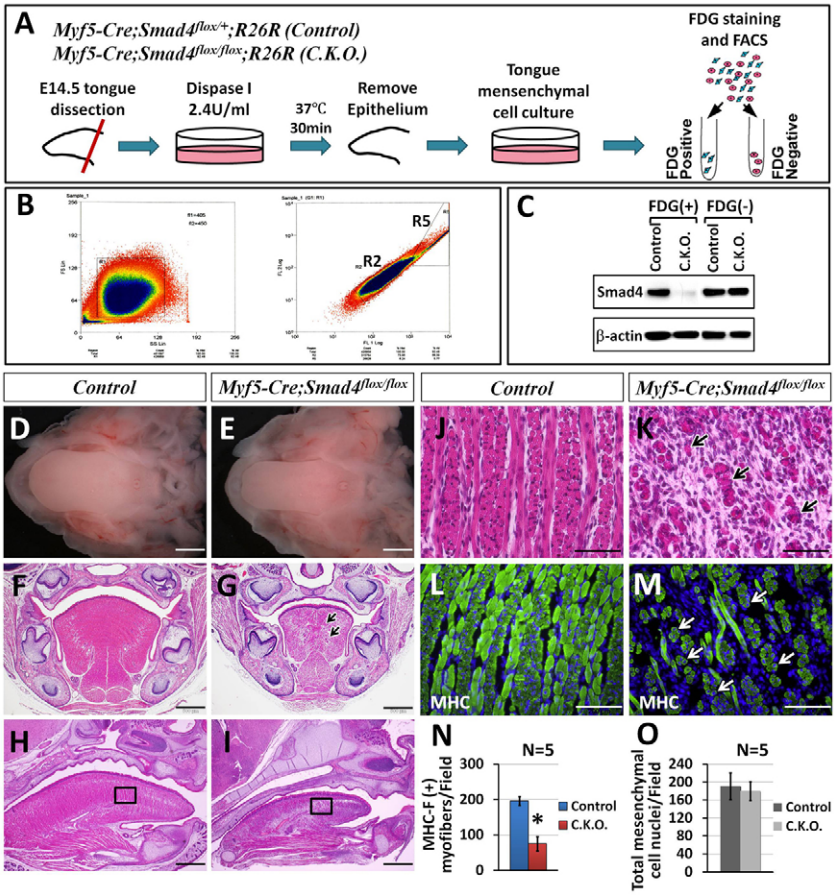

Fig. 2. *Myf5-Cre;Smad4$^{flox/flox}$* mice exhibit microglossia and a reduction of muscle fibers. (**A**) Schematic diagram of fluorescence activated cell sorting (FACS) approach based on fluorescein di-β-D-galactopyranoside (FDG) staining. (**B**) FACS plots of FDG-positive (myogenic cells, R5) and -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;Smad4$^{flox/flox}$;R26R* (C.K.O.) mice. (**D-M**) Macroscopic appearance (D,E), Hematoxylin and Eosin staining (F-K) and MHC immunofluorescence (L,M) of tongues from *Myf5-Cre;Smad4$^{flox/+}$* control (D,F,H,J,L) and *Myf5-Cre;Smad4$^{flox/flox}$* (E,G,I,K,M) newborn mice. (F-K) Black arrows indicate disorganized muscle fibers in *Myf5-Cre;Smad4$^{flox/flox}$* 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;Smad4$^{flox/flox}$* 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.

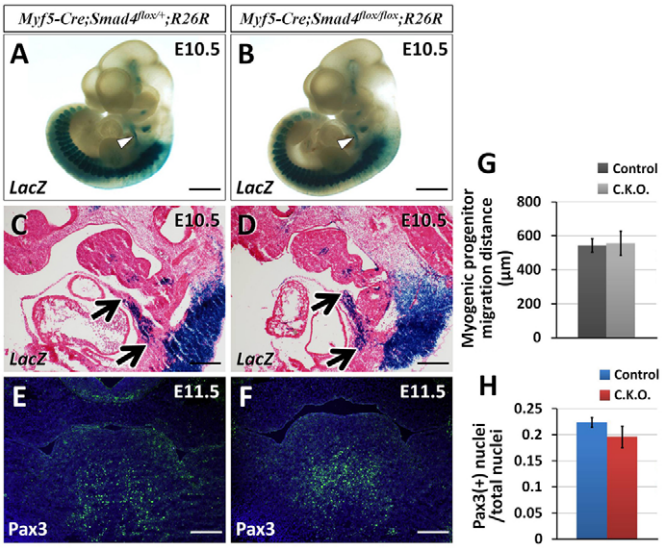

Fig. 3. Loss of *Smad4* in myogenic cells does not affect myogenic progenitor cell migration. (**A-D**) Whole-mount *lacZ* staining (A,B) and *lacZ* staining in sections (C,D) of E10.5 *Myf5-Cre;Smad4*$^{flox/+}$*;R26R* control (A,C) and *Myf5-Cre;Smad4*$^{flox/flox}$*;R26R* mouse embryos (B,D). White arrowheads in A and B indicate the myogenic progenitor cells migrating from the occipital somite to the tongue primordium through the hypoglossal cord. Black arrows in C and D indicate the starting points for the measurement of myogenic progenitor cell migration distance. (**E**,**F**) Immunofluorescence of Pax3 (Pax3, green; DAPI, blue) in E11.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (E) and *Myf5-Cre;Smad4*$^{flox/flox}$ (F) tongue primordia. (**G**,**H**) Quantitation of myogenic progenitor cell migration distance (G) from C and D, and the ratio of Pax3-positive nuclei (H) from E and F. Five randomly selected non-overlapping samples were used from each experimental group (n=3). Graphs show average ± s.d. Scale bars: 1 mm in A,B; 300 μm in C,D; 200 μm in E,F.

was no significant difference between control and *Myf5-Cre;Smad4*$^{flox/flox}$*;R26R* mice (Fig. 3B,D,G). In order to quantify the number of progenitor cells that arrived in the tongue primordium, we analyzed the expression of Pax3 at E11.5, because the migrating progenitor cells in the hypoglossal cord express Pax3 (Relaix et al., 2004). Pax3-positive cells were detectable by immunofluorescence in the tongue primordium (Fig. 3E,F), and the number of Pax3-positive cells in *Myf5-Cre;Smad4*$^{flox/flox}$ mice was comparable with that of control mice (Fig. 3H), indicating that myogenic progenitor cell migration is not compromised in the tongue of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. In order to investigate the cellular mechanism responsible for microglossia in *Myf5-Cre;Smad4*$^{flox/flox}$ mice, we examined cell proliferation and apoptosis. We found that proliferation of both myogenic and CNC-derived cells and apoptosis were unaffected in the tongue of *Myf5-Cre;Smad4*$^{flox/flox}$ mice at E12.5, E13.5 and E14.5 (Fig. 4A-N, supplementary material Fig. S1). Our data indicate that loss of *Smad4* in myogenic cells does not affect myogenic progenitor cell migration, proliferation or apoptosis during tongue myogenesis.

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

To define the progression of tongue myogenesis in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice at E13.5 (Fig. 5A-B′), and the number of MyoD1-positive cells in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice at E12.5 and E13.5 (Fig. 5D), indicating that the determination of myoblasts was unaffected in *Myf5-Cre;Smad4*$^{flox/flox}$ 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,E′). In *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice. We observed that the length of muscle fibers was significantly decreased in *Myf5-Cre;Smad4*$^{flox/flox}$ mice (Fig. 6A-C). Moreover, the number of nuclei contained in each muscle fiber was significantly reduced in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice (Fig. 6E-L). Statistical analyses revealed that the myoblast fusion index and myotube length were significantly reduced in *Myf5-Cre;Smad4*$^{flox/flox}$ samples (Fig. 6M,N); however, the number of Myod1-positive cells in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice at E13.5 and E14.5 (Fig. 6P-R), consistent with a fusion defect in tongue myoblasts of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. Moreover, the expression level of cyclin D1, a cell cycle progression marker, in tongues of *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice is not the consequence of reduced myoblast number or decreased proliferation. In order to analyze whether the observed phenotype in *Myf5-Cre;Smad4*$^{flox/flox}$ mice is due to loss

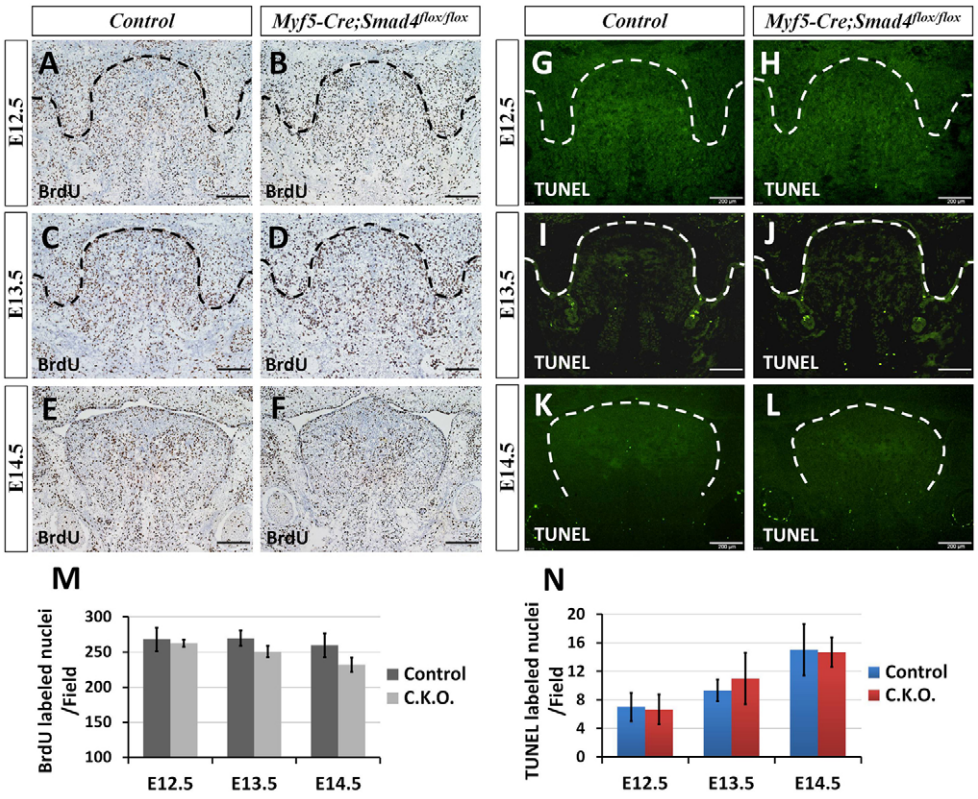

Fig. 4. Loss of *Smad4* in myogenic cells does not affect myogenic progenitor cell proliferation or apoptosis. (**A-F**) BrdU incorporation analysis of E12.5, E13.5 and E14.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (A,C,E) and *Myf5-Cre;Smad4*$^{flox/flox}$ (B,D,F) mice. Dashed lines indicate outline of the tongue. (**G-L**) TUNEL assays of E12.5, E13.5 and E14.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (G,I,K) and *Myf5-Cre;Smad4*$^{flox/flox}$ mice (H,J,L). Dashed lines indicate outline of the tongue. (**M**,**N**) Quantitation of BrdU incorporation (M) and TUNEL (N) data from A-L. Five randomly selected non-overlapping samples were analyzed from each experimental group (n=3). Graphs show average ± s.d. Scale bars: 200 μm.

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;Tgfbr2*$^{flox/flox}$ mice exhibit microglossia, but the tongue of *Myf5-Cre;Bmpr1a*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice at E13.5 (Fig. 7G); however, no significant difference was detectable between control and *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice at E13.5. We detected significant reductions in expression of several members of the FGF family, including *Fgf4*, *Fgf5*, *Fgf6*, *Fgf7* and *Fgfr4*. (All data are available at the NCBI GEO repository: www.ncbi.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

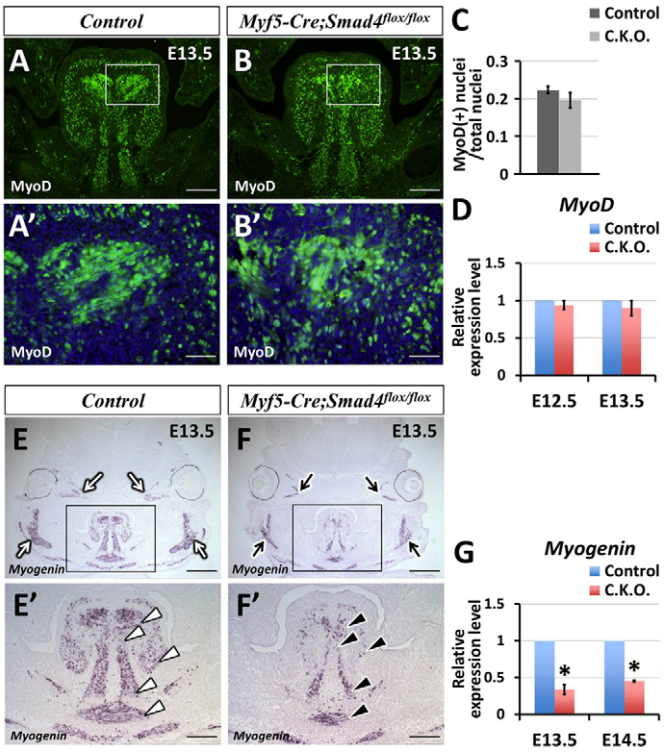

Fig. 5. Myogenic differentiation is compromised in the tongues of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**A-B'**) Immunofluorescence of MyoD1 (MyoD1, green; DAPI, blue) in the tongue primordia of E13.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (A,A') and *Myf5-Cre;Smad4*$^{flox/flox}$ (B,B') mice. Boxed areas in A and B are shown magnified in A' and B'. (**C**,**D**) Quantitation of MyoD1-positive nuclei number (C) and real-time PCR for *Myod1* relative expression level (D) using tongue primordia from E12.5 and E13.5 *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice. (**E-F'**) In situ hybridization of myogenin in E13.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (E,E') and *Myf5-Cre;Smad4*$^{flox/flox}$ mice (F,F') tongue primordia. Boxed areas in E and F are shown magnified in E' and F'. (E) White arrows indicate myogenin expression in masseter and extraocular muscles in *Myf5-Cre;Smad4*$^{flox/+}$ control mice. (F) Black arrows indicate diminished expression of myogenin in masseter and extraocular muscles in *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (E') White arrowheads indicate myogenin expression in intrinsic and extrinsic muscles of tongue in *Myf5-Cre;Smad4*$^{flox/+}$ control mice. (F') Black arrowheads indicate diminished expression of myogenin in *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**G**) Real-time PCR for myogenin relative expression level using tongue primordia from E13.5 and E14.5 *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice. Values are expressed relative to control. Graphs show average ± s.d. **P*<0.05; *n*=3. Scale bars: 200 μm in A,B,E',F'; 50 μm in A',B'; 500 μm in E,F.

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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ samples (Fig. 9D). Statistical analyses revealed that myoblast fusion and myotube length increased in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ samples. Although exogenous Fgf6 treatment significantly increased the expression levels of these myogenic differentiation and myoblast fusion-related genes in *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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; Droguett et al., 2010).

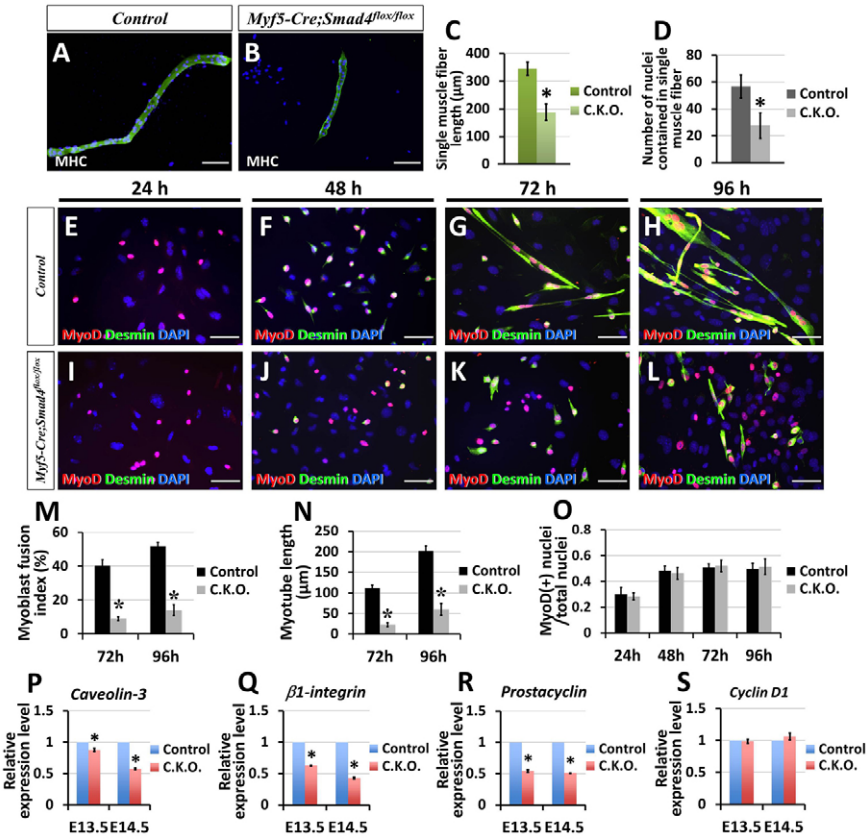

Fig. 6. Defective myoblast fusion in the tongues of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**A**,**B**) Immunofluorescence of MHC (MHC, green; DAPI, blue) in single tongue muscle fibers from E18.5 *Myf5-Cre;Smad4*$^{flox/+}$ control (A) and *Myf5-Cre;Smad4*$^{flox/flox}$ (B) mice. (**C**,**D**) Quantitation of the length of single muscle fibers (C) and the number of nuclei contained in single muscle fibers (D) isolated from E18.5 control and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice. **P*<0.05; *n*=3. (**E-L**) Immunofluorescence of MyoD1 and desmin (MyoD1, red; desmin, green; DAPI, blue) in *Myf5-Cre;Smad4*$^{flox/+}$ control (E-H) and *Myf5-Cre;Smad4*$^{flox/flox}$ (I-L) tongue mesenchymal cells at 24 hours, 48 hours, 72 hours and 96 hours after plating. (**M-O**) Quantitation of myoblast fusion index (M), myotube length (N) and the ratio of MyoD1-positive nuclei number (O) in *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) primary tongue mesenchymal cell culture. **P*<0.05; *n*=5. (**P-S**) Real-time PCR analysis of caveolin 3 (P), β1-integrin (Q), prostacyclin (R) and cyclin D1 (S) expressed by myoblasts in tongue primordia from *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice at E13.5 and E14.5. Values are expressed relative to control. Graphs show average ± s.d. **P*<0.05; *n*=3. Scale bars: 100 μm in A,B; 50 μm in E-L.

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 the

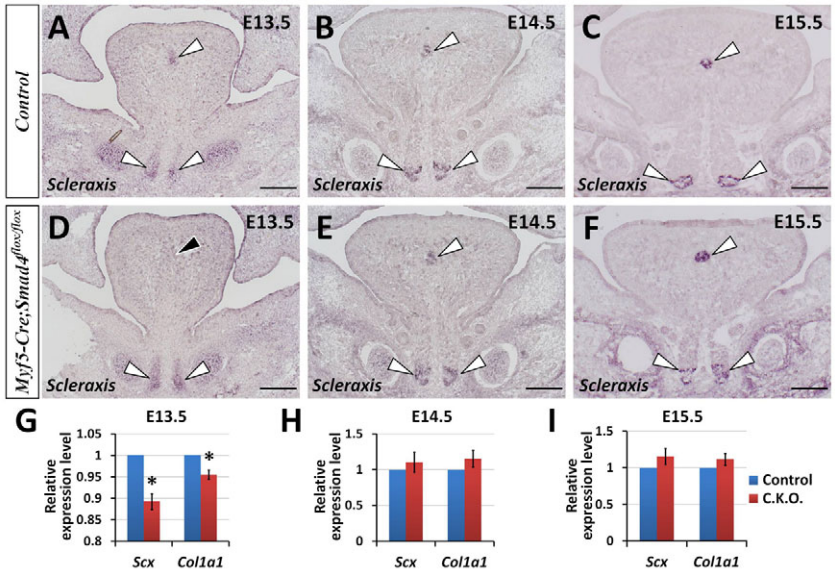

Fig. 7. CNC-derived tendon cell differentiation is unaffected in tongues of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**A-F**) In situ hybridization of scleraxis in *Myf5-Cre;Smad4*$^{flox/+}$ control (A-C) and *Myf5-Cre;Smad4*$^{flox/flox}$ (D-F) mice at E13.5, E14.5 and E15.5. (A-C) White arrowheads indicate scleraxis expression in the tongue septum of the intrinsic muscles and tendons of the genioglossus in control mice. (D-F) Black arrowhead indicates the lack of scleraxis expression in the tongue septum of the intrinsic muscles in E13.5 *Myf5-Cre;Smad4*$^{flox/flox}$ mice (D), but white arrowheads show scleraxis expression in *Myf5-Cre;Smad4*$^{flox/flox}$ mice that is comparable with control. (**G-I**) Real-time PCR analysis of scleraxis (*Scx*) and type I collagen (*Col1a1*) expressed by CNC-derived cells using tongue primordia from *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice at E13.5 (G), E14.5 (H) and E15.5 (I). Values are expressed relative to control. Graphs show average ± s.d. **P*<0.05; *n*=3. Scale bars: 200 μm.

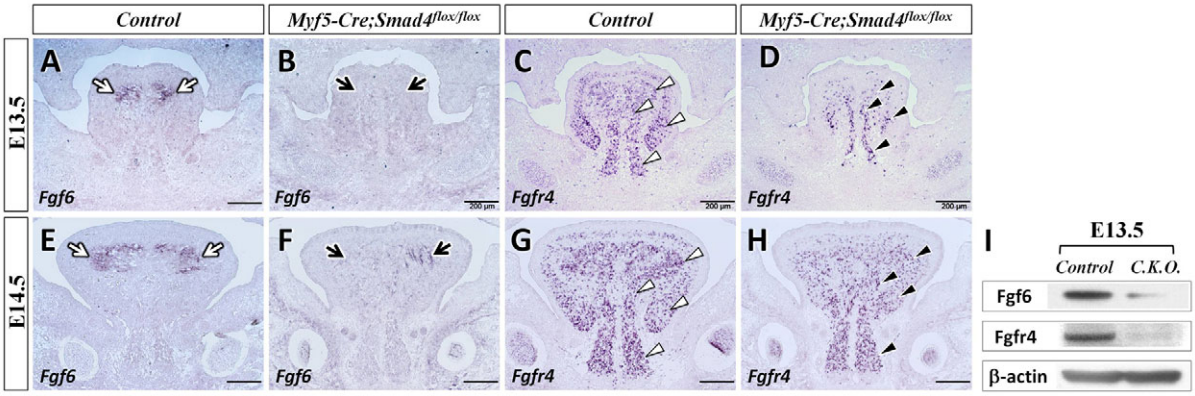

Fig. 8. *Fgf6* and *Fgfr4* expression is altered in the tongue primordia of *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**A-H**) In situ hybridization of *Fgf6* (A,B,E,F) and *Fgfr4* (C,D,G,H) in *Myf5-Cre;Smad4*$^{flox/+}$ control (A,C,E,G) and *Myf5-Cre;Smad4*$^{flox/flox}$ (B,D,F,H) mice at E13.5 (A-D) and E14.5 (E-H). (A,B,E,F) White arrows indicate *Fgf6* expression restricted to developing myotubes of tongue transverse muscles in control mice (A,E), black arrows indicate the lack of *Fgf6* expression in *Myf5-Cre;Smad4*$^{flox/flox}$ mice (B,F). (C,D,G,H) White arrowheads indicate wide expression of *Fgfr4* in the myogenic cells and developing myotubes of the tongue intrinsic muscles and genioglossus in control mice (C,G), but *Fgfr4* expression appears significantly reduced in *Myf5-Cre;Smad4*$^{flox/flox}$ mice (black arrowheads; D,H). (**I**) Western blot analysis of Fgf6 and Fgfr4 in the tongue mesenchyme of E13.5 *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) mice. Scale bars: 200 μm.

terminal differentiation of committed myoblasts (Braun and Gautel, 2011). In *Myf5-Cre;Smad4*$^{flox/flox}$ mice, myogenin expression is compromised, but MyoD1 expression is not affected, suggesting that early myoblast determination does not rely on Smad4-mediated TGFβ signaling, but Smad4-mediated TGFβ signaling is crucial for myoblast terminal differentiation during tongue myogenesis. Moreover, in *Myf5-Cre;Smad4*$^{flox/flox}$ 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 TGFβ 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 contain 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ 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 tongue tendon development and muscle-tendon interaction may differ from that of trunk or head. Strikingly, our data suggest that CNC-derived tendon differentiation in the tongue is independent of Smad4-mediated signals from the muscle. Because *Myf5-Cre;Smad4*$^{flox/flox}$ 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;Smad4*$^{flox/flox}$ mice or a universal mechanism during tongue morphogenesis. Nevertheless, muscle-independent tendon

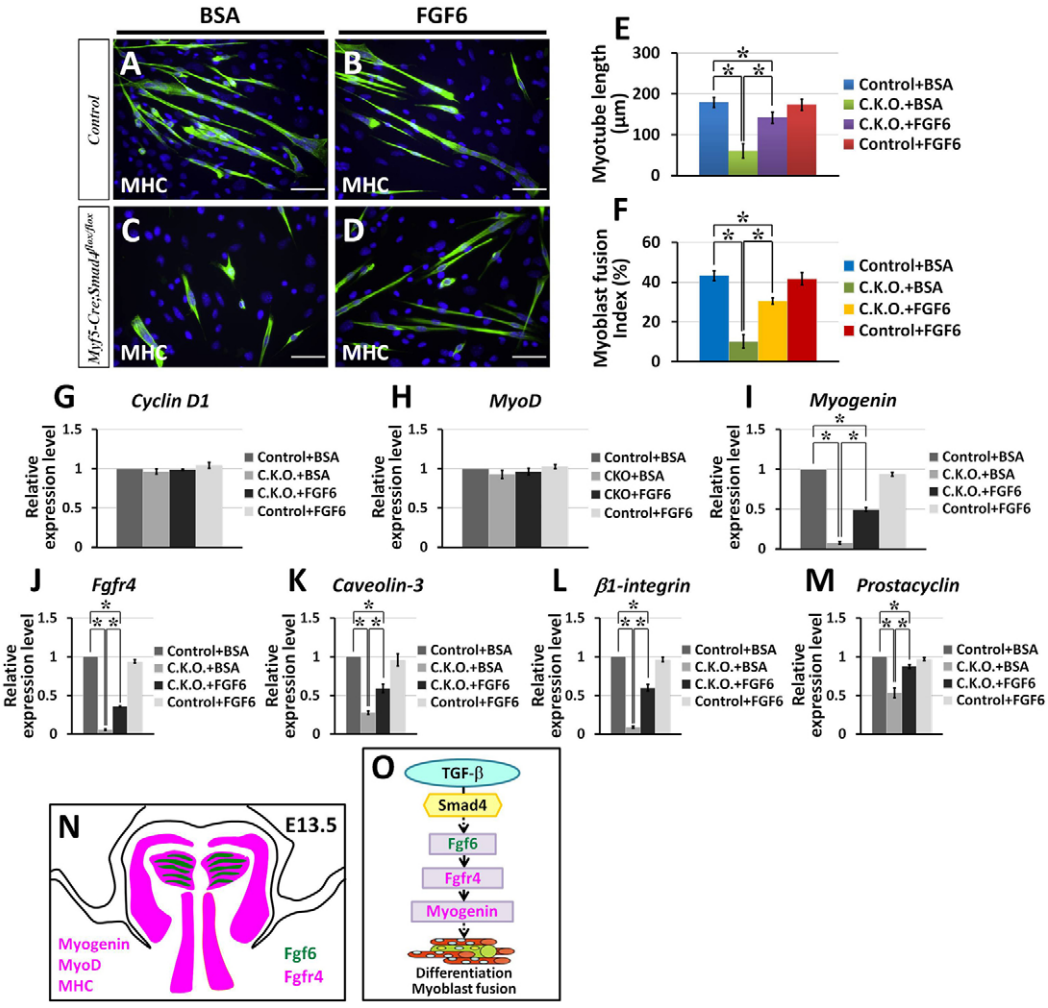

Fig. 9. Exogenous Fgf6 partially rescues the myoblast fusion defect in *Myf5-Cre;Smad4*$^{flox/flox}$ mice. (**A-D**) MHC immunofluorescence (MHC, green; DAPI, blue) in *Myf5-Cre;Smad4*$^{flox/+}$ control (A,B) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C,D) primary cell culture treated with BSA or Fgf6. (**E**,**F**) Quantitation of myotube length (E) and myoblast fusion index (F) in *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) primary cell culture treated with BSA or Fgf6. **P*<0.05; *n*=5. (**G-M**) Real-time PCR analysis of cyclin D1 (G), Myod1 (H), myogenin (I), *Fgfr4* (J), caveolin 3 (K), β1-integrin (L) and prostacyclin (M) in *Myf5-Cre;Smad4*$^{flox/+}$ (control) and *Myf5-Cre;Smad4*$^{flox/flox}$ (C.K.O.) primary cell culture after BSA or Fgf6 treatment. Values are expressed relative to *Myf5-Cre;Smad4*$^{flox/+}$ (control) samples treated with BSA. **P*<0.05; *n*=3. (**N**) The expression pattern in E13.5 mouse embryo tongue of genes and proteins involved in regulating tongue myogenesis that have been investigated in this study: Fgf6 (green), and Fgfr4, myogenin, MyoD1 and MHC (pink). (**O**) Summary diagram of a TGFβ-Smad4-Fgf6 signaling cascade in regulating tongue myogenesis. Dashed lines indicate that regulation might be indirect. Graphs show average ± s.d. Scale bars: 50 μm.

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 *Fgf6* 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

pattern, even considering diffusion, Fgf6 seems unlikely to be the only ligand that activates Fgfr4 to control myogenesis in tongue. Whether these other Fgf ligands are also under the control of Smad4-mediated TGFβ signaling remains to be determined. Significantly, we have demonstrated that expression of Fgf6 and Fgfr4 mRNA and protein was dramatically downregulated following the loss of *Smad4* in vivo, suggesting that both *Fgf6* and *Fgfr4* 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$^{flox/flox}$* 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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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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