Noncanonical Transforming Growth Factor β (TGFβ)
Signaling in Cranial Neural Crest Cells Causes Tongue Muscle
Developmental Defects*″§

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Background: TGFβ signaling is required in cranial neural crest (CNC) cells during tongue development. Results: TGFβ-mediated ABL1 activation in CNC cells results in altered Fgf4 and Fst gene expression and a failure of muscle development. Conclusion: TGFβ activates the ABL1 pathway in the absence of TGFβ receptor type II (TβRII) during tongue development. Significance: Activation of noncanonical TGFβ signaling causes microglossia.

Microglossia is a congenital birth defect in humans and adversely impacts quality of life. In vertebrates, tongue muscle derives from the cranial mesoderm, whereas tendons and connective tissues in the craniofacial region originate from cranial neural crest (CNC) cells. Loss of transforming growth factor β (TGFβ) type II receptor in CNC cells in mice (Tgfbrii−/−;Wnt1-Cre) causes microglossia due to a failure of cell-cell communication between cranial mesoderm and CNC cells during tongue development. However, it is still unclear how TGFβ signaling in CNC cells regulates the fate of mesoderm-derived myoblasts during tongue development. Here we show that activation of the cytoplasmic and nuclear tyrosine kinase 1 (ABL1) cascade in Tgfbrii−/−;Wnt1-Cre mice results in a failure of CNC-derived cell differentiation followed by a disruption of TGFβ-mediated induction of growth factors and reduction of myogenic cell proliferation and differentiation activities. Among the affected growth factors, the addition of fibroblast growth factor 4 (FGF4) and neutralizing antibody for follistatin (FST; an antagonist of bone morphogenetic protein (BMP)) could most efficiently restore cell proliferation, differentiation, and organization of muscle cells in the tongue of Tgfbrii−/−;Wnt1-Cre mice. Thus, our data indicate that CNC-derived fibroblasts regulate the fate of mesoderm-derived myoblasts through TGFβ-mediated regulation of FGF and BMP signaling during tongue development.

Craniofacial development requires the orchestrated integration of multiple tissue-tissue interactions (1). Previous fate-mapping experiments have identified the derivatives of CNC3 cells and cranial mesoderm in the craniofacial region (1); however, the spatial and temporal relationships and interactions between these cell types are not completely understood. In vertebrates, tendons and connective tissues are derived from the lateral mesoderm, except in the craniofacial region where CNC-derived cells give rise to tendons and connective tissues in the skeletal muscles (2). Therefore, the genetic program controlling craniofacial muscle specification is distinct from that underlying trunk and limb myogenesis.

The tongue is a muscular organ in which CNC and cranial mesodermal cells interact, and it has important physiological functions in suckling, swallowing, speech, gustation, and mastication (3). The tongue is composed of intrinsic (situated within the tongue) and extrinsic (originating outside the tongue) muscles and has the capacity for complicated movements due to the muscles’ unique feature of having only one end tethered to bony structures. CNC cells give rise to tenocytes and fibroblasts that overlie the muscle fibers and provide the connective tissue in the tongue (4, 5). Twenty-two medical syndromes, including acrorenal mandibular syndrome, aglosi-adactyilia syndrome, and Freeman-Sheldon syndrome, among others, exhibit microglossia without muscular defects in the rest of the body (6). Therefore, it is crucial to investigate the regulatory mechanism of tongue morphogenesis. Furthermore, it appears that CNC and myogenic progenitor cells interact to regulate tongue development. This process provides the opportunity to investigate the functional significance of cell-cell interactions during organogenesis.

TGFβ signaling plays a role in craniofacial development, and loss of Tgfb2 in CNC cells causes craniofacial defects including microglossia (1, 7). TGFβ transmits signals through canonical and noncanonical pathways in physiological and pathological

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All scaled gene expression scores and .cel files are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository www.ncbi.nih.gov/geo/ under Series Accession Number GSE45968.

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3 The abbreviations used are: CNC, cranial neural crest; ABL1, cytoplasmic and nuclear tyrosine kinase 1; AZAN, azocarmine G and aniline blue; BMP, bone morphogenetic protein; E13.5, embryonic day 13.5; FDR, false discovery rate; FST, follistatin; TβRII, TGFβ receptor type I; TβRIII, TGFβ receptor type II; TβRIII, TGFβ receptor type III; microCT, microcomputed tomography; MyHC, myosin heavy chain.
conditions (8–10), targeting a variety of genes in a developmental stage-dependent and cell type-specific manner (11, 12). Nevertheless, it is still unclear how TGFβ signaling in CNC cells is regulated during tongue development and how CNC cells interact with mesoderm-derived myoblasts.

In this study, we found that noncanonical TGFβ signaling through the type I receptor resulted in ABL1 activation in Tgfbr2 mutant CNC cells, a failure of CNC cell differentiation, and compromised TGFβ-mediated gene expression of growth factors followed by a failure of tongue muscle development. Significantly, a haploinsufficiency of Tgfbr1 (also known as Alk5) normalized altered ABL1 activation in Tgfbr2 mutant CNC cells and rescued defects in muscle cell proliferation and differentiation via BMP and FGF pathways. Our findings indicate that tissue-specific TGFβ signaling in CNC cells regulates the fate of mesoderm-derived myoblasts during tongue development.

EXPERIMENTAL PROCEDURES

Animals—To generate Tgfbr2fl/fl;Wnt1-Cre mice, we mated Tgfbr2fl/fl;Wnt1-Cre with Tgfbr2fl/fl mice. To generate Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice, we mated Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ with Tgfbr2fl/fl;Alk5fl/+ mice. Genotyping was performed using PCR primers as described previously (9).

Phosphoprotein Profiling by the TGFβ Signaling Phospho-specific Antibody Microarray—The TGFβ signaling phospho-specific antibody microarray, which was designed and manufactured by Full Moon Biosystems, Inc. (Sunnyvale, CA), contains 176 highly specific and well characterized phosphorylation antibodies. Each of the antibodies has six replicates that are printed on a coated glass microscope slide, along with the non-phospho pairs of the phospho-specific antibodies to compare expression levels based on the phosphorylation state. The antibody array experiment was performed according to the manufacturer’s established protocol (Full Moon Biosystems, Inc.). A 95% confidence interval was used to quantify the precision of the phosphorylation signal ratio change based on analysis of the replicates.

Microarray Analysis—Total RNA samples (1 μg/sample) were converted into biotin-labeled cRNA using the EnzoTMBioArray terminal labeling kit with biotin-ddUTP and standard protocols recommended by Affymetrix (Santa Clara, CA). Fragmented cDNA was applied to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) that contain probe sets designed to detect over 39,000 transcripts. Microarrays were hybridized, processed, and scanned, as described previously (9). WebArray software was used to generate scaled log2 transformed gene expression values using the robust multiarray average algorithm (13, 14). Probe sets showing ≥1.5-fold differential expression with a <5% false discovery rate (FDR) were identified through Linear Models for Microarray Data (LIMMA)-based linear model statistical analysis (15), and FDR calculations made using the spacings LOESS histogram (SPLOSH) method (16).

Cell Culture—Primary mouse tongue mesenchymal cells were isolated from embryonic day 13.5 (E13.5) tongue of Tgfbr2fl/fl; Wnt1-Cre and Tgfbr2fl/fl mice and then cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplemented with penicillin, streptomycin, l-glutamate, sodium pyruvate, and nonessential amino acids. Primary mouse tongue mesenchymal cells were treated with TGFβ1 (10 ng/ml) or nilotinib (10 μM) for the indicated time (17).

Histological Examination—Hematoxylin and eosin (H&E) staining and BrdU staining were performed as described previously (9). Gomori’s aldehyde fuchsin staining and azocarmine G and aniline blue (AZAN) staining were performed to investigate the distribution of elastic and collagen fibers, respectively. Immunohistochemical staining was performed as described previously (17–19). Antibodies used for immunohistochemistry were rabbit polyclonal antibody against tenascin C (Abcam), mouse monoclonal antibody against myosin heavy chain (Sigma), and rat monoclonal antibody against BrdU (Abcam). Fluorescence images were obtained using a fluorescence microscope (Model IX71, Olympus).

Immunoblotting Analysis—Immunoblots were performed as described previously (17, 18). Antibodies used for immunoblotting were as follows: rabbit polyclonal antibodies against ABL, phosphorylated ABL, protein kinase C (PKC)-θ, phosphorylated PKC-θ (Cell Signaling Technology), and tenasin C (Abcam); and mouse monoclonal antibody against GAPDH (Millipore).

Tongue Organ Culture—Timed-pregnant mice were sacrificed at E13.5. Genotyping was carried out as described above. Tongues were micro-dissected and cultured in serum-free chemically defined medium as previously described (9). Tongues were treated with beads containing BMP5, FGF4, FGF5, FGF6 (10 μg/ml), neutralizing antibody for FST or WNT10A (20 μg/ml), or a medium containing nilotinib (50 μM) for 24 or 72 h in culture and then harvested, fixed, and processed, for 4% p-formaldehyde/0.1% x glutaraldehyde, and processed to analyze histology.

MicroCT Analysis—MicroCT analysis was performed using a SCANCO μCT50 device at the University of Southern California Molecular Imaging Center. The microCT images were acquired with the x-ray source at 70 kilovoltage peak and 114 μA. The data were collected at a resolution of 10 μm. The reconstruction was done with AVIZO 7.1 (Visualization Sciences Group).

Quantitative RT-PCR—Total RNA was isolated from E14.5 mouse embryonic tongue dissected with the QIAshredder and RNeasy mini extraction kit (Qiagen), as described previously (17, 20). The following PCR primers were used: Bmp5, 5′-AGCTCTGTGCAGAGCGGGG-3′ and 5′-ATGATCCA-GTCTCGCCATCCACA-3′; Fgf4, 5′-GAAAGCAGACCGG-AAGAGCTT-3′ and 5′-GCCAGCGGTTCTCTTG-3′; Fgf5, 5′-GCAAAGTAAGTTGCTCCAC-3′ and 5′-ATTTGGGCA-TTTCATGGAGTTT-3′; Fgf6, 5′-AGGGTGGGGAGCCAC-CTGTGTG-3′ and 5′-GTCGTTGAAAGGGGGTGTG-3′; Wnt10a, 5′-TGTCGCCCAGTGTCGACCCACAC-3′ and 5′-ACAGAGGCGAGTAGGTTGGACACAC-3′; Fst, 5′-ATCCCTCGG-GTTTGTGTTAC-3′ and 5′-TGTGCCCTCTACCTCTCTC-3′; and Gapdh, 5′-AACATTGGCATTGGAAGG-3′ and 5′-ACAATGAGGAGGTAGAACA-3′. PCR primers for Tnc were purchased from Santa Cruz Biotechnology Inc.

Statistical Analysis—Two-tailed Student’s t tests were applied for statistical analysis. A p value ≤ 0.05 was considered statistically significant. For all graphs, data are represented as means ± S.D.
**RESULTS**

Identification of Altered TGFβ Signaling Activation in the Absence of TβRII in the Tongue—We have recently reported that loss of Tgfb2 induces abnormal p38 mitogen-activated protein kinase (MAPK) activation through the TGFβ2-mediated TβRI/TβRIII pathway and causes cleft palate in Tgfb2Δ/Δ, Wnt1-Cre mutant mice (9). This clearly suggests that loss of Tgfb2 may result in the activation of an alternative pathway during embryogenesis. To explore what TGFβ signaling pathway is altered in the tongue of Tgfb2Δ/Δ, Wnt1-Cre mice, we performed protein array analysis for phosphorylated proteins using E14.5 tongues of Tgfb2Δ/Δ control and Tgfb2Δ/Δ, Wnt1-Cre mice (Fig. 1, supplemental Table S1, and supplemental Table S2). As expected, SMAD3 phosphorylation on both Ser-213 and Ser-425 was decreased in E14.5 tongue of Tgfb2 mutant mice as compared with littermate control mice (1.24- and 1.52-fold changes, respectively). In addition, phosphorylation of p38 MAPK (Tyr-182 and Tyr-322) and MKK6 (Ser-207) was increased in E14.5 tongue of Tgfb2 mutant mice as compared with littermate controls (1.69- and 1.59-fold change, respectively), indicating that noncanonical TGFβ signaling is regulated in a tissue-specific manner (Fig. 1A). We confirmed the phosphorylation status of the proteins by immunoblotting (Fig. 1B).

To test whether the activation of the ABL1 and PKC pathways is induced by TGFβ, we treated primary mouse tongue mesenchymal cells with TGFβ1 (Fig. 1C). Tgfb2Δ/Δ control cells were responsive to TGFβ1 stimulation with a progressive increase in both ABL1 and PKC phosphorylation. In Tgfb2 mutant cells, ABL1, but not PKC, phosphorylation was increased after TGFβ1 stimulation. Thus, our data indicate that TGFβ primarily activates the ABL1 pathway in the absence of TβRII during tongue development.

CNC-derived cells form connective tissues composed of extracellular matrix including elastic and collagen fibers and glycoproteins to provide a niche for surrounding cells, including muscle cells (2). Previous studies have demonstrated that a niche is crucial for regulating the movement and function of growth differentiation factors (21–24). The mature connective tissue of limbs and trunk as well as their progenitors expresses specific molecules and plays a critical role in the morphogenesis of the embryo during embryonic development (25, 26).

We hypothesized that the formation of the niche for muscle cells is regulated by canonical or noncanonical TGFβ signaling, both of which are altered in Tgfb2Δ/Δ, Wnt1-Cre mice, resulting in defects in muscle cell proliferation, differentiation and organization. To explore the status of the niche for muscle cells, we analyzed the formation of the extracellular matrix including elastic and collagen fibers and tenascin C. Tenascin C is a large hexameric glycoprotein expressed in the extracellular matrix in developing vertebrate embryos (27). We investigated the distribution of elastic fibers by Gomori’s aldehyde fuchsin staining...
at E14.5 and E18.5 (Fig. 2A). In \textit{Tgfbr2}^fl/fl control mice, elastic fibers formed along with the muscle and epithelial layer. In contrast, elastic fibers were disorganized in the tongue of E18.5 \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice. In addition, elastic fibers in the tongue of \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice were thinner than those of the controls.

Similarly, to investigate the distribution of collagen fibers, we performed AZAN staining at E14.5 and E18.5 (Fig. 2B). In the tongues of E18.5 \textit{Tgfbr2}^fl/fl control mice, collagen fibers were well organized and formed along with the muscle and epithelial layer. In contrast, we found that collagen fibers were immature and disorganized in E18.5 \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice. Moreover, protein and gene expression of \textit{tenascin C} (\textit{Tnc}) was compromised in \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} tongue (Fig. 3, A–C), suggesting that loss of \textit{Tgfbr2} in CNC cells results in a defect in extracellular matrix formation. Taken together, the microenvironment of muscle cells was poorly developed and disorganized in the tongue of \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice.

To identify downstream targets of dysfunctional TGFβ signaling during tongue muscle development, we performed microarray analysis of the tongue tissue of E14.5 \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mutant and \textit{Tgfbr2}^fl/fl control mice (\textit{n} = 3 per genotype). In this comparison, we uncovered 294 probe sets representing transcripts that were differentially expressed (1.5-fold, <5% FDR), 163 more abundant in \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} and 131 more abundant in \textit{Tgfbr2}^fl/fl control mice (supplemental Table S3 and supplemental Table S4). The genes identified as being altered in \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} tongue were consistent with muscle defects, with significant reductions in the expression level of transcripts related to myoblast proliferation and differentiation.

To explore how CNC-derived fibroblasts regulate cell proliferation and differentiation of muscle fibers, we examined the expression of growth differentiation factors. Based on our

**FIGURE 3. Decreased tenascin C expression in the tongue of \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice.** A, immunohistochemical analysis of tenascin C (brown) in the tongue of E14.5 \textit{Tgfbr2}^fl/fl control and \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice. Boxed areas are shown enlarged below. Sections are counterstained with 0.03% methylene blue. Top images, scale bars 200 µm; lower images, scale bars, 50 µm. B, immunoblotting analysis of tenascin C in tongues from E14.5 \textit{Tgfbr2}^fl/fl control (lane 1) and \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} (lane 2) mice. C, quantitative RT-PCR analysis of tenascin C (\textit{Tnc}) in E14.5 tongues of \textit{Tgfbr2}^fl/fl control (white bars) and \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} (black bars) mice. *, \textit{p} < 0.05.

**FIGURE 4. Identification of molecules with altered expression in the tongue of \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice.** A, list of differentially expressed genes related to growth differentiation factors based on genome-wide expression profiling analysis of E14.5 tongues of \textit{Tgfbr2}^fl/fl and \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} mice. The ratio is based on the geometric means of expression scores derived from \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} and \textit{Tgfbr2}^fl/fl control mice. The FDR was calculated based on the genome-wide expression profiling analysis, as described under “Experimental Procedures.” All relevant transcripts with a ≥1.5-fold change with <5% FDR are shown. AFFY ID, affinity ID. B, quantitative RT-PCR analyses of the indicated genes in E14.5 tongues of \textit{Tgfbr2}^fl/fl control (white bars) and \textit{Tgfbr2}^fl/fl;\textit{Wnt1-Cre} (black bars) mice. *, \textit{p} < 0.05.
microarray data, transcript levels of Fst (BMP antagonist follistatin) and Wnt10a were significantly up-regulated, whereas transcript levels of Bmp5, Fgf4, Fgf5, and Fgf6 were significantly reduced in the tongues of Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice (Fig. 4A). We confirmed these changes by quantitative RT-PCR analysis (Fig. 4B). These results suggest that compromised TGF\(\beta\)-mediated BMP and FGF pathways in the tongue of Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice are candidates for involvement in defects of muscle cell proliferation and differentiation.

To test the functional significance of these growth factors during tongue development, we treated tongue explants with each growth factor that was decreased in the tongue of Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice (BMP5, FGF4, FGF5, and FGF6) or with neutralizing antibodies for molecules that were increased in the tongue of Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice (FST and WNT10A) in an \textit{ex vivo} organ culture system (Fig. 5, A and B). We found that implantation of beads containing FGF4 or neutralizing antibody for follistatin could partially rescue the cell proliferation defect in Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice. In contrast, implantation of beads containing BMP5, FGF5, FGF6, or neutralizing antibody for WNT10A failed to rescue the cell proliferation defect.

**FIGURE 5.** BMP and FGF family members rescue defects in cell proliferation and muscle differentiation in Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre tongue. A, BrdU staining (green) in the tongue of Tgfbr2\textsuperscript{fl/fl} (wild type control) or Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice after treatment with BSA, BMP5, FGF4, FGF5, or FGF6, or neutralizing antibody (NAb) for FST or WNT10A. Dotted line outlines bead. Nuclei are counterstained with DAPI. \(n=5\) per each treatment. Scale bar, 100 \(\mu\)m. B, quantitation of the percentage of BrdU-labeled nuclei in the tongues of Tgfbr2\textsuperscript{fl/fl} (white bar) and Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre (black bar) mice treated with the indicated molecules for 24 h. \(n=5\) per each treatment. *, \(p<0.05\). C, immunohistochemical analysis of MyHC (red) in the tongue of Tgfbr2\textsuperscript{fl/fl} (wild type control) or Tgfbr2\textsuperscript{fl/fl};Wnt1-Cre mice after treatment with indicated molecules. Nuclei are counterstained with DAPI. Dotted line outlines bead. \(n=5\) per each treatment. Scale bar, 100 \(\mu\)m.
Next, we tested whether these molecules play a role in the induction of muscle differentiation in the tongue. Exogenous FGF4, FGF5, FGF6, or neutralizing antibody for follistatin partially rescued the defect in muscle formation in Tgfbr2fl/fl;Wnt1-Cre mice, but beads containing BMP5 or neutralizing antibody for WNT10A failed to rescue the muscle formation (Fig. 5C). Collectively, these data suggest that TGFβ-mediated FGFs and BMPs originating from CNC cells are key regulators for tongue muscle development.

**Noncanonical TGFβ Signaling in CNC Cells Causes Microglossia in Tgfbr2 Mutant Mice**—We have recently reported that formation of a TβRI-TβRIII complex in the absence of TβRII induces noncanonical TGFβ signaling during palatal development (9). We hypothesized that a reduction of altered noncanonical TGFβ signaling may rescue microglossia in Tgfbr2fl/fl;Wnt1-Cre mice by blocking TβRI/TβRIII complex formation. In fact, a haploinsufficiency of Tgfbr1/Alk5 rescued microglossia in E18.5 Tgfbr2fl/fl;Wnt1-Cre mice (Fig. 6A). We measured the tongue volume of E18.5 mouse embryos from control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice by scanning microCT images for soft tissue (Fig. 6B). The microCT images were reconstructed into three-dimensional microCT images to investigate the volume of the tongue (n = 3 per each genotype). The volume of the tongue from Tgfbr2fl/fl;Wnt1-Cre mice was significantly smaller than that of controls, and tongue volume reduction in Tgfbr2fl/fl;Wnt1-Cre mice was partially rescued in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice (Fig. 6C).

To explore the cellular mechanism of the rescue of microglossia in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice, we analyzed cell proliferation and apoptosis activities in control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice. We found that cell proliferation was rescued in the tongue of Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice at E14.0 and E14.5 (Fig. 6D and E). We detected no change in apoptosis during tongue development in

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**FIGURE 6. Rescue of microglossia in Tgfbr2fl/fl;Wnt1-Cre mice by reduction of TβRI/ALK5.** A, morphology of the tongues of newborn Tgfbr2fl/fl control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice. B, microCT images (top and side views) of tongues from E18.5 Tgfbr2fl/fl control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice. The tongue is shown in red. C, volumetric analysis of the intrinsic tongue muscles using three-dimensional reconstruction images from microCT images of E18.5 Tgfbr2fl/fl control (blue), Tgfbr2fl/fl;Wnt1-Cre (red), and Tgfbr2fl/fl;Wnt1-Cre;Alk55+/+ (green) mice. **, p < 0.01. D, BrdU staining (brown) of tongues from Tgfbr2fl/fl control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk55+/+ mice at E14.0 and E14.5. Boxed areas are shown enlarged below. Sections are counterstained with 0.03% methylene blue. Top images, scale bars, 200 µm; lower images, scale bars, 50 µm. E, quantification of the number of BrdU-labeled nuclei in the tongues of control, Tgfbr2fl/fl;Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk55+/+ mice at E14.0 and E14.5. *, p < 0.05.
these mice (data not shown). Taken together, our data indicate that an activated alternative TGFβ signaling cascade causes a defect in cell proliferation in the tongue of Tgfbr2fl/fl;Wnt1-Cre mice.

Next, we assessed whether muscle fiber formation is rescued in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice using histological and microCT analyses (Fig. 7). Although the mass of tongue muscle was mostly restored in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice, Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice still have a defect in muscle fiber alignment similar to that seen in Tgfbr2fl/fl;Wnt1-Cre mice. To compare muscle development, we performed immunohistochemical analysis for myosin heavy chain (MyHC), a differentiation marker for myofibers, in control, Tgfbr2fl/fl; Wnt1-Cre, and Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice at E14.5 to E18.5 (Fig. 8). Although the number of myofibers in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice was almost comparable with that of control mice, we detected disorganized myofibers in Tgfbr2fl/fl;Wnt1-Cre mice. These results suggest that noncanonical TGFβ signaling affects myoblast proliferation and differentiation, whereas canonical TGFβ signaling is required for muscle organization.

Based on this hypothesis, we investigated ABL1 activation (Fig. 9A) and expression of target molecules (Fig. 9, B–F) in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice. We found that altered ABL1 activation and gene expression of Bmp5, Fgf4, and Fst were restored in Tgfbr2fl/fl;Wnt1-Cre;Alk5fl/+ mice, suggesting that
they are regulated by noncanonical TGFβ signaling. Gene and protein expression of tenascin C was also normalized in Tgfb2Δ/Δ; Wnt1-Cre, Wnt1-Cre; Alk5Δ/Δ mice (Fig. 9, B and C). In contrast, gene expression of Fgf5, Fgf6, and Wnt10a was not restored in Tgfb2Δ/Δ; Wnt1-Cre; Alk5Δ/Δ mice, suggesting that the expression of these genes is regulated by canonical TGFβ signaling. Taken together, our data indicate that loss of Tgfb2 in CNC cells induces altered ABL1 activation and results in the failure of expression of genes related to niche formation and muscle cell development.

Finally, we tested whether inhibition of ABL1 signaling restored cell proliferation and differentiation defects in Tgfb2Δ/Δ; Wnt1-Cre mice (Fig. 10). We treated primary mouse tongue mesenchymal cells from Tgfb2Δ/Δ control and Tgfb2Δ/Δ; Wnt1-Cre mice with an ABL1 inhibitor (nilotinib) and TGFβ1. Nilotinib blocked the TGFβ1-induced ABL1 phosphorylation in both Tgfb2Δ/Δ control and Tgfb2Δ/Δ; Wnt1-Cre cells (Fig. 10A). Gene expression of Fgf4 and Tnc, but not Bmp5 and Fst, was restored after the treatment with nilotinib, indicating that Fgf4 and Tnc are downstream targets of ABL1 signaling. The cell proliferation defect, but not the differentiation defect, was restored after the treatment with nilotinib (Fig. 10, C and D). Thus, our data indicate that an altered TGFβ/ABL1 pathway causes microglossia via regulating cell proliferation activity in the absence of Tgfb2 during tongue development.

**Discussion**

In this study, we investigated how TGFβ signaling in CNC cells regulates the fate of mesoderm-derived myoblasts during tongue development. We found that noncanonical TGFβ signaling is crucial for myoblast proliferation and differentiation, whereas canonical TGFβ signaling is required for muscle organization. Among the candidate growth factors, exogenous FGF4 could most efficiently restore cell proliferation and differentiation in the tongue of Tgfb2Δ/Δ; Wnt1-Cre mice.

FGF family members function in the myogenic cells of limb muscles and the tongue (4, 28). In particular, Fgf4 is expressed in muscle and tendon boundary regions during limb development, and Fgf4-knockout mice exhibit mortality during early embryonic development (29). Fgf5 knock-out mice are viable and show no gross abnormalities (30). Fgf6 knock-out mice have a severe muscle regeneration defect with fibrosis and myotube degeneration, indicating that Fgf6 is a critical component
TGFβ Signaling Mechanism during Tongue Development

In the craniofacial region of vertebrates, tendons and connective tissue are derived from CNC cells, whereas mesoderm-derived cells give rise to muscle cells (39–41). Interactions between fibroblasts and muscle cells during myogenesis are crucial for their development (42). CNC cells provide a niche for muscle cells via the extracellular matrix and growth differentiation factors (4, 43). In this study, we found that extracellular matrix formation and gene expression of growth differentiation factors were compromised in Tgfb2<sup>−/−</sup>;Wnt1-Cre mice and restored by disrupting the noncanonical TGFβ signaling pathway. Our results suggest that noncanonical TGFβ signaling activation in CNC cells results in a failure of niche formation for muscle cells.

Although cleft palate in Tgfb2<sup>−/−</sup>;Wnt1-Cre mice is rescued by a haploinsufficiency of either TGFβ2 ligand (Tgfb2<sup>−/−</sup>;Wnt1-Cre:Tgfb2<sup>+/−</sup>) or TβRI (Tgfb2<sup>−/−</sup>;Wnt1-Cre:Alk5<sup>ΔIκB</sup>), these mice exhibit different tongue phenotypes (9), suggesting that tissue-specific TGFβ signaling may contribute to abnormal tongue development in the absence of Tgfb2 (data not shown). Previous in vitro studies demonstrated that TGFβ2 signaling controls proliferation and fusion of myoblasts (44). We confirmed that TGFβ2 was specifically expressed in the region of myoblast-to-myotube fusion, suggesting that myoblasts may induce TGFβ2 to stimulate the fusion of adjacent myoblasts (45). Consistent with these findings, the defects in cell proliferation and microglossia were not rescued in Tgfb2<sup>−/−</sup>;Wnt1-Cre:Tgfb2<sup>+/−</sup> mice. In contrast, Tgfb2/Alk5 haploinsufficiency largely rescued the cell proliferation and differentiation defects as well as microglossia in Tgfb2<sup>−/−</sup>;Wnt1-Cre mice. One possible explanation is that TGFβ ligands other than TGFβ2 can signal through the TβRI–TβRII complex to cause adverse effects during tongue development. Therefore, a reduction of TGFβ2 signaling alone does not prevent these developmental defects, but a reduction of Alk5 does. This also indirectly confirms that activated noncanonical TGFβ signaling is responsible for muscle cell proliferation and differentiation in Tgfb2<sup>−/−</sup>;Wnt1-Cre mice. Our results highlight that TGFβ signaling via TβRI–TβRII is a widely used mechanism and can induce a variety of noncanonical TGFβ signaling in a spatial and temporal specific manner during craniofacial development. Moreover, the function of and requirement for TGFβ signaling may be different in each tissue. Our data indicate that altered noncanonical TGFβ signaling causes defects in cell proliferation and differentiation in the tongue, whereas the organization of muscle is regulated by canonical TGFβ signaling. TGFβ-mediated ABL activation has been found in leukemia and renal fibrosis (46, 47). Inhibiting the ABL1 pathway restored gene expression of Fgf4 and Tnc followed by restored cell proliferation activity in Tgfb2<sup>−/−</sup>;Wnt1-Cre tongue. Interestingly, ABL1 inhibitor failed to restore muscle differentiation in Tgfb2<sup>−/−</sup>;Wnt1-Cre tongue, suggesting that the ABL pathway mainly contributes to cell proliferation activity and that a combination of noncanonical TGFβ signaling involving ABL1 and PKC likely regulates muscle differentiation during tongue development.

In patients with microglossia, TGFβ signaling may be altered due to genetic and/or epigenetic cause(s). We have shown that ectopic activation of noncanonical TGFβ signaling in CNC...
cells is responsible for adversely affecting cell proliferation and differentiation in the tongue muscle and that reduction of Alk5 expression rescues microglossia in Tgfb2 Δ/Δ,Wnt1-Cre mice. Our findings may be relevant in human disease, given that altered TGFβ signaling is implicated in multiple malformations and syndromes in humans.

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