The tongue is one of the major structures involved in human food intake and speech. Tongue malformations such as aglosisia, microglossia, and ankyloglossia are congenital birth defects, greatly affecting individuals’ quality of life. However, the molecular basis of the tissue-tissue interactions that ensure tissue morphogenesis to form a functional tongue remains largely unknown. Here we show that Shh-mediated epithelial deletion of Wntless (Wls), the key regulator for intracellular Wnt trafficking, leads to lingual hypoplasia in mice. Disruption of epithelial Wnt production by Wls deletion in epithelial cells led to a failure in lingual epidermal stratification and loss of the lamina propria and the underlying superior longitudinal muscle in developing mouse tongues. These defective phenotypes resulted from a reduction in epithelial basal cells positive for the basal epidermal marker protein p63 and from impaired proliferation and differentiation in connective tissue and paired box 3 (Pax3)- and Pax7-positive muscle progenitor cells. We also found that epithelial Wnt production is required for activation of the Notch signaling pathway, which promotes proliferation of myogenic progenitor cells. Notch signaling in turn negatively regulated Wnt signaling during tongue morphogenesis. We further show that Pax7 is a direct Notch target gene in the embryonic tongue. In summary, our findings demonstrate a key role for the lingual epithelial signals in supporting the integrity of the lamina propria and muscular tissue during tongue development and that a Wnt/Notch/Pax7 genetic hierarchy is involved in this development.

The vertebrate tongue, a complex muscular organ located in the oral cavity, plays a crucial role in food intake and is one of the important structures involved in human speech (1). The mammalian tongue is composed of a stratified, squamous, non-keratinized epithelium and the underlying cranial neural crest cell (CNCC)-derived connective tissue and striated muscle (2–4). The fibrous connective tissue beneath the lingual epithelium in the process of tongue development still remains elusive.

Tongue development begins at E10.5 in mice by formation of the median lingual swelling of the first branchial arch, followed by the formation and fusion of lateral lingual swellings. As early as this stage, CNCCs populate the tongue primordium. At E11.5, the myogenic progenitors enter the tongue primordium and establish intimate contact with the CNCC (3, 5). The myogenic progenitor cells expressing Pax3 or Pax7, the paired-box transcription factors, are maintained as a proliferating population in embryonic and fetal muscles of the trunk and limb throughout development (3, 9, 10). Moreover, ectopic expression of either Pax3 or Pax7 is able to stimulate cell proliferation in myoblasts (11, 12). Pax3 and Pax7 have distinct and overlapping functions in muscle development (13, 14). Pax3 knock-out embryos exhibit defective limb and tongue muscle development, and Pax7 knock-out mice grow with small skeletal muscles displaying a complete absence of functional satellite cells, whereas in Pax3/Pax7 double-knock-out mice, only the primary myotome forms, with all of the subsequent phases of myogenesis compromised (10, 15–17). In vitro studies have identified Pax7 as a direct target gene of the Notch signaling pathway, which plays critical roles in the quiescence/activation, proliferation, and differentiation of satellite cells and myoblasts and the maintenance of embryonic myogenic progenitor cells (18–20). Conditional deletion of RBP-J, the principle effector of Notch signaling, leads to reduced Pax7-positive muscle cells in developing mouse embryos followed by decreased myogenic cell proliferation and differentiation (19). It is clear that Notch signaling is required for maintenance of Pax7-expressing stem
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cells; however, it still remains to be investigated how Notch/Pax7 signaling is regulated in particular organ development.

The Wnt signaling pathway is an evolutionarily conserved pathway that plays critical roles in cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (21–23). The biologic function of Wnt signaling in regulating tongue development has been largely focused on its function in tongue taste papillae development. Activation of canonical Wnt signaling has been shown to initiate lingual taste papillae development (24–26). Here, we find that epithelial deletion of Wntless (Wls), the key regulator of Wnt trafficking, leads to the formation of a hypoplastic tongue. This result suggests that epithelial Wnt ligand production mediated by Wls plays an important role in regulating tongue development. By using the conditional knock-out mouse model of Wls, we demonstrate that epithelial Wnt ligand production is required for the maintenance of epithelial p63-positive basal cells to form a stratified oral epithelium, the lamina propria, and the underlying muscle. Furthermore, our results reveal a genetic loop in which Wnt signaling regulates the proliferation of Pax7-positive myogenic cells through positive regulation of the Notch signaling pathway that negatively feeds back to regulate Wnt signaling activity to promote embryonic tongue development. Our findings thus provide a mechanism for patterning the tongue lamina propria and muscle in which a Wnt/Notch/Pax7 genetic hierarchy is involved.

Results

Epithelial Wnt production is essential for embryonic tongue development

To investigate the role of epidermal Wnt production in developmental regulation of tongue composition, we deleted Wls, the gene encoding the main regulator of Wnt protein trafficking, in the mandibular arch using an Shh-Cre mouse in which Cre is active in the dorsal epithelium of the tongue (24, 27) (supplemental Fig. S1). Immunohistochemical staining on sections of tongue showed that deletion of epithelial Wls leads to microglossia (E and F), epithelial hypoplasia (white arrows), and compromised muscle formation. SLM and transverse and vertical myofibers (TVM) are indicated by arrows and arrowheads, respectively. Boxed regions in E and F are shown magnified in G and H. E–H, frontal sections; I–L, sagittal sections. M–P, immunostaining of myosin (green) for muscle fibers in E14.5 tongues. Boxed regions in M and N are shown magnified in O and P. Q, statistical analysis of lingual epithelial thickness from histological sections (I–L). R, comparison of the number of SLM and transverse and vertical myofibers in the designated area (O and P) of control and Wls mutant tongues. Data are shown as scatter plots. **, p < 0.01. Scale bars, 200 μm (A–D, M, and N), 500 μm (E and F), and 100 μm (G–L, O, and P).
displayed severe deformities in both the epithelium and the underlying muscle (Fig. 1, E–I). The epithelium of Wls mutant tongues were not stratified compared with the control tongues (Fig. 1, G–L, white arrows), and their epidermal thickness was significantly reduced (Fig. 1Q). In the control tongues, the muscle fibers were well organized and interwoven with each other (Fig. 1, G, I, and K). By contrast, muscle fibers in mutant embryos were severely reduced in number and disorganized (Fig. 1, H, J, and L), which was also revealed by immunostaining of myosin antibody and quantification of myofiber numbers (Fig. 1, M–P and R). It is noteworthy that the superior longitudinal muscle (SLM) was completely absent from tongues of Wls<sup>Shh-Cre</sup> embryos (Fig. 1, black arrows in G–L and white arrows in O). Taken together, these data indicate that epithelial Wls-mediated production of Wnt plays a critical role in maintaining the integrity of the epithelium and the underlying muscle during tongue organogenesis.

Epithelial Wls regulates epithelial cell proliferation and lamina propria formation in the embryonic tongue

To decipher the role of Wls-mediated epithelial Wnt production in regulating embryonic tongue development, we first investigated lingual epithelial abnormalities. Because epithelial stratification requires proper proliferation of basal layer cells, we examined whether basal cell division was disrupted in epithelial Wls mutant tongues. Immunofluorescence analysis showed that the number of K5-labeled lingual epithelial basal layer cells was significantly decreased in tongues lacking epidermal Wls (Fig. 2A and supplemental Fig. S2A). Next, we examined whether cell proliferation in the dorsal epithelium of Wls-deficient tongues is compromised using sections double-stained with BrdU and p63, a transcription factor expressed in epidermal basal cells required for epidermal stem cell maintenance and regulation. Consistent with the K5 immunostaining result, the number of p63-positive basal cells was decreased in Wls<sup>Shh-Cre</sup> tongues (Fig. 2B and supplemental Fig. S2B). The ratio of epithelial BrdU-positive cells in p63-positive basal cells in tongues of Wls<sup>Shh-Cre</sup> embryos at E13.5–E15.5 was significantly reduced (Fig. 2C), suggesting that epithelial Wnt production is required for the proliferative potential of lingual epithelial basal cells, similar to that of the embryonic skin (28).

The development of multiple organs, including the skin, requires reciprocal interactions between the epithelium and the underlying mesenchyme (29–32). Thus, the development of the lamina propria, the dense connective tissue beneath the epithelium, is probably affected in Wls mutants. We noted that the lamina propria cores (Fig. 2A, arrows) and the epithelial undulations protruding into the lamina propria were missing in Wls<sup>Shh-Cre</sup> tongues (Fig. 2A). In addition, mesenchymal cell proliferation just beneath the epithelium was also significantly decreased (Fig. 2, B and D). Transmission electron microscopy (TEM) micrographs of embryonic tongues at E15.5 reveal the presence of the lamina propria, which consists of abundant connective tissue cells (asterisks) and fibers (arrowheads), in control tongues (Fig. 2E). By contrast, the number of connective tissue cells and fibers was greatly reduced in the tissue just beneath the epithelium in tongues lacking epithelial Wls (Fig. 2, E and F), suggesting that the development of the lamina propria is severely inhibited due to a lack of epithelial Wnt production.

In situ hybridization showed that the expression of type I collagen and tenasin C, main extracellular matrix proteins in connective tissue, is greatly decreased in Wls<sup>Shh-Cre</sup> embryos at E12.5 and E14.5 (Fig. 2G). Of note, type I collagen was expressed in the lamina propria and the underlying tissue in control embryos at E12.5, whereas its expression region was narrowed down in Wls mutants. At E14.5, type I collagen was expressed surrounding and intertwining with the SLM in the control, but not in the Wls<sup>Shh-Cre</sup> tongues. Transcripts of tenascin C were found in the lingual lamina propria in control embryos at E12.5; however, no expression of tenasin C was detected in the comparable region in Wls<sup>Shh-Cre</sup> embryos (Fig. 2G, arrow). Scleraxis is a transcription factor that plays critical roles in condensation, determination, and differentiation of tendon progenitors (33). Scleraxis was expressed in the tissue beneath the lamina propria in the control tongues at E12.5 (Fig. 2G, asterisk). By contrast, in Wls mutant tongues at E12.5, scleraxis was expressed immediately beneath the lingual epithelium (Fig. 2G, arrow). The distance between the scleraxis-expressing region and the epithelium was also dramatically reduced in E14.5 Wls<sup>Shh-Cre</sup> tongues (Fig. 2G). These results suggest that the lamina propria in tongues lacking epithelial Wls is not properly formed.

Cell proliferation is significantly reduced but apoptosis is unaffected in tongues of Wls<sup>Shh-Cre</sup> mice

To investigate cellular processes underlying the defective tongue development in Wls<sup>Shh-Cre</sup> mice, we performed BrdU labeling assays. The samples were double-stained with BrdU and Desmin, a muscle-specific intermediate filament protein, to distinguish connective tissue and muscle in the embryonic tongue. The number of BrdU-positive cells in the tongue was greatly reduced in Wls<sup>Shh-Cre</sup> tongues between E13.5 and E15.5 compared with the control littersmates (Fig. 3, A and B). Quantification of BrdU-positive cells in the Desmin-marked cells and in non-Desmin-positive cells delineated a significant reduction of cell proliferation in both myogenic and CNC-derived mesenchyme cells (Fig. 3C). In parallel, we found by TUNEL assay no evidence for increased cell death in Wls<sup>Shh-Cre</sup> tongues (supplemental Fig. S3). Taken together, the loss of epithelial Wnt production causes a globally decreased cell proliferation in tongues of Wls<sup>Shh-Cre</sup> mice and leads to severe defects in tongue development.

Epithelial Wls is required for maintaining myogenic progenitor cells in the embryonic tongue

Given that the cell proliferation rate is reduced in tongue muscle, we investigated whether the expression of Pax3 and Pax7, two transcription factors critical for proliferation of myoblasts, was affected in the developing embryonic tongues. Immunostaining analysis shows that Pax3-positive myogenic progenitors reached the bilateral upper-tongue region (lamina propria and the lateral superior longitudinal muscle region) at E11.5 in both control and Wls mutant tongues (Fig. 4A). The number of Pax3-positive cells was comparable in both control and Wls<sup>Shh-Cre</sup> tongues at E11.5 (Fig. 4, A and B). These data
suggest that the migration of muscle precursor cells is not affected in Wls mutant tongues. In contrast, the number of Pax3-positive cells was significantly reduced in the E12.5 Wls<sup>Shh-Cre</sup> tongue, in comparison with the wild type tongue, where the number of Pax3-positive muscle precursor cells was dramatically increased and the most abundant Pax3-positive cells were found in bilateral upper-tongue regions in control embryos (Fig. 4, A and B). Immunofluorescent analysis using a Pax7-specific antibody at later embryonic stages revealed that Pax7-positive muscle progenitor cells are also severely reduced in epithelial Wls mutant tongues (Fig. 4, C and D).

We have also examined whether myogenic determination and differentiation processes are disrupted in Wls<sup>Shh-Cre</sup> tongues. MyoD1 and myogenin were expressed in Wls<sup>Shh-Cre</sup> tongues during E12.5–E16.5; however, the expression level was relatively lower than those of the control tongues (supplemental Fig. S4). Taken together, these data suggest that epithelial Wnt production is critical for the regulation of proliferation and differentiation processes of tongue muscle development.
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Figure 3. Epithelial Wnt production mediated by Wls regulates cell proliferation in embryonic tongues. A, immunofluorescence with antibodies against desmin (green) and BrdU (red) shows BrdU-labeled cells in muscle (desmin-positive) and the surrounding tissue. Boxed areas are enlarged on the right. B, percentage of BrdU-positive nuclei in the total cell population in tongues from E13.5 to E15.5. C, percentage of BrdU-positive nuclei in desmin-positive cells (myogenic cells) and desmin-negative mesenchyme cell in E13.5 tongues. Data are shown as scatter plots. **, p < 0.01. Scale bars, 100 µm (enlarged images of boxed regions in A); 250 µm (others).

Figure 4. Significant decrease in the number of muscle progenitor cells in the absence of the epithelial Wls. A, immunofluorescence of Pax3 expression (red) in embryonic tongues at E11.5 and E12.5. B, percentage of Pax3-positive cells in the total cell population in E11.5 and E12.5 tongues. C, immunostaining of Pax7 for myogenic progenitor cells in E13.5 and E15.5 tongues. D, quantitation of Pax7-expressing cells in E13.5 and E15.5 tongues. Boxed areas are enlarged on the right. Data are shown as scatter plots. **, p < 0.01. Scale bars, 200 µm (A and C) and 100 µm (boxed regions in A and C).
Wls-mediated canonical Wnt signaling regulates Notch signaling activity

Reciprocal interaction between Wnt and Notch signaling pathways plays important roles in regulating many aspects of metazoan development (34). Notch signaling is required for maintaining myogenic progenitor cells during mouse fetal development (19). Thus, the decrease of myogenic cells in WlsShh-Cre tongues might be attributed to inhibition of the Notch signaling pathway. To test this possibility, we examined the effect of Wnt signaling on the activity of Notch signaling in WlsShh-Cre tongues.

First, we tested the effect of Wls loss on Wnt signaling during tongue development. TopGal staining showed that deletion of epidermal Wls significantly impaired canonical Wnt signaling in E12.5 WlsShh-Cre tongues (Fig. 5A). The canonical Wnt signaling was manifest in both the epithelium and the superior longitudinal muscle region in the E14.5 wild-type control, but not in the WlsShh-Cre tongues (Fig. 5B). The expression of Axin2 and Lef1, two downstream targets of canonical Wnt signaling, was also significantly decreased in tongues lacking epidermal Wls at E12.5 (Fig. 5B), especially in the lingual epithelium and the underlying tissue (Fig. 5B, insets). These results suggest that epithelial Wnt ligands production is required for canonical Wnt signaling activation in the lingual epithelium and the underlying tissue.

Next, we examined the activity of the Notch signaling pathway in response to epithelial Wnt deficiency. Immunofluorescent analysis of Jag1, one of the Notch ligands, shows that the most abundant Jag1 protein was expressed in the lingual epithelium and the adjacent tissue in control tongues at E12.5 (Fig. 5C). However, the expression of Jag1 was significantly decreased in Wls-deficient tongues (Fig. 5C). The expression of Notch3 and Hes1, two downstream targets of Notch signaling, was also significantly decreased in Wls-deficient tongues (Fig. 5D). These results suggest that Wnt signaling is required for Notch signaling activation in the lingual epithelium and the underlying tissue.
However, the Jag1 protein level was severely reduced in mutant tongues lacking epithelial Wls (Fig. 5C). As revealed by immunostaining of the active form of Notch1, activation of Notch signaling was present in the lingual epithelium, the adjacent mesenchyme, and muscles in E13.5 control embryos but was inhibited in Wls mutant tongues, especially in the epithelium and the underlying tissue (Fig. 5C). At E15.5, active Notch signaling was obviously detected in the lingual epithelium and muscle in control embryos; however, its level is greatly reduced in WlsShh-Cre tongues (Fig. 5C), indicating the repression of Notch signaling activity in Wls mutant tongues. Taken together, these results suggest that canonical Wnt signaling mediated by epithelial Wls is required for activation of Notch signaling during embryonic tongue development.

**Notch is involved in embryonic tongue muscle development by regulating Pax7**

To ask the question of whether impaired Notch signaling accounts for the decrease in number of myogenic progenitor cells in Wls mutant tongues, we investigated whether the expression of myogenic progenitor marker Pax7 can be induced in response to activation of Notch signaling pathway in developing tongues. A bead implantation experiment revealed that exogenously applied active Jag1 peptide was able to induce Pax7 expression in mutant tongues (Fig. 5D), suggesting the repression of Notch signaling activity in Wls mutant tongues. Taken together, these results suggest that canonical Wnt signaling mediated by epithelial Wls is required for activation of Notch signaling during embryonic tongue development.
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expression in \(\text{Wls}^{\text{Shh-Cre}}\) tongue explants (Fig. 6, A and C). Meanwhile, cell proliferation in \(\text{Wls}\) mutant tongue explants was successfully restored with implantation of Jag1-soaked beads (Fig. 6, B and D). Next, we investigated the effect of Notch inhibition on Pax7 expression in cultured tongue explants. The addition of DAPT, the Notch inhibitor, to the culture medium led to significant down-regulation of Notch target genes, including \(\text{Hes}1, \text{Hes}5, \text{Hes}7,\) and \(\text{Notch3}\) (supplemental Fig. S5), suggesting that the Notch signaling activity was efficiently inhibited by DAPT. Expression of Pax7 was strikingly down-regulated in response to DAPT treatment, associated with decreased expression of myogenin, \(\text{MyoD1}\), and desmin (Fig. 6E). By contrast, the expression of Pax3, the paralogue of Pax7 with partially overlapping functions during development, is almost unaffected in DAPT-treated tongue explants (Fig. 6E).

Analysis of the genome sequence of Pax7 indicates two consensus sites of RBP-J present within the 8-kb promoter region of the Pax7 gene (supplemental Fig. S6). In an effort to test whether Pax7 is a Notch target gene in the developing tongue, we performed a ChIP assay using embryonic tongue samples with an antibody against active Notch1, which functions together with RBP-J to regulate expression of Notch target genes. Both binding sites are significantly enriched by > 5-fold in the ChIP assay (Fig. 6F), suggesting that Notch signaling regulates Pax7 expression directly by binding these two sequences in the Pax7 promoter region. Taken together, these results support the notion that the Notch signaling pathway plays a critical role in embryonic tongue development by regulating Pax7.

Notch signaling pathway feeds back to negatively regulate Wnt signaling activity during tongue development

To further investigate the cross-talk between the Notch and Wnt signaling pathways in the embryonic tongue, we examined readouts of Wnt signaling in tongue explants with inhibited Notch signaling. We performed organ culture experiments using tongues from \(\text{BATGAL}\) transgenic mice, a reporter strain for canonical Wnt signaling activity. E11.5 \(\text{BATGAL}\) mandibles were cultured in the presence of DAPT to inhibit Notch signaling and then subjected to X-gal staining analysis. Upon DAPT treatment, the canonical Wnt signaling activity in both mandibles (arrows) and tongues (arrowheads) was greatly increased (Fig. 6G), indicating that Notch signaling feeds back to negatively regulate Wnt signaling during tongue morphogenesis.

Discussion

In the present study, we address the question of how the Wnt production of the lingual epithelium is involved in the developmental regulation of lingual lamina propria and muscle during tongue morphogenesis. Based on our results, we propose a genetic hierarchy model that integrates Wnt and Notch signaling between the lingual epithelium and the internal tongue tissues in tongue development (Fig. 6H). In this model, Wnt ligands from the tongue epithelium play an inductive role in regulating the embryonic tongue development by activating the canonical Wnt signaling pathway. Wnt signaling acts upstream of Notch signaling to maintain the proliferation of muscle progenitor cells by Pax7. Notch in turn represses Wnt signaling for normal tongue development. This model suggests a regulatory network from the epithelium to the CNC-derived connective tissue and muscle in ensuring the integrity of the epidermis, connective tissue, and underlying muscular tissues in tongue organogenesis.

It is well known that the development of the skin and its derivatives, such as the hair follicle, depend on reciprocal epidermal-dermal interactions (35–37). In the process of embryonic skin stratification, an essential BMP-FGF signaling axis in the dermis responds to the epidermal Wnts and feeds back to regulate basal progenitors marked by p63 (28). Similar to skin development, the development and homeostatic maintenance of the mucosa, which consists of a superficial epithelium and the underlying lamina propria, also require interactions between the epithelium and the lamina propria (29, 30, 38, 39). During intestinal mucosa development, epithelium-derived growth factor Hh plays a critical role in the formation of the lamina propria mesenchymal cells, which in turn contribute to the supportive microenvironment of the epithelial stem cell niche (38). In the epithelium of \(\text{Wls}\) mutant tongues, the number of p63-positive basal cells is decreased, and they are associated with loss of lamina propria. Together with previous reports, it is reasonable to deduce that defective lamina propria in turn inhibits normal development of the tongue epithelium in \(\text{Wls}\) mutants.

The tongues of Pax3 knock-out mice lack both intrinsic and extrinsic muscles. In \(\text{Pax3}^{\text{{\text{neo/neo}}}}\) mutants, where Pax3 is partially reduced, intrinsic tongue muscles are partially absent, whereas extrinsic muscles are intact (40). In tongues lacking myogenic RBP-J, the development of limb and tongue muscles are also affected and are associated with decreased Pax7+ cells (19). In \(\text{Wls}\) mutant tongues, Pax7-positive myogenic progenitor cells are significantly reduced and associated with a reduction of intrinsic muscle fibers. These results suggest that the muscle defects in \(\text{Wls}\) mutant tongues directly result from the ablation of myogenic progenitor cells.

The Pax7-marked stem cells regulated by Notch signaling are satellite cells crucial for muscle regeneration after fetal development (9, 10, 12, 15, 18, 20). In \(\text{Wls}^{\text{Shh-Cre}}\) tongues, the number of Pax7+ cells greatly decreased due to epithelial Wnt deficiency, indicating the requirement of epithelial Wnt signaling for the maintenance of the lingual myogenic satellite cell pool. Our results have shown that Pax7 is downstream of Wnt-mediated Notch signaling during tongue development. Unlike Pax7, the expression of Pax3 seems not to be regulated by the Notch signaling pathway in developing embryonic tongue because the inhibition of Notch signaling pathway does not affect the Pax3 level. These results indicate the existence of distinct mechanisms that are involved in the regulation of Pax7- and Pax3-positive myogenic progenitors during tongue development.

The ability of the tongue to move flexibly depends on the unique arrangement of its eight interwoven muscles. The four intrinsic muscles act to change the shape of the tongue, while the four extrinsic muscles function to change the position of the tongue (4). It is intriguing to address how these lingual muscles are formed during tongue development. It has been well elucidated that extrinsic signals from adjacent tissues orchestrate the myogenic initiation, differentiation, movement, proliferation,
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In summary, our results reveal that epidermal production of Wnt ligands in the tongue is essential for preserving lingual epithelium integrity, promoting lamina propria development, and maintaining muscle progenitor cells. Our results illustrate a mechanism of how the epithelium is involved in tissue-tissue interactions to regulate tongue development by serving as a pool of Wnt ligands. These findings highlight a Wnt/Notch/Pax7 genetic hierarchy during embryonic tongue muscle development.

Experimental procedures

Animals

The Wls conditional knock-out mice (Wls<sup>−/−</sup>) were crossed with Shh<sup>CreGFP</sup> mice to generate mice with lingual epidermal loss of function of Wls (Wls<sup>Ssh-Cre</sup>). Shh<sup>CreGFP</sup>, R26R-LacZ, TopGal, and BATGAL mouse lines were purchased from the Jackson Laboratory (Bar Harbor, ME). Wls<sup>−/−</sup> mice were crossed with TopGal or BATGAL mice to obtain Wls<sup>−/−</sup> mice carrying the TopGal or BATGAL allele, which were backcrossed with Wls<sup>−/−</sup> to produce Wls<sup>−/−</sup> mice carrying the TopGal or BATGAL allele. In all experiments, the respective controls were Wls<sup>+/+</sup>:Shh-Cre littermates. The animal experimental protocols involved in this report were approved by the Animal Users Committee of Hangzhou Normal University, China.

Histology, in situ hybridization, X-gal staining, and TEM

Standard hematoxylin/eosin staining and nonradioactive in situ hybridization were performed on paraffin sections as described previously. X-gal staining was performed on cryostat sections as described previously (24). For TEM analysis, embryonic tongues were dissected, fixed with 2.5% glutaraldehyde, and processed according to standard protocols.

Immunohistochemistry

For immunohistochemistry, embryonic heads were fixed in 4% paraformaldehyde for 15–30 min, washed in PBS, and then processed for paraffin sections. Immunostaining was performed with 7-μm paraffin samples using antibodies against myosin (Sigma, M4276), Wls (31), Jag1 (Santa Cruz Biotechnology, Inc., sc-6011), active Notch1 (Abcam, ab8925), Pax3 (DSHB, AB_528426), Pax7 (DSHB, AB_528428), and phosphohistone H3 (Cell Signaling Technology, 3458S).

Cell proliferation and apoptosis assays

For the cell proliferation assay, timed pregnant mice were injected intraperitoneally with BrdU (Sigma) solution (3 mg/100 g of body weight) 1 h before embryo collection. For immunofluorescent analysis, paraffin sections were co-stained with antibody against p63 (Abcam, ab53039) or desmin (Abcam, ab185033) and anti-BrdU antibody (Roche Applied Science, 1170376001). Cell apoptosis was detected with the TUNEL BrightGreen apoptosis detection kit (Vazyme) according to the manufacturer’s instructions. At least three embryonic heads for each genotype were used for data analysis according to the manufacturer’s instructions.
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**Tongue organ culture**

Embryonic tongues from timed pregnant mice were micro-dissected, placed on a Nucleopore Track-Etch membrane in a Trowell-type organ culture dish, and cultured as described previously. For the bead implantation experiment, explanted tongues at E14.5 were implanted with Affi-Gel blue agarose beads carrying Jag1 protein active peptide fragment (AnaSpec), harvested after 1 day in culture, and processed for paraffin sections for immunohistochemistry analysis. For the DAPT treatment experiment, E11.5 mandibles were cultured for 3 days in the presence of 23 μM DAPT (Sigma) with or without 10 mM LiCl, harvested, and processed for quantitative RT-PCR analysis or X-gal staining. The culture medium was changed daily with the fresh addition of chemicals as described above. Three or four embryos were used for each group in experiments.

**ChiP**

Tongues from E14.5 mouse embryos were cut into small pieces and processed for ChiP analysis as described previously. ChiP was performed with antibody against active Notch1 (Abcam, ab8925) or normal rabbit IgG (Beyotime, A7016) using the Magna ChiP G tissue kit (Millipore) according to the user manual. For the detection of the enriched Pax7 promoter region, eluted DNA was used as template for quantitative real-time PCR analysis with primers specific for RBP-J binding sites as described previously (18).

**Quantitative real-time PCR**

For analysis of gene expression, quantitative real-time PCR was performed in triplicate using SsoFast EvaGreen Supermix with the CFX96 real-time PCR detection system (Bio-Rad) as described previously. Briefly, total RNA was isolated from explanted tongues using an RNAqueous-4PCR kit (Ambion), and cDNA was synthesized and used as template for real-time PCR. The real-time PCR primer sequences were obtained from PrimerBank (52): Hes1, 5′-CCAGCGCAGTGTCAACACAGA-3′ and 5′-ATATGGCCGGCACTCTTCTTCT-3′; Hes5, 5′-AGTCCCAAGGAGAAAAACCGA-3′ and 5′-GCTGTGGTTTTCAGGTGCTGAC-3′; Hes7, 5′-CGGGGAGCGAGCTGAGAAG-3′ and 5′-CACGGCGAACTCAGATCTCT-3′; Notch3, 5′-TGCCAGAGTTTAGGTGTTGG-3′ and 5′-CACAGGCAATGCCGCACC-3′; Pax7, 5′-TCTCCAAGATGCTGCTCTG-3′ and 5′-CGGGGTTCTCTCTCTTCATCC-3′; myogenin, 5′-GAGAACATCCCCCTATTTCACCA-3′ and 5′-GTCCTAGTTGCCATAGGCC-3′; MysD1, 5′-CAAGCTGACGTAGAAGAGAC-3′; Pax3, 5′-GGCACTGCTGCTGCTG-3′ and 5′-AAAGCGCAGGTCTGTGTGA-3′; desmin, 5′-GTCCTAGTGCAGCCACT-3′ and 5′-GTGGATGCAGCCACTC-3′; and 5′-GTCCTAGTGCAGCCACT-3′ and 5′-GTGGATGCAGCCACTC-3′. 18S rRNA was used as a reference gene.

**Statistical analysis**

For quantification of proliferation, BrdU-positive cells within a defined area were counted from ≥ 15 consecutive fields from three samples for both control and mutant embryos. Cells labeled with other antibodies were counted in a way similar to that described above. Numbers of total cells were calculated as DAPI-positive cells within a defined area. Student’s t test was used to determine statistical significance. A p value < 0.05 was considered statistically significant. Data are represented as scatter plots with the average and S.D.

**Author contributions**—X.-J. Z. contributed to design, data acquisition, and analysis and drafted and critically revised the manuscript. X. Yuan contributed to design, data acquisition, and analysis and critically revised the manuscript. M. W., Y. F., Y. L., and X. Z. contributed to data acquisition and analysis and critically revised the manuscript. X. Yang and Y. L. contributed to data acquisition and critically revised the manuscript. Zunyi Zhang contributed to conception, design, data acquisition, analysis, and interpretation and drafted and critically revised the manuscript.

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**References**

1. Iwasaki, S. (2002) Evolution of the structure and function of the vertebrate tongue. J. Anat. 201, 1–13
2. Parada, C., and Chai, Y. (2015) Mandible and tongue development. Curr. Top. Dev. Biol. 115, 31–58
3. Parada, C., Han, D., and Chai, Y. (2012) Molecular and cellular regulatory mechanisms of tongue myogenesis. J. Dent. Res. 91, 528–535
4. Bailey, E. F., and Fregosi, R. F. (2004) Coordination of intrinsic and extrinsic tongue muscles during spontaneous breathing in the rat. J. Appl. Physiol. 96, 440–449
5. Han, D., Zhao, H., Parada, C., Hacia, J. G., Bringas, P., Jr., and Chai, Y. (2012) A TGF-β-Smad4-Fgfg signaling cascade controls myogenic differentiation and myoblast fusion during tongue development. Development 139, 1640–1650
6. Hosokawa, R., Oka, K., Yamaza, T., Iwata, J., Urata, M., Xu, X., Bringas, P. Jr, Nonaka, K., and Chai, Y. (2010) TGF-β-mediated FGF10 signaling in cranial neural crest cells controls development of myogenic progenitor cells through tissue-tissue interactions during tongue morphogenesis. Dev. Biol. 341, 186–195
7. Iwata, J., Suzuki, A., Pelikan, R. C., Ho, T. V., and Chai, Y. (2013) Noncanonical transforming growth factor β (TGFβ) signaling in cranial neural crest cells causes tongue muscle developmental defects. J. Biol. Chem. 288, 29760–29770
8. Lin, C., Fisher, A. V., Yin, Y., Maruyama, T., Veith, G. M., Dhandha, M., Huang, G. J., Hsu, W., and Ma, L. (2011) The inductive role of Wnt-β-catenin signaling in the formation of oral apparatus. Dev. Biol. 356, 40–50
9. Yokoyama, S., and Asahara, H. (2011) The myogenic transcriptional network. Cell. Mol. Life Sci. 68, 1843–1849
10. Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005) A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. J. Cell. Physiol. 206, 2990–2997
11. Collins, C. A., Gnocchi, V. F., White, R. B., Boldrin, L., Perez-Ruiz, A., Relaix, F., Morgan, J. E., and Zammit, P. S. (2009) Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation. PLoS One 4, e4475
12. Messina, G., and Cossu, G. (2009) The origin of embryonic and fetal myoblasts: a role of Pax3 and Pax7. Genes Dev. 23, 902–905
13. Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2004) Differential functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev. 18, 1088–1105
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15. Oustanina, S., Hause, G., and Braun, T. (2004) Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J. 23, 3430–3439
16. Mansouri, A., Pla, P., Larue, L., and Gruss, P. (2001) Pax3 acts cell autonomously in the neural tube and somites by controlling cell surface properties. Development 128, 1995–2005
17. Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M. A. (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102, 777–786
18. Wen, Y., Bi, P., Liu, W., Asakura, A., Keller, C., and Kuang, S. (2012) Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. Mol. Cell. Biol. 32, 2300–2311
19. Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A., and Birchmeier, C. (2007) RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and to generate satellite cells. Proc. Natl. Acad. Sci. U.S.A. 104, 4443–4448
20. Conboy, I. M., and Rando, T. A. (2002) The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev. Cell 3, 397–409
21. MacDonald, B. T., Tamai, K., and He, X. (2016) Wnt signal transduction pathways: modules, development and evolution. BMC Syst. Biol. 10, 44
22. MacDonald, B. T., Tamai, K., and He, X. (2009) Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev. Cell 17, 9–26
23. Komiya, Y., and Habas, R. (2008) Wnt signal transduction pathways. Organogenesis 4, 68–75
24. Zhu, X., Liu, Y., Zhao, P., Dai, Z., Yang, X., Li, Y., Qiu, M., and Zhang, Z. (2014) Gpr177-mediated Wnt signaling is required for fungiform placode initiation. J. Dent. Res. 93, 582–588
25. Liu, F., Thirumangalathu, S., Gallant, N. M., Yang, S. H., Stoick-Cooper, C. L., Reddy, S. T., Andl, T., Takeo, M. M., Dlugosz, A. A., Moon, R. T., Barlow, L. A., and Millar, S. E. (2007) Wnt-β-catenin signaling initiates taste papilla development. Nat. Genet. 39, 106–112
26. Iwatsuki, K., Liu, H. X., Grönder, A., Singer, M. A., Lane, T. F., Grosschedl, R., Mistretta, C. M., and Margolskee, R. F. (2007) Wnt signaling interacts with Shh to regulate taste papilla development. Proc. Natl. Acad. Sci. U.S.A. 104, 2253–2258
27. Li, F., Fu, G., Liu, Y., Miao, X., Li, Y., Yang, X., Zhang, Y., Xu, D., Gan, L., Qiu, M., Chen, Y., Zhang, Z., and Zhang, Z. (2017) ISLET1-dependent β-catenin/Hedgehog signaling is required for outgrowth of the lower jaw. Mol. Cell. Biol. 10.1128/MCB.00590-16
28. Zhu, X. J., Liu, Y., Dai, Z. M., Zhang, X., Yang, X., Li, Y., Qiu, M., Fu, J., Hsu, W., Chen, Y., and Zhang, Z. (2014) BMP-FGF signaling axis mediates Wnt-induced epidermal stratification in developing mammalian skin. PLoS Genet. 10, e1004687
29. Santosh, A. B., and Jones, T. J. (2014) The epithelial-mesenchymal interactions: insights into physiological and pathological aspects of oral tissues. Oncol. Rev. 8, 239
30. Ribatti, D., and Santoiemma, M. (2014) Epithelial-mesenchymal interactions: a fundamental developmental biology mechanism. Int. J. Dev. Biol. 58, 303–306
31. Zhu, X., Zhao, P., Liu, Y., Zhang, X., Fu, J., Ivy Yu, H. M., Qiu, M., Chen, Y., Hsu, W., and Zhang, Z. (2013) Intra-epithelial requirement of canonical Wnt signaling for tooth morphogenesis. J. Biol. Chem. 288, 12080–12089
32. Thesleff, I. (2003) Epithelial-mesenchymal signalling regulating tooth morphogenesis. J. Cell Sci. 116, 1647–1648
33. Murchison, N. D., Price, B. A., Conner, D. A., Keene, D. R., Olson, E. N., Tabin, C. J., and Schweitzer, R. (2007) Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development 134, 2697–2708
34. Collu, G. M., Hidalgo-Sastre, A., and Brennan, K. (2014) Wnt-Notch signalling crosstalk in development and disease. Cell. Mol. Life Sci. 71, 3553–3567
35. Estrach, S., Ambler, C. A., Lo Celso, C., Hozumi, K., and Watt, F. M. (2006) Jagged 1 is a β-catenin target gene required for ectopic hair follicle formation in adult epidermis. Development 133, 4427–4438
36. Kishimoto, J., Ehamma, R., Wu, L., Jiang, S., Jiang, N., and Burgeson, R. E. (1999) Selective activation of the visceran promotor by epithelial-mesenchymal interactions during hair follicle development. Proc. Natl. Acad. Sci. U.S.A. 96, 7336–7341
37. Blanpain, C., Horsley, V., and Fuchs, E. (2007) Epithelial stem cells: turning over new leaves. Cell 128, 445–458
38. Powell, D. W., Pinchuk, I. V., Saada, J. I., Chen, X., and Mifflin, R. C. (2011) Mesenchymal cells of the intestinal lamina propia. Annu. Rev. Physiol. 73, 213–237
39. Liu, J., Mao, J. J., and Chen, L. (2011) Epithelial-mesenchymal interactions as a working concept for oral mucosa regeneration. Tissue Eng. Part B Rev. 17, 25–31
40. Zhou, H. M., Wang, J., Rogers, R., and Conway, S. J. (2008) Lineage-specific responses to reduced embryonic Pax3 expression levels. Dev. Biol. 315, 369–382
41. Cisternas, P., Henriquez, J. P., Brandon, E., and Inestrosa, N. C. (2014) Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis. Mol. Neurobiol. 49, 574–589
42. von Maltzahn, J., Chang, N. C., Bentzinger, C. F., and Rudnicki, M. A. (2012) Wnt signaling in myogenesis. Trends Cell Biol. 22, 602–609
43. Bentzinger, C. F., Wang, Y. X., and Rudnicki, M. A. (2012) Building muscle: molecular regulation of myogenesis. Cold Spring Harb. Perspect. Biol. 10.1101/cshperspect.a008342
44. Mennerich, D., and Braun, T. (2001) Activation of myogenesis by the homeobox gene Lbx1 requires cell proliferation. EMBO J. 20, 7174–7183
45. Rodilla, V., Villanueva, A., Obraj-B-Hevia, A., Robert-Moreno, A., Fernández-Majada, V., Grilli, A., López-Bigas, N., Bellora, N., Albà, M. M., Torres, F., Duitach, M., Sanjuan, X., Gonzalez, S., Gridley, T., Capella, G., et al. (2009) Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. Proc. Natl. Acad. Sci. U.S.A. 106, 6315–6320
46. Hayward, P., Kalmar, T., and Arias, A. M. (2008) Wnt/Notch signalling and information processing during development. Development 135, 411–424
47. Cheng, X., Huber, T. L., Chen, V. C., Gadue, P., and Keller, G. M. (2008) Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast. Development 135, 3447–3458
48. Klein, T., and Arias, A. M. (1999) The vestigial gene product provides a molecular context for the interpretation of signals during the development of the wing in Drosophila. Development 126, 913–925
49. Galceran, J., Sustmann, C., Hsu, S. C., Folberth, S., and Grosschedl, R. (2004) LEF1-mediated regulation of Delta-like links Wnt and Notch signaling in somitogenesis. Genes Dev. 18, 2718–2723
50. Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., and Rando, T. A. (2008) A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2, 50–59
51. Wang, R., Sun, Q., Wang, P., Liu, M., Xiong, S., Luo, J., Huang, H., Du, Q., Geller, D. A., and Cheng, B. (2016) Notch and Wnt/β-catenin signaling pathway play important roles in activating liver cancer stem cells. Oncotarget 7, 5754–5768
52. Wang, X., Sippolados, A., Wang, H., and Seed, B. (2012) PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. Nucleic Acids Res. 40, D1144–D1149