

## RESEARCH ARTICLE

# Dynamic activation of Wnt, Fgf, and Hh signaling during soft palate development

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## Abstract

The soft palate is a key component of the oropharyngeal complex that is critical for swallowing, breathing, hearing and speech. However, complete functional restoration in patients with cleft soft palate remains a challenging task. New insights into the molecular signaling network governing the development of soft palate will help to overcome these clinical challenges. In this study, we investigated whether key signaling pathways required for hard palate development are also involved in soft palate development in mice. We described the dynamic expression patterns of signaling molecules from well-known pathways, such as Wnt, Hh, and Fgf, during the development of the soft palate. We found that Wnt signaling is active throughout the development of soft palate myogenic sites, predominantly in cells of cranial neural crest (CNC) origin neighboring the myogenic cells, suggesting that Wnt signaling may play a significant role in CNC-myogenic cell-cell communication during myogenic differentiation in the soft palate. Hh signaling is abundantly active in early palatal epithelium, some myogenic cells, and the CNC-derived cells adjacent to the myogenic cells. Hh signaling gradually diminishes during the later stages of soft palate development, indicating its involvement mainly in early embryonic soft palate development. Fgf signaling is expressed most prominently in CNC-derived cells in the myogenic sites and persists until later stages of embryonic soft palate development. Collectively, our results highlight a network of Wnt, Hh, and Fgf signaling that may be involved in the development of the soft palate, particularly soft palate myogenesis. These findings provide a foundation for future studies on the functional significance of these signaling pathways individually and collectively in regulating soft palate development.

## OPEN ACCESS

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## Introduction

The vital functions of the craniofacial region are facilitated by a complex system of “tubes” and “cavities” [1]. Two major cavities of the craniofacial region are divided by the palate, which serves as the floor of the nasal cavity as well as the roof of the oral cavity. The palate itself is a heterogeneous structure with complex developmental origins. The primary palate is formed by the posterior expansion of the frontonasal process, whereas the secondary palate is formed by the fusion of paired palatal shelves [2–4]. The secondary palate can be further divided into the

hard palate (the palatine process of the maxilla and the palatine bone) and soft palate (consisting of muscles). The soft palate is the more posterior portion of the secondary palate and forms part of a bigger functional system, the oropharyngeal complex, which functions in swallowing, speech, breathing, and hearing [5]. These functions are affected by soft palate clefting and have a life-long impact on the health, social integration and overall quality of life of these patients [6, 7]. Although isolated cleft of the soft palate is considered a mild form of cleft palate, restoring the proper functions of the soft palate is a very challenging task for surgeons because the soft palate muscle fibers in such cases are few in number, disoriented and low in regenerative capacity, and their function may be compromised by fibrosis [8, 9]. In light of the disrupted crucial functions that arise from soft palate clefts, understanding the molecular signaling network that controls soft palate development is critical for addressing longstanding challenges in the clinical treatment of cleft soft palate.

The soft palate consists of five muscles in humans and four in mice: the tensor veli palatini (TVP), levator veli palatini (LVP), palatoglossus (PLG) and palatopharyngeus (PLP) are present in both species, whereas the musculus uvulae is only found in humans [10]. At the cellular level, the soft palate region is comprised of CNC-derived cells, cranial paraxial mesoderm and pharyngeal ectoderm [10, 11]. Close interaction between CNC-derived mesenchyme and myogenic cells derived from cranial paraxial mesoderm is required during the development of craniofacial muscles, as signals from CNC-derived mesenchyme guide myogenic progenitors into the soft palate region and instruct myogenic cells to differentiate [10].

Multiple signaling pathways, for example Wnt, Tgf- $\beta$ , Hh and Fgf, have been shown to regulate palatogenesis [4, 12–14]. Wnt signaling is crucial for regulating craniofacial development; loss or gain of Wnt signaling function can cause severe craniofacial malformations, including cleft palate, indicating that precisely regulated Wnt signaling is a prerequisite for normal craniofacial morphogenesis [15–19]. In particular, Wnt3 and Wnt9b are associated with orofacial clefts in both humans and mice [20–22]. Wnt signaling also plays a role during muscle development, including tongue development [14, 23, 24]. Canonical Wnt signaling is a necessary regulator of progenitor cells and myofibers during early fetal development, myoblast differentiation and myoblast migration in the tongue muscles [24, 25]. Recently, canonical Wnt signaling was also identified to be crucial in the final stage of myogenesis, myoblast fusion, through regulating nephrin [23]. In soft palate development, Wnt signaling is downregulated after conditional deletion of Tgf- $\beta$  signaling from the palatal epithelium, which is associated with soft palatal muscle defects [12]. Hh signaling is critical for early embryonic development and plays a key role in patterning, survival and proliferation of early CNC and other cell populations [26, 27] and is involved in the development of multiple craniofacial tissues [27–29]. During hard palate development, Hh signaling establishes the oro-nasal and antero-posterior axes through epithelial-mesenchymal interactions [30–33]. Hh signaling also plays a role during muscle development, including that of the tongue, and is crucial for post-migratory CNC proliferation in the craniofacial region [14, 27]. Both loss- and gain-of-function mutations in Fgf receptors and ligands are associated with numerous congenital disorders affecting the craniofacial region, including non-syndromic and syndromic cleft palate [34–39]. Syndromes associated with cleft palate include those arising from mutations in *FGFR1-3*; e.g. Apert syndrome (*FGFR2*), Muenke syndrome (*FGFR3*), Crouzon syndrome (*FGFR2, FGFR3*) and Hartsfield syndrome (*FGFR1*) [39]. The role of Fgf signaling has also been shown in skeletal muscle development, especially in repressing the differentiation of myoblasts [40]. Together with Wnt signaling, Fgf signaling is essential for specification of paraxial mesoderm during early fetal myogenesis [41]. Recently, Fgf signaling was shown to play a role in Dlx5-mediated development of the soft palate muscles [13]. There is a close interaction among the Wnt, Hh and Fgf signaling pathways during the development of the hard palate [4, 42]. Wnt signaling acts

upstream of Hh during hard palate development [14, 43]. Hh cooperates with Fgf signaling via epithelial-mesenchymal interactions, especially through Fgfr2/Fgf10, during the development of the hard palatal shelves [31]. Fgf signaling is also modulated by canonical Wnt signaling during early craniofacial development [18] and there is a regulatory feedback loop between Wnt11 and Fgfr1 during hard palate development [44].

Since most previous molecular studies have focused on the hard palate, the regulatory mechanism of soft palate development has just started to be unveiled. In order to investigate and characterize the signaling network that controls the interactions between CNC-derived mesenchyme, mesoderm-derived myogenic cells and pharyngeal ectoderm-derived epithelium, our current study focuses on the expression of active Wnt, Hh and Fgf signaling during soft palate development in mice. We have analyzed the expression and activation patterns of Wnt, Hh and Fgf pathway members throughout the soft palate region in cells of ectodermal, CNC and mesodermal origin during embryonic development and revealed their dynamic involvement in soft palate muscle development. This study identifies dynamic signaling expression patterns during the development of the soft palate and lays the groundwork for improved treatment of clefts of the soft palate.

## Material and methods

### Animals and procedures

Animal studies were completed in accordance with federal regulations and approval from the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California (Protocol Number: 9320). C57BL/6J and reporter mouse lines *Gli1-LacZ*<sup>+/-</sup> [JAX#008211, [45]] and *Axin2-LacZ*<sup>+/-</sup> [JAX#009120, [46]] were used for this study. Animals were euthanized by carbon dioxide overdose followed by cervical dislocation. Embryos collected on embryonic day (E)14.5, E16.5 or E18.5 were fixed overnight in 10% neutral buffered formalin solution (Sigma, HT501128), decalcified in ethylenediaminetetraacetic acid (EDTA, pH 7.1–7.3, Alfa Aesar, A15161) for 1 week and processed for cryosectioning or paraffin embedding and sectioning (8 μm). Briefly, for the paraffin sectioning protocol, samples were washed in DEPC-PBS three times for 5 minutes, dehydrated through an increasing ethanol series (4 hours each of 30%, 50%, 70%, 80%, and 95% EtOH followed by 2 x 4 hours in 100% EtOH), then treated with xylene (for 3 hours or until clear), xylene/paraffin for 45 minutes, and finally paraffin (six 1-hour baths, one bath overnight). For cryosectioning, samples were washed in DEPC-PBS three times for 5 minutes each, dehydrated in 15% sucrose solution (Sigma, S0389), diluted in DEPC-PBS and 30% sucrose/50% OCT solution, and embedded in Tissue-Tek<sup>®</sup> OCT Compound (Sakura, 4583). In the case of *LacZ*<sup>+/-</sup> samples, 0.2% glutaraldehyde with 2mM MgCl<sub>2</sub> was used for fixation overnight, followed by decalcification in EDTA (pH 7.1–7.3) with 2mM MgCl<sub>2</sub> for one week. Samples were dehydrated in sucrose solutions with 2mM MgCl<sub>2</sub> as described above and embedded in OCT.

### Histological and X-gal staining

Hematoxylin and eosin staining was performed on deparaffinized slides according to standard procedure. Sections from *LacZ* reporter mice were subjected to X-gal staining. After rinsing the slides with PBS and 2mM MgCl<sub>2</sub> to remove the OCT, they were placed in X-gal staining solution (1M MgCl<sub>2</sub>, 1% NaDOC, 1% NP-40, 50 mM K Ferri, 50 mM K Ferro, 1 M Tris (pH 7.3), 40 mg/ml X-gal, 1x PBS) at 37°C overnight or over two nights, if necessary. After staining, slides were washed twice with PBS for 5 minutes each, fixed in formalin for 30 minutes, counterstained with Nuclear Fast Red for 3 minutes, and washed with distilled water and 70% EtOH twice for 2 minutes each. Slides were stained in eosin for 75 seconds, then subjected to

two 95% EtOH rinses and two 2-minute washes in 100% EtOH before final clearance in xylene (two times 2 minutes) and mounted.

### RNAscope and immunohistochemistry

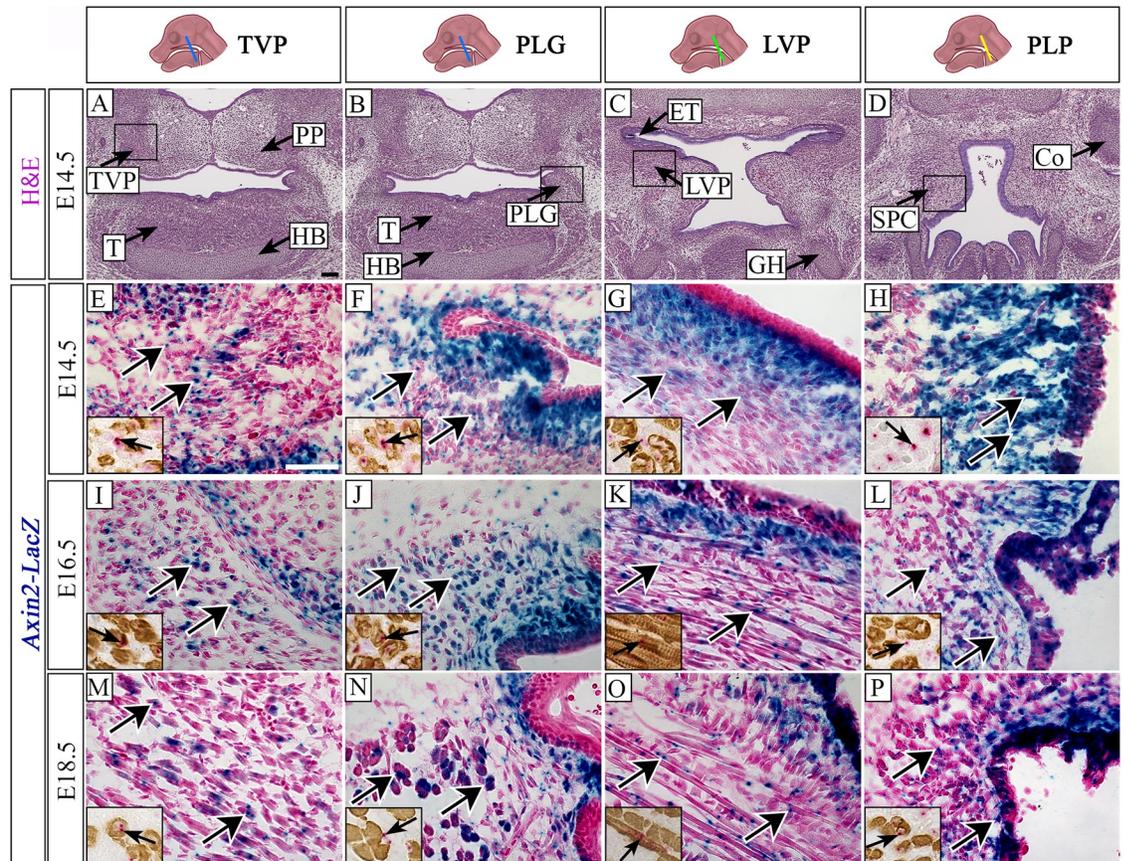
Sections were air dried for 15 minutes at room temperature and 15 minutes at 37°C. OCT was removed from the slides with a 5-minute wash in distilled water and slides were placed into pre-heated target retrieval reagent (Advanced Cell Diagnostics, ACD, 322000) for 15 minutes at 99°C. For *in situ* hybridization, RNAscope<sup>®</sup> 2.5 HD detection reagent–red kit was used (ACD, 322360) according to the manufacturer's instructions. The probes used for this study were Mm-Axin2 (400331), Mm-Etv4 (458121), Mm-Etv5 (316961), Mm-Fgfr1 (443491), Mm-Fgfr2 (443501), Mm-Fgfr3 (440771), Mm-Fgfr4 (443511), and Mm-Gli1 (311001). After detection of the mRNA signal, slides were fixed in formalin for 10 minutes, and washed in PBS. Immediately after that, pre-heated citrate-based antigen unmasking solution (Vector, H-3300) was applied for 15 minutes at 99°C. After washes in 0.1% Tween20/PBS (PBST), sections were incubated with blocking reagent (Perkin Elmer, FP1012) for 1 hour followed by primary antibody (myosin heavy chain, MHC, Developmental Studies Hybridoma Bank: MF20) diluted 1:10 overnight at 4°C. The second day, after three five-minute washes in PBS, secondary antibody from the M.O.M. kit (Vector, MP-2400) was applied for 10 minutes followed by detection of the signal using peroxidase substrate kit DAB (Vector, SK-4105). After signal development, slides were counterstained with haematoxylin, fixed for 10 minutes in formalin, and mounted with aqueous mounting medium (Vector, H-5501). For MHC immunofluorescence, after incubation of the primary antibody overnight, secondary antibody (Alexa Fluor 488 anti-mouse, 1:200, Invitrogen, A11001) was applied for two hours at room temperature, followed by three washes in PBST and 5 minutes counterstaining with DAPI (Invitrogen, D1306). For immunofluorescence staining of active  $\beta$ -catenin and its co-localization with MHC, samples were treated with 3% hydrogen peroxide solution, pre-heated in citrate-based antigen unmasking solution (Vector, H-3300, 15 minutes at 99°C) and treated with blocking reagent (Invitrogen, B40922) for 1 hour at room temperature. After incubation of the primary antibody overnight (myosin heavy chain, MHC, Developmental Studies Hybridoma Bank: MF20, 1:10 and active beta-catenin, Cell Signaling: S45, D2UBY, 1:100), secondary antibody (Alexa Fluor 568 anti-mouse, 1:200, Invitrogen, A11004 and anti-rabbit IgG poly HRP conjugate) was applied for two hours at room temperature, followed by three washes in PBST, tyramide development for 3 minutes (Alexa Fluor 488 Tyramide SuperBoost, Invitrogen, B40922) and counterstaining with DAPI (Invitrogen, D1306).

## Results

### Activation of Wnt signaling during soft palate development

As previously described, in mice, the TVP and PLG are the most anterior soft palate muscles [9, 10], and can be seen at E14.5 in coronal section with the tongue (T), hyoid bone (HB) and pterygoid plate (PP) (Fig 1A and 1B). The LVP is located posterior to the TVP, where the greater horns of the hyoid bone (GH) can be observed in histological sections, together with the opening of the Eustachian tube (ET) (Fig 1C). The PLP is located in the most posterior part of the soft palate at the level of the cochlea (Co) and superior pharyngeal constrictor (SPC), but it is not yet differentiated at E14.5 (Fig 1D).

Previous studies have demonstrated that Wnt signaling, particularly canonical Wnt signaling, is involved during the development of the palate and during muscle development [14, 16, 20, 23–25, 47, 48]. Therefore, we hypothesized that Wnt signaling may also be active during soft palate myogenesis and evaluated the expression of *Axin2*, which plays an important role in



**Fig 1. *Axin2* is expressed throughout the embryonic development of the murine soft palate region.** (A-D) Hematoxylin and eosin staining of frontal sections of a mouse head at E14.5. Schematic drawings of the mouse head in the top panel depict the position and angle of the sections. Boxed areas in A-D respectively indicate approximate locations of higher magnification images (E-P) of TVP (E,I,M), PLG (F,J,N), LVP (G,K,O) and PLP (H,L,P). (E-P) Visualization of *Axin2-LacZ* reporter mice at E14.5 (E-H), E16.5 (I-L) and E18.5 (M-P). Insets in E-P show expression of *Axin2* mRNA detected by RNAscope (pink) and MHC protein in comparable regions to E-P visualized by immunohistochemistry (brown). Arrows indicate *Axin2*-positive cells. Co, cochlea; ET, Eustachian tube; GH, greater horns of the hyoid bone; HB, hyoid bone; H&E, hematoxylin and eosin staining; LVP, levator veli palatini; PLG, palatoglossus; PLP, palatopharyngeus; PP, pterygoid plate; SPC, superior pharyngeal constrictor; T, tongue; TVP tensor veli palatini. Scale bar in A = 100  $\mu$ m (A-D), scale bar in E = 50  $\mu$ m (E-P), 27  $\mu$ m (insets in E-P).

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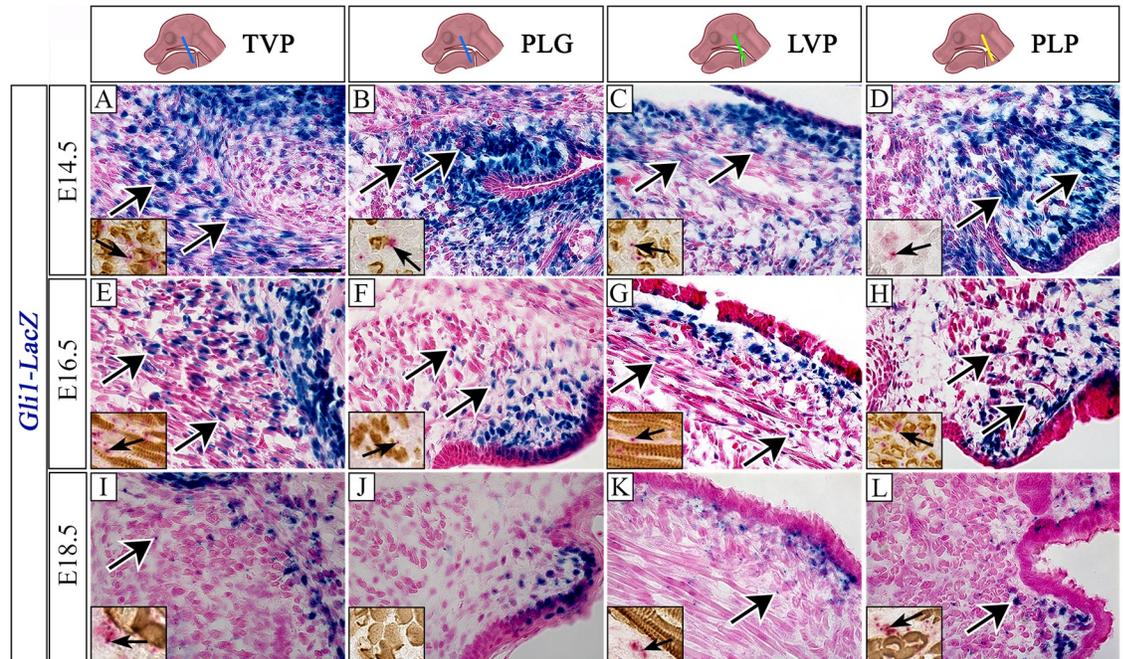
the regulation of  $\beta$ -catenin and can be used as a readout of active canonical Wnt signaling, by analyzing *Axin2-LacZ* reporter mice and by analyzing *Axin2* mRNA expression. LacZ staining of *Axin2-LacZ* mice indicated *Axin2* expression as early as E14.5 in the whole soft palate region. In the TVP region at E14.5, *Axin2* expression was predominantly present in CNC-derived mesenchymal cells and in a few MHC<sup>+</sup> muscle fibers (Fig 1E). CNC-derived mesenchymal cells also showed abundant expression of *Axin2* in the region surrounding the PLG, with the highest expression in the region close to the epithelium (Fig 1F). A few *Axin2*<sup>+</sup> myogenic cells were also found in the PLG region (Fig 1F). More posteriorly, *Axin2* expression was detectable predominantly in the CNC-derived mesenchymal cells surrounding initial LVP muscle fibers (Fig 1G). At E14.5, although the muscle fibers of PLP were not yet differentiated, extensive expression of *Axin2* was detectable in the putative region of the PLP, mainly consisting of CNC-derived mesenchymal cells (Fig 1H). Later in development, muscles of the soft palate increase in size and number [10]. In the TVP and PLG region at E16.5, *Axin2* expression

was detectable primarily in the CNC-derived mesenchymal cells (Fig 1I and 1J). In the region of the LVP and PLP, the expression pattern was similar: *Axin2* was widely expressed in the CNC-derived mesenchymal cells surrounding the myogenic cells, and a few MHC+ cells were also *Axin2*+ (Fig 1K and 1L). At E18.5, *Axin2* expression persisted in CNC-derived mesenchymal cells and in the MHC+ myogenic fibers of individual soft palatal muscles at the level of the TVP (Fig 1M), PLG (Fig 1N), LVP (Fig 1O) and PLP (Fig 1P). At all examined stages, expression of *Axin2* was also observed in the palatal epithelium (Fig 1F–1H, 1J–1L and 1N–1P). These results were confirmed also by assessing the active  $\beta$ -catenin signaling (S1 Fig).  $\beta$ -catenin is the mediator of canonical Wnt signaling, transducing the signal upon specific Wnt ligand and receptor binding, mediating cellular response. The detection of active  $\beta$ -catenin therefore indicates activation of canonical Wnt signaling. Similar to *Axin2* expression, activated  $\beta$ -catenin was predominantly present in the CNC-derived mesenchymal cells and less expression was seen in the myogenic cells (S1 Fig).

These results demonstrate the broad and persistent expression of Wnt signaling in the course of embryonic soft palate development, predominantly in the CNC-derived mesenchymal cells surrounding the myogenic fibers, but also in the MHC+ and epithelial cells of the soft palate region. Consistent with previous studies, our analysis suggests that Wnt signaling is likely to be significant for soft palate muscle establishment, differentiation, and fusion.

### Hh signaling activity during soft palate development

Hh signaling is involved in early embryonic development and plays an indispensable role in reciprocal epithelial-mesenchymal interactions guiding palatal outgrowth and tongue muscle development [14, 27, 32, 33]. To test our hypothesis that Hh signaling may be involved in soft palate muscle development, we analyzed the expression of *Gli1*, a transcription factor activated by Hh signaling, via *Gli1-LacZ* mice and *Gli1* mRNA expression. At E14.5, *Gli1* expression was plentiful at the level of the TVP in the CNC-derived mesenchymal cells surrounding myogenic cells, and a few myogenic MHC+ cells were also *Gli1*+ (Fig 2A). In the PLG region, *Gli1* expression was also abundant in the CNC-derived mesenchymal cells, and only a few *Gli1*+ MHC+ myogenic cells were visible (Fig 2B). More posteriorly, at the level of the LVP, where the first myogenic fibers of LVP could be observed, *Gli1* signal was abundant in the CNC-derived mesenchyme surrounding the myogenic fibers close to the epithelium, whereas almost no *Gli1*+MHC+ myogenic cells were detectable (Fig 2C). In the putative region of the PLP primordia, abundant expression of *Gli1* was present in the CNC-derived mesenchymal cells (Fig 2D). Later in development, at E16.5, overall fewer *Gli1*+ cells were observed in the soft palatal region. The majority of the *Gli1*+ cells persisted in the CNC-derived mesenchyme both surrounding the myogenic fibers and adjacent to the epithelium (Fig 2E–2H). At E18.5, overall *Gli1* expression remained low, except a few *Gli1*+ cells were detected at the level of the TVP in the CNC-derived mesenchyme (Fig 2I). The PLG region showed scattered *Gli1* expression in CNC-derived mesenchyme surrounding the muscle cells (Fig 2J). The LVP region was nearly absent of *Gli1*+ cells, with only few *Gli1*+ cells present in the CNC-derived mesenchyme close to the epithelium (Fig 2K). In the PLP region, scattered *Gli1* expression persisted in the CNC-derived mesenchymal cells close to the epithelium (Fig 2L). *Gli1* expression was also observed in the epithelium of all the soft palate regions throughout development (Fig 2B–2D, 2F–2H and 2J–2L). This dynamic spatiotemporal expression pattern of *Gli1* suggests the requirement of Hh signaling pathway in the early stages of soft palate development, predominantly in CNC-derived mesenchymal cells, to regulate myogenesis through cell-cell interaction.



**Fig 2. *Glial* is dynamically expressed in the soft palate region.** (A-L) Visualization of *Glial-LacZ* reporter mice at E14.5 (A-D), E16.5 (E-H), and E18.5 (I-L). Insets in A-L show expression of *Glial* by RNAscope (pink) and MHC immunohistochemistry (brown) in comparable regions to A-L. Arrows indicate *Glial* positive cells. Schematic drawings of the mouse head in the top panel depict the position and angle of the sections. LVP, levator veli palatini; PLG, palatoglossus; PLP, palatopharyngeus; TVP, tensor veli palatini. Scale bars = 50  $\mu$ m (A-L), 27  $\mu$ m (A-L).

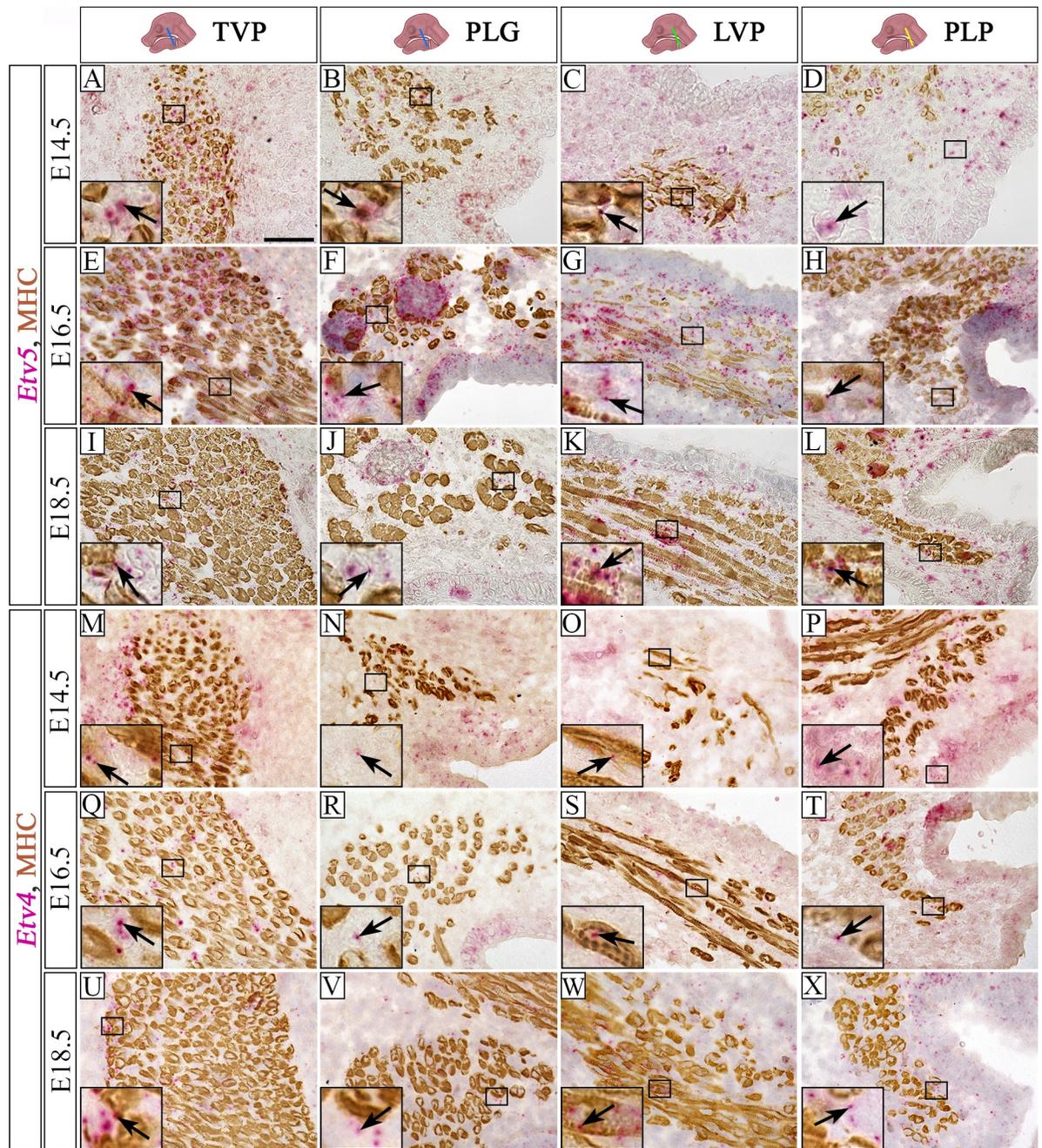
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### Expression of Fgf signaling during soft palate development

The role of Fgf signaling has been shown in both palatal clefting and skeletal muscle development, especially in repressing the differentiation of myoblasts [34, 39, 40]. We therefore hypothesized that Fgf signaling might play a role in the development of soft palate muscles. We analyzed the expression of *Etv5* and *Etv4*, downstream targets of Fgf signaling, and Fgf receptors (*Fgfr1-4*) to assess whether Fgf signaling is activated during soft palate development.

Early in embryonic soft palate development at E14.5, *Etv5* and *Etv4* expression could be observed predominantly in the CNC-derived mesenchymal cells closely interacting with muscle fibers of the TVP (Fig 3A and 3M) and PLG (Fig 3B and 3N). Few MHC+ myogenic cells of the TVP and PLG expressed *Etv5* (Fig 3A, 3B, 3M and 3N). Initial muscle fibers of the LVP were surrounded by active Fgf signaling in the CNC-derived mesenchymal cells (Fig 3C and 3O). The putative region of the PLP, where only CNC-derived mesenchymal cells could be observed at E14.5, also showed activation of Fgf signaling (Fig 3D and 3P). Later in development, at E16.5 and E18.5, Fgf signaling remained active predominantly in the CNC-derived mesenchymal cells with rare *Etv5* expression in MHC+ cells (Fig 3E–3L and 3Q–3X). Fgf signaling activity was also detected in the epithelium throughout the embryonic development of the soft palate (Fig 3B–3D, 3F–3H, 3J–3L, 3N, 3P, 3R, 3S and 3V–3X).

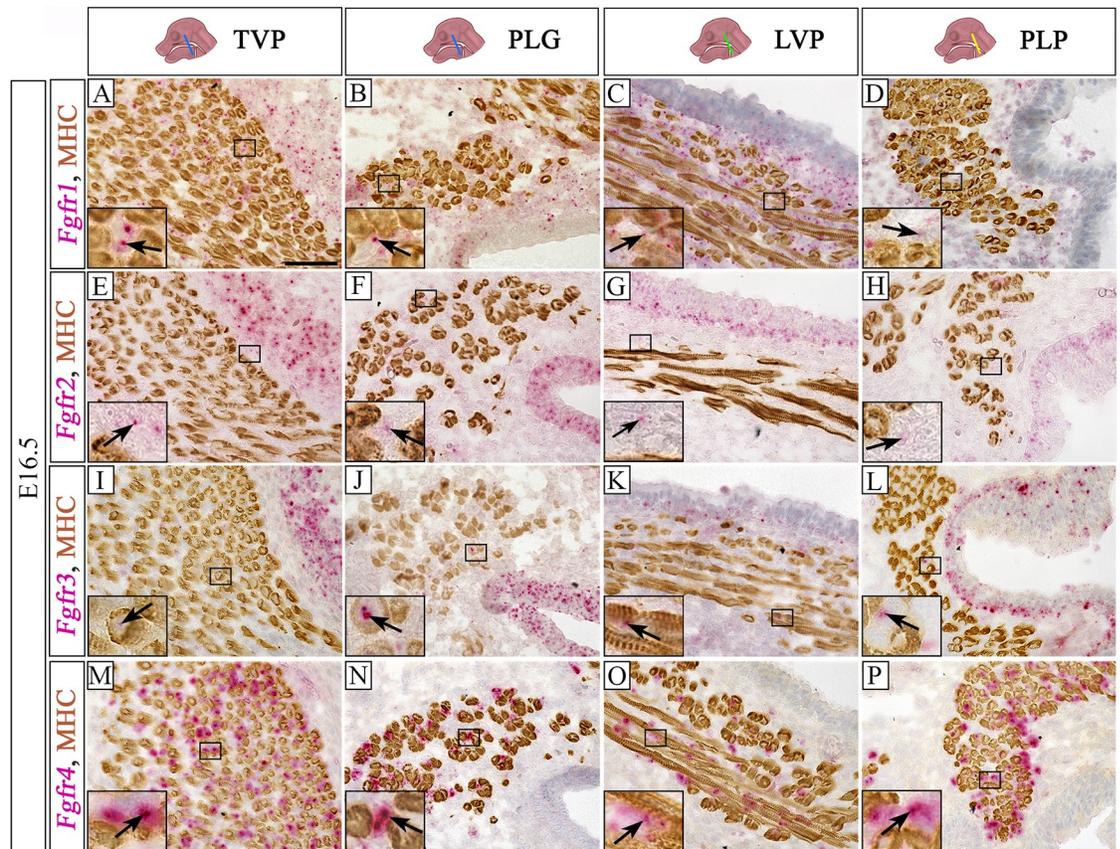
We further analyzed Fgf receptor activity at E16.5. The expression of *Fgfr1* was predominantly present in the CNC-derived mesenchymal cells and in the epithelium (Fig 4A–4D). Few scattered *Fgfr2* positive cells were in the CNC-derived mesenchyme and epithelium (Fig 4E–4H). Expression of *Fgfr3* was detected in few CNC-derived mesenchymal cells and epithelium (Fig 4I–4L). Among the four Fgf receptors we assessed, *Fgfr4* appeared to be the most



**Fig 3. *Etv5* and *Etv4*, downstream targets of Fgf signaling, are abundantly expressed throughout embryonic soft palate muscle development.** (A-L) *Etv5* and (M-X) *Etv4* RNAscope (pink) and MHC immunohistochemistry (brown) in the soft palate region of C57BL/6J mice at E14.5 (A-D, M-P), E16.5 (E-H, Q-T), and E18.5 (I-L, U-X). Insets (A-X) show magnified views of *Etv5* (A-L) and *Etv4* (M-X) signal detected by RNAscope (pink) and MHC immunohistochemistry (brown). Arrows indicate *Etv5* and *Etv4* positive cells. Schematic drawings of the mouse head in the top panel depict the position and angle of the sections. LVP, levator veli palatini; MHC, myosin heavy chain; PLG, palatoglossus; PLP, palatopharyngeus; TVP, tensor veli palatini. Scale bars = 50  $\mu$ m (A-X), 12  $\mu$ m (insets in A-X).

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abundantly expressed during soft palate development. The majority of the *Fgfr4* signal was in the CNC-derived mesenchymal cells surrounding the muscle fibers at the level of the TVP (Fig 4M), PLG (Fig 4N), LVP (Fig 4O) and PLP (Fig 4P) at E16.5; little expression of *Fgfr4* was detectable in the myogenic fibers. Interestingly, the expression of *Fgfr4* was not detected in the

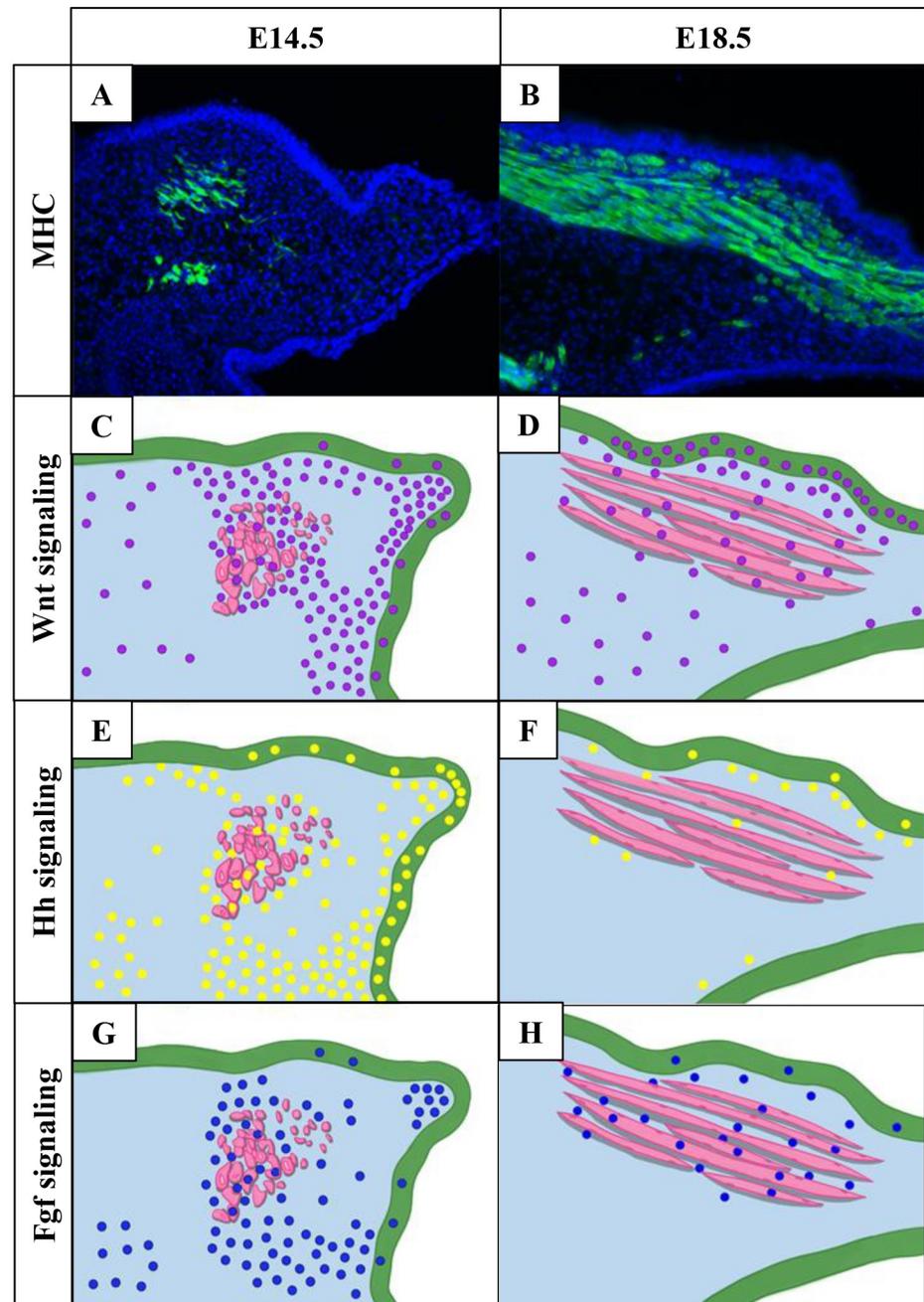


**Fig 4. Expression of *Fgfr1*, *Fgfr2*, *Fgfr3*, and *Fgfr4* in the soft palate myogenic sites.** (A–P) Co-staining of MHC immunohistochemistry (brown) and Fgf receptor RNAscope (pink) in the soft palate myogenic sites of C57BL/6J mice at E16.5: *Fgfr1* (A–D), *Fgfr2* (E–H), *Fgfr3* (I–L), *Fgfr4* (M–P). Arrows indicate positive signal. Insets (A–P) show magnified views of *Fgfr1* (A–D), *Fgfr2* (E–H), *Fgfr3* (I–L) and *Fgfr4* (M–P) signal detected by RNAscope (pink) and MHC immunohistochemistry (brown). Schematic drawings of the mouse head in the top panel depict the position and angle of the sections. LVP, levator veli palatini; MHC; myosin heavy chain; PLG, palatoglossus; PLP, palatopharyngeus; TVP, tensor veli palatini. Scale bars = 50  $\mu$ m (A–P), 13.5  $\mu$ m (insets in A–P).

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palatal shelf epithelium (Fig 4N–4P). Our results describe the spatiotemporal activation pattern of Fgf signaling in soft palate development and suggest its role in this process.

It is particularly important to understand the molecular signaling that controls the development of the LVP, which is the major muscle of the soft palate and performs important functions as a part of the oropharyngeal complex [5]. For this reason, we highlight the LVP in summarizing the molecular signaling network that regulates soft palate muscle development (Fig 5A and 5B). We found that Wnt signaling was abundant in all cell types of the soft palate, especially in the CNC-derived mesenchyme, at E14.5 (Fig 5C) and persisted until E18.5 (Fig 5D). Hh signaling was active mainly in the CNC-derived mesenchymal cells, as well as in a few myogenic and epithelial cells at E14.5 (Fig 5E) and diminished at E18.5 (Fig 5F). Fgf signaling was widespread in the CNC-derived mesenchymal cells but scattered in the myogenic cells and palatal epithelium at E14.5 (Fig 5G) and persisted in the soft palate region at E18.5 (Fig 5H). Taken together, our studies suggest a dynamic signaling network of Wnt, Hh, and Fgf pathways that mediate the interactions between CNC-derived mesenchymal and myogenic cells and/or interactions between epithelium and mesenchyme during soft palate development.



**Fig 5. Schematic drawing summarizing the expression of Wnt, Hh, and Fgf signaling in the LVP region.** (A-B) Visualization of myogenic fibers by MHC immunofluorescence at E14.5 (A) and E18.5 (B). (C-D) Schematic drawings summarizing Wnt signaling activity (purple dots) at E14.5 (C) and E18.5 (D); (E-F) Hh signaling activity (yellow dots) at E14.5 (E) and E18.5 (F); and (G-H) Fgf signaling activity (dark blue dots) at E14.5 (G) and E18.5 (H). Light blue indicates the CNC-derived mesenchymal cells and green indicates soft palatal epithelium. MHC, myosin heavy chain.

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## Discussion

In this study, we show for the first time the detailed spatiotemporal expression patterns of Wnt, Hh and Fgf signaling pathways during embryonic soft palate muscle development with a focus on potential interactions among cells derived from CNC, paraxial mesoderm and

pharyngeal ectoderm. We highlight the importance of these signaling pathways during soft palate formation and suggest that they may instruct cell-cell interactions to guide embryonic soft palate muscle development.

### Expression patterns of Wnt, Hh, and Fgf signaling pathways suggest their role in regulating soft palate development

Wnt, Hh, and Fgf are multifunctional signaling pathways and their specificity in regulating organogenesis is achieved through different combinations of their specialized activities, interactions with other signaling molecules/pathways and the specific cellular environments where they reside [49, 50]. The Wnt, Hh and Fgf signaling networks are highly conserved across species, as well as across the development of various organs. For instance, the interplay of these signaling pathways has been shown to regulate the development of scales in zebrafish [51]. In mice, the interplay of this signaling network regulates the growth/patterning of teeth [52], limbs [50], salivary glands [53, 54] and airway smooth muscle [55], as well as the early development of the face [18] and the thalamus [49]. These signaling pathways are also co-expressed during the development of the hard palate [14, 33, 39, 56].

Conventional or conditional knockout of Wnt, Hh, or Fgf signaling in the CNC cells, palatal mesenchyme or epithelium causes severe craniofacial phenotypes, most of which include cleft palate [16, 18, 19, 27, 28, 31, 36, 48, 57–59]. Furthermore, during hard palate development, Wnt signaling acts upstream of both Fgf and Hh signaling [14, 57], and Hh is downstream of Fgf signaling [31]. The co-expression of Wnt, Hh and Fgf signaling pathways we observed in our study might imply their interaction and shared function in embryonic development of the soft palate, especially at early stages, since Hh signaling diminishes in later stages of soft palate development.

Despite the fact that roles for Wnt, Hh and Fgf signaling are also well established in the development of the hard palate, the involvement of these signaling pathways in the soft palate development has not previously been described. Revealing the interaction of the signaling pathways regulating soft palate myogenic development is critical for understanding this process since the complexity of signaling pathways involved reflects the high incidence of cleft palate. It will be crucial to understand how these signaling pathways achieve their specificity in regulating soft palate development. This information will guide us in potential tissue engineering approaches to improve soft palate repair and regeneration.

### Cell-cell interactions in soft palate development

Cell-cell and tissue-tissue interactions are fundamental for the development and function of various organs. Epithelial-mesenchymal interactions are some of the best-studied inductions and are well characterized in the development of numerous organs, such as the tooth [60], lung, kidney [61], mammary gland [62] and many others, including the palate [12, 31]. In the craniofacial region, interactions between CNC cells and mesodermal/myogenic cells are of special interest [4, 11]. The cooperation of different cell types through signaling molecules has been previously suggested for regulation of soft palate development as well [10, 13, 63]. Our data are in agreement with these suggestions and indicate that Wnt, Hh and Fgf signaling are active in all three components of soft palate: CNC-derived mesenchymal cells, myogenic cells, and epithelium. The activity of all these signaling pathways is predominant in the CNC-derived mesenchymal cells during early soft palate development, when the myogenic cells of the soft palate are not differentiated yet (e.g., in the presumptive region of PLP at E14.5), and Wnt and Fgf signaling activity persists in the CNC-derived mesenchymal cells until the later stages of soft palate development. Previously published results revealed that Wnt signaling

mediates cell-cell interactions of fibroblasts with myocytes and other cell populations after cardiac injury [64]. Furthermore, defective soft palate muscle formation is observed subsequent to decreased Wnt signaling in CNC-derived cells [12]. Hh and Fgf signaling play important roles in cells of CNC origin, interacting with tongue myogenic progenitor cells [27, 65]. In hard palate development, epithelial Hh signaling closely interacts with Fgf signaling molecules in the mesenchyme, specifically Fgf10 and Fgfr2 [31]. Similarly, downregulation of Fgf signaling in Dlx5+ cells disrupts tissue-tissue interactions, leading to aberrant development of the muscles of the soft palate [13]. Our findings implicate both autocrine and paracrine signaling in the soft palate region, since expression of Wnt, Hh, and Fgf signaling was present in epithelial, CNC-derived and myogenic cells, implying that these signaling pathways may regulate early soft palate development through epithelial-to-mesenchymal and mesenchymal-to-myogenic cell-cell interactions.

Precise understanding of the signaling pathways involved in the development of the soft palate is necessary to understand signaling instruction for the formation and patterning of the soft palate muscles, which is critical for the repair and regeneration of myogenic tissues. The pathway analysis presented in this study provides an important foundation for better understanding of the development of the soft palate myogenic cells and their close interaction with CNC-derived cells. Future studies using tissue-specific conditional knockout mice are necessary to analyze in detail the function of the suggested signaling network in this region. The integration of knowledge gained from expression patterns and conditional knockout mouse models will elucidate the complex regulatory networks and their specific functions controlling palate development.

## Supporting information

**S1 Fig. Active  $\beta$ -catenin is expressed throughout the embryonic development of the murine soft palate.** (A-L) Immunofluorescence for active  $\beta$ -catenin and MHC at E14.5 (A-D), E16.5 (E-H) and E18.5 (I-L). Schematic drawings of the mouse head in the top panel depict the position and angle of the sections. LVP, levator veli palatini; MHC, myosin heavy chain; PLG, palatoglossus; PLP, palatopharyngeus; TVP, tensor veli palatini. Scale bar (A-L) = 50  $\mu$ m. (TIF)

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