© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev180075. doi:10.1242/dev.180075

RESEARCH ARTICLE

Temporospatial sonic hedgehog signalling is essential for neural crest-dependent patterning of the intrinsic tongue musculature

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ABSTRACT

The tongue is a highly specialised muscular organ with a complex anatomy required for normal function. We have utilised multiple genetic approaches to investigate local temporospatial requirements for sonic hedgehog (SHH) signalling during tongue development. Mice lacking a *Shh cis*-enhancer, *MFCS4* (*ShhMFCS4/–*), with reduced SHH in dorsal tongue epithelium have perturbed lingual septum tendon formation and disrupted intrinsic muscle patterning, with these defects reproduced following global *Shh* deletion from E10.5 in *pCag-CreERTM; Shhflox/flox* embryos. SHH responsiveness was diminished in local cranial neural crest cell (CNCC) populations in both mutants, with SHH targeting these cells through the primary cilium. CNCC-specific deletion of orofaciodigital syndrome 1 (*Ofd1*), which encodes a ciliary protein, in *Wnt1-Cre; Ofdfl/Y* mice led to a complete loss of normal myotube arrangement and hypoglossia. In contrast, mesoderm-specific deletion of *Ofd1* in *Mesp1-Cre; Ofdfl/Y* embryos resulted in normal intrinsic muscle arrangement. Collectively, these findings suggest key temporospatial requirements for local SHH signalling in tongue development (specifically, lingual tendon differentiation and intrinsic muscle patterning through signalling to CNCCs) and provide further mechanistic insight into the tongue anomalies seen in patients with disrupted hedgehog signalling.

KEY WORDS: Neural crest, Sonic hedgehog, Tendon, Tongue

INTRODUCTION

The mammalian tongue is a highly specialised muscular organ situated in the oral cavity and oropharynx that contributes to multiple essential functions, including airway maintenance, phonetic articulation, oral sensation, mastication and swallowing. Normal function of the tongue requires the coordinated activity of both extrinsic and intrinsic muscles and their associated tendons, an extensive vasculature and complex sensory and motor innervation. The morphological and functional variation seen in the tongue of mammals is reflective of the significant environmental adaptation observed amongst these organisms (Iwasaki, 2002).

In the mouse embryo, tongue formation is heralded by the appearance of paired buds on the oral side of the first (mandibular) pharyngeal arch around embryonic day (E)10.0. These buds grow and ultimately fuse with a medial lingual swelling to produce an early primordium situated in the midline of the oral cavity floor by E11.0. The primordium is initially populated by cranial neural crest cells (CNCCs) (Han et al., 2012); however, myogenic progenitor cells subsequently enter this region from E11.5 following their migration from the caudal occipital somites as the hypoglossal cord (Czajkowski et al., 2014; reviewed by Noden and Francis-West, 2006). Further development of the tongue requires interaction between the overlying oropharyngeal epithelium, CNCCs and myogenic progenitors to regulate cell proliferation, differentiation and survival. By E13.5, a prototype tongue structure is established, with a symmetrically arranged intrinsic musculature and a specific group of extrinsic muscles suspending the tongue between the skull, palate, mandible and hyoid – all supported by the midline lingual septum and peripheral aponeurosis linguae tendons within the tongue dorsum (Standring, 2016). Although the gross embryological processes involved in forming the tongue have been understood for many years (Sadler, 2012) it is only relatively recently that some insight has been gained into the complex molecular signalling events that coordinate development (Parada et al., 2012).

Sonic hedgehog (SHH) is a secreted signalling protein that plays a key role in many diverse biological events extending from early development through to postnatal tissue homeostasis (reviewed by Briscoe and Thérond, 2013; Ingham and McMahon, 2001; Ingham et al., 2011; McMahon et al., 2003). SHH signals from three key regions of ectoderm in the early head, which includes the ventral forebrain, frontonasal process and oropharynx (reviewed by Helms et al., 2008; Marcucio et al., 2011; Petryk et al., 2015; Tapadia et al., 2005; Xavier et al., 2016). Specifically, *Shh* is expressed from E9.5 in the pharyngeal endoderm and oropharyngeal epithelium (Billmyre and Klingensmith, 2015; Firulli et al., 2014; Xu et al., 2019) with this expression maintained in the primordial tongue epithelium before localising to fungiform papillae of the anterior tongue from E12.5 (Jung et al., 1999; Sagai et al., 2009) (Fig. S1), where SHH plays a key role in regulating lingual epithelial cell fate (Castillo et al., 2014; Miura et al., 2014). Blocking SHH in embryonic rat pharyngeal explant culture arrests development of the early tongue primordium (Liu et al., 2004) and *Nkx2.5-Cre;Shh$^{flox/flox}$* mice lacking SHH from the early pharyngeal endoderm and oropharyngeal epithelium have aglossia and micrognathia (Billmyre and Klingensmith, 2015). Moreover, *Gas1$^{-/-}$; Boc$^{-/-}$* mice with reduced SHH signalling secondary to loss of co-receptor function have a diminutive and cleft tongue (Seppala et al., 2014). SHH signal transduction to CNCCs is essential for tongue development. *Wnt1-Cre; Smo$^{n/c}$* and *Wnt1-Cre; Kif3$^{flox/flox}$* mice lack SHH responsiveness in CNCCs from their point

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Received 20 May 2019; Accepted 17 August 2019

Development (2019) 146, dev180075. doi:10.1242/dev.180075

of migration through abrogated function of the essential smoothened (SMO) transducer or the primary cilium, respectively, and both have aglossia (Jeong et al., 2004; Millington et al., 2017). In addition, *Hand2-Cre; Smo$^{c/c}$* embryos lacking SMO function in the mandibular arch from around E9.5 display a tongue that is only rudimentary and associated with significantly reduced numbers of muscle precursor cells (Xu et al., 2019). Collectively, these studies have demonstrated a role for SHH transduction within CNCCs during establishment of the early tongue primordium, although the local contribution of SHH during subsequent development of this organ is less clear.

The long-range *cis*-regulatory enhancers mammal fish conserved sequence 4 (*MFCS4*) and mammal reptile conserved sequence 1 (*MRCS1*) located 600-900 Kb upstream of the mouse *Shh* locus drive regional *Shh* expression in the epithelial lining of the oral cavity and pharynx (Sagai et al., 2017, 2009). *MFCS4* is conserved between mammals and teleost fishes (Sagai et al., 2009) and is an orthologue of the human-fugu conserved sequence *SHH_1* (Goode et al., 2005; Woolfe et al., 2005), whereas *MRCS1* is conserved amongst mammals, chicken and lizard but not *Xenopus* or teleost fishes (Sagai et al., 2009). *MFCS4* directs *Shh* expression in epithelium of the primordial tongue, pharynx and larynx from E11.0, with strong expression in the epiglottis and arytenoid swelling by E13.5, whereas *MRCS1* regulates expression in the incisor and molar teeth at E12.5, and the palatal rugae and anterior fungiform papillae at E13.5. Targeted deletion of *MFCS4* in mice (*MFCS4*$^{-/-}$) results in severely downregulated *Shh* expression in epithelia of the pharyngeal and laryngeal regions, leading to truncation of the soft palate, slight deformation of the tongue, loss or reduced size of the epiglottis and hypotrophy of the arytenoid accompanied by hypoplasia of multiple cartilaginous laryngeal elements (Sagai et al., 2009). However, some hedgehog signalling activity does remain in anterior regions of the tongue in these mice, consistent with the predominantly posterior pharyngeal and laryngeal phenotype, and suggestive of a requirement for SHH signalling in tongue development after establishment of the early primordium. These mice therefore represent a useful experimental tool for investigating the role of SHH during tongue formation.

In this study, we have utilised a series of genetic approaches to investigate the temporospatial contribution of SHH signalling during tongue formation, focusing on timing and the local effect on tissues responding to this pathway within the early tongue. We find that production of SHH ligand in tongue epithelium from E10.5 to E12.5 is crucial for lingual septum tendon formation and a prerequisite for normal patterning of the intrinsic musculature, occurring primarily through local signalling to CNCCs within the tongue primordium. Moreover, we provide further insight into the tongue phenotypes seen in humans with disrupted hedgehog signalling, including the ciliopathies, for which the relative contribution of local signalling interactions and global timing of developmental events during tongue development is currently unclear.

RESULTS

SHH in the oropharyngeal epithelium is required for intrinsic muscle organisation in the developing tongue

To explore the function of SHH signalling within the tongue primordium, we first utilised *Shh*$^{MFCS4/-}$ mutant mice, in which one allele lacks *MFCS4*, a key *cis*-enhancer element for *Shh* expression in tongue epithelium activated at E11.0 (Sagai et al., 2009) and *Shh* is conventionally deleted in the other allele. It was predicted that an almost complete loss of *Shh* would occur in tongue and pharyngeal epithelia of the mouse, whereas the remaining tissues would be heterozygous for *Shh* expression. We confirmed that, in comparison to wild-type (WT) littermates, SHH signalling was significantly decreased in the developing tongue of *Shh*$^{MFCS4/-}$ mice by E11.5, as demonstrated by reduced *Shh* and patched 1 (*Ptch1*) expression (Fig. 1A-D) and consistent with the known period of *MFCS4* activity (Sagai et al., 2009). In addition, we also observed slightly decreased *Gli1* expression in the mutant (Fig. 1E,F) (Millington et al., 2017). At this stage of development, *Myf5*-positive occipital somite-derived myoblasts had reached the developing tongue primordia in both WT and *Shh*$^{MFCS4/-}$ mice (Fig. 1G,H), confirming that myoblast migration into the tongue primordium was not affected in these mice and in contrast to mice with disrupted SHH signalling in CNCCs through loss of primary cilia (Millington et al., 2017). The presence of myoblast differentiation was also confirmed in WT tongue through the detection of *Myod1* and desmin in anterior and posterior regions at E11.5 (Fig. 1I-L). Interestingly, myoblasts present anteriorly expressed *Myod1* but not desmin at this stage (Fig. 1I,K, anterior panels), whereas those located more posteriorly expressed both markers (Fig. 1I,K, posterior panels). Desmin is a marker of more advanced myoblast differentiation than *Myod1*, thus myoblast differentiation progresses from posterior to anterior during normal tongue development. The expression pattern of *Myod1* and desmin was not altered in *Shh*$^{MFCS4/-}$ mutants (Fig. 1J,L). At E12.5, analysis confirmed that SHH signalling was decreased but myogenic differentiation was not affected (data not shown). These observations were confirmed by quantitative RT-PCR (Fig. 1M).

At E13.5, the establishment of myotube organisation in both intrinsic and extrinsic muscles was indicated by the expression pattern of *Myod1* and desmin in WT (Fig. 1N,P). In *Shh*$^{MFCS4/-}$ mice, this arrangement was disrupted and distinct in the superior and inferior longitudinal, vertical and transverse intrinsic muscles, whereas in lateral regions where the bilateral extrinsic styloglossus muscles run, there was no significant disruption (Fig. 1O,Q). All *Shh*$^{MFCS4/-}$ mutants died between postnatal day (P)0 and P0.5 with cleft palate, and at this stage the mutant also exhibited disorganised intrinsic muscle fibres based upon anti-smooth muscle actin (SMA) immunohistochemistry (Fig. 1R-U). Interestingly, the disorganised intrinsic muscle arrangement was rarely found in the tongues of *MFCS4* homozygous-deleted mice (*Shh*$^{MFCS4/MFCS4}$) (Fig. S2).

The cleft palate observed in *Shh*$^{MFCS4/-}$ animals was caused by a failure of palatal shelf elevation, which requires a combination of intrinsic elevation forces within the shelves and extrinsic factors, such as clearing of the tongue from the roof of the mouth due to involuntary and swallowing movements. Maxillary organ culture showed that, although the soft palate was shorter in an anterior-posterior direction, the palatal shelves of *Shh*$^{MFCS4/-}$ foetuses successfully elevated and were able to fuse in the midline (Fig. S3). Based on these data as well as previous studies on tongue obstruction leading to cleft palate (Iseki et al., 2007; Tsunekawa et al., 2005), we hypothesised that decreased SHH signalling in the tongue results in disorganised intrinsic muscle arrangement, which leads to cleft palate. This idea was supported by the observation that the penetrance of cleft palate was limited in *Shh*$^{MFCS4/MFCS4}$ mice [13.5% in a previously published study (Sagai et al., 2009) versus 100% in *Shh*$^{MFCS4/-}$].

The finding of some residual *Shh* expression in the *Shh*$^{MFCS4/-}$ tongue after the stage at which *MFCS4* was activated (E11.0) suggested the presence of other factors regulating *Shh* in this region. Thus, use of the *Shh*$^{MFCS4/-}$ mutant in this study allowed us to examine the consequences of severely decreased epithelial *Shh* expression combined with a decrease of SHH signalling within the tongue primordium.

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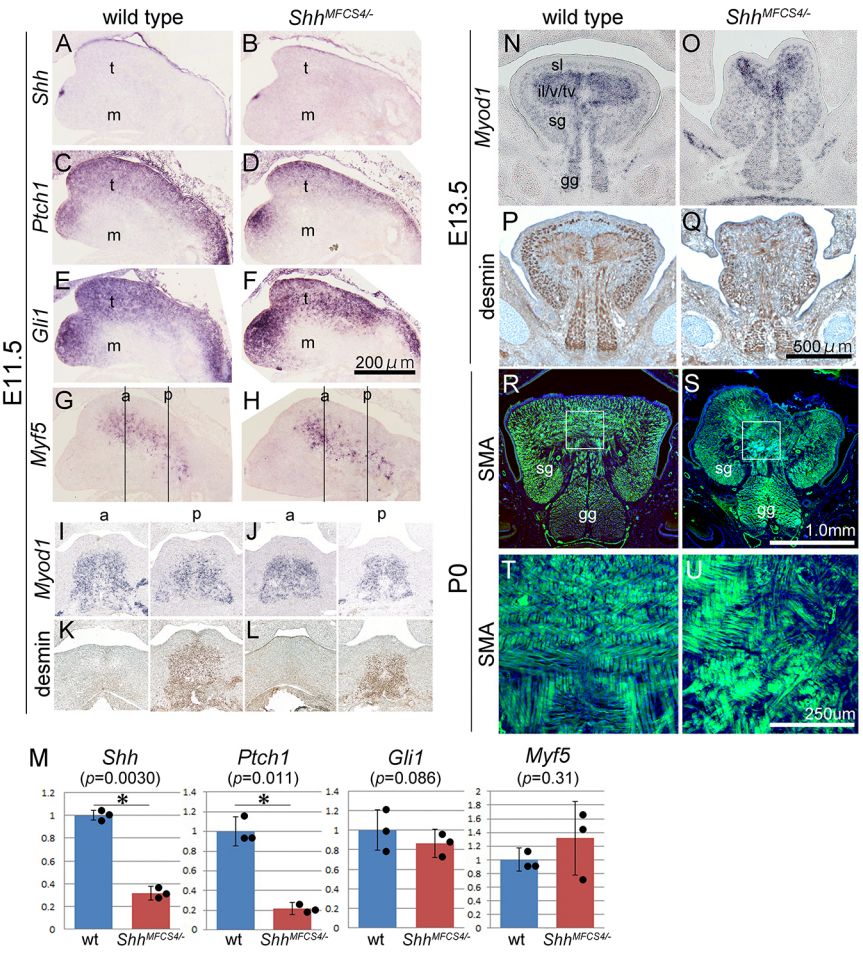

Fig. 1. Reduced SHH signalling in lingual epithelium results in impaired myotubule arrangement. (A-H) Expression of *Shh* (A,B), *Ptch1* (C,D), *Gli1* (E,F) and *Myf5* (G,H) on sagittal sections of the developing tongue in WT (A,C,E) and $Shh^{MFCS4/-}$ (B,D,F) heads at E11.5 (anterior is to the left for A-H). (I-L) Expression of *Myod1* (I,J) and immunohistochemistry for desmin (K,L) on coronal sections of the developing tongue in WT (I,K) and $Shh^{MFCS4/-}$ (J,L) heads at E11.5. Coronal plane of section indicated in G,H is anterior (a) and posterior (p) for I-L. Scale bar for A-L is in F. (M) Semi-quantitative RT-PCR analysis of *Shh*, *Ptch1*, *Gli1* and *Myf5* transcription in WT and $Shh^{MFCS4/-}$ tongue at E11.5. All experiments were performed three times, each one in triplicate. Data are shown as mean±s.d. and compared by two-tailed *t*-test (*P<0.05). (N-Q) Expression of *Myod1* and immunohistochemistry for desmin on coronal sections of E13.5 WT (N,P) and $Shh^{MFCS4/-}$ (O,Q). Scale bar for N-Q is in Q. (R-U) Immunohistochemistry for SMA on coronal sections of P0 WT (R,T) and $Shh^{MFCS4/-}$ (S,U) tongues. The lingual septum (white boxes) is magnified in T,U. Scale bar for R,S is in S and T,U is in U. A-J and N,O are all digoxigenin-labelled ISH. gg, genioglossus; il/v/tv, inferior longitudinal, vertical and transverse muscle; m, mandible; sg, styloglossus muscle; sl, superior longitudinal muscle; t, tongue.

SHH from the tongue epithelium is required for lingual septum tendon formation

CNCCs within the developing tongue have been suggested to act as a scaffold during the organisation of lingual myoblasts and myotubes (Parada et al., 2012). Patterning of the tongue musculature occurs simultaneously with tendon development and includes formation of the midline lingual septum tendon within the tongue dorsum as a flat broad vertical sheath of midline fibrous tissue. We investigated expression of the tenocyte marker scleraxis (*Scx*) (Schweitzer et al., 2001) using section *in situ* hybridisation and found low-level expression from E11.5 in the posterior tongue, but not the anterior (Fig. 2A and data not shown), consistent with the pattern of muscle localisation in the WT. At E12.5, an M-shaped expression domain was seen (Fig. 2C), coinciding with the expression pattern seen in whole mounts (Fig. 2E). In contrast, there was little evidence of *Scx* transcripts in $Shh^{MFCS4/-}$ mice at E11.5 (Fig. 2B) and only faint expression at E12.5 (Fig. 2D), which was confirmed in whole mounts (Fig. 2F). In addition to expression in the midline at the developing lingual septum tendon (Fig. 2G, double-headed arrow), a thin layer of bilateral widespread low-level expression was also found in the tongue dorsum at E13.5 in the WT, indicating early formation of the tendinous aponeurosis linguae (Fig. 2G, dotted line). Beneath the tongue, there was strong bilateral expression of *Scx* at sites of tendon formation associated with the paired extrinsic genioglossus muscles (Fig. 2G, arrowheads). In contrast, $Shh^{MFCS4/-}$ mice failed to develop *Scx*-positive tendons for the intrinsic muscles at E13.5 (Fig. 2H), despite clear expression in the extrinsic genioglossus muscle tendons (Fig. 2H, arrowheads). Expression of *Scx* in the tongue dorsum was significantly decreased; however, low-level *Scx* transcripts were occasionally observed in lateral regions of the mutant tongue dorsum (Fig. 2F,H, arrows). As *Shh* expression and *MFCS4* activation were found in the dorsal lingual epithelium, these observations suggested involvement of other factors in tenocyte differentiation on the lateral sides of the tongue. Detection of CD31 (Pecam1) and synaptophysin, markers for vascularisation and innervation, respectively, did not demonstrate any particular differences between WT and $Shh^{MFCS4/-}$ mice at E13.5 (Fig. S4).

CNCC-derived differentiation but not proliferation is affected in the tongue of $Shh^{MFCS4/-}$ mice

We further analysed CNCC differentiation in $Shh^{MFCS4/-}$ mice through the expression of *Sox9*, a marker for CNCCs as well as a common representative transcription factor for chondrocyte, ligament cell and tenocyte differentiation (Mori-Akiyama et al., 2003; Spokony et al., 2002). *Sox9* expression was transiently evident during the early stages of tongue development in the WT, with strong expression established in the future lingual septum-forming region by E12.5 (Fig. 2I, arrow); however, by E13.5 *Sox9* expression had dissipated in the WT and been replaced by *Scx* marking the lingual tendon (data not shown). In contrast, *Sox9* was only weakly expressed at E12.5 in the mutant (Fig. 2J). Collectively, these data suggest that in the process of tenocyte differentiation from CNCC-derived tongue primordium mesenchyme there is a transition stage, during which WT mesenchyme expresses both *Sox9* and *Scx*, whereas the mutant fails to properly express either gene.

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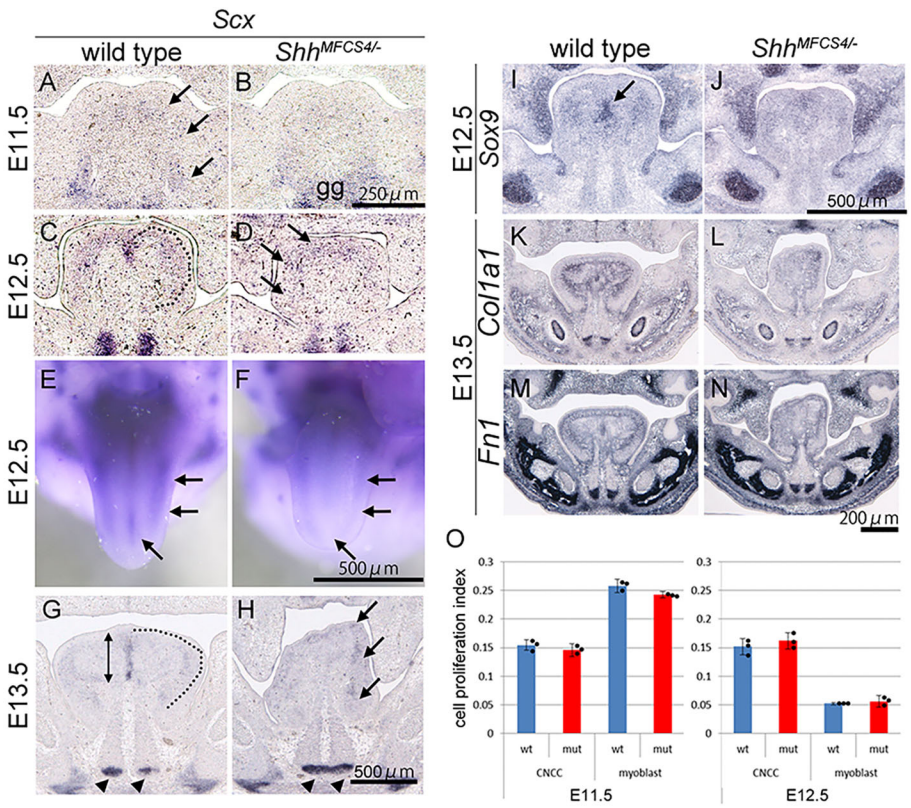

Fig. 2. SHH signalling is required for differentiation of lingual CNCCs to tenocytes. (A-H) Expression of *Scx* on coronal sections of the developing tongue (A-D,G,H) and on whole mounts (E,F) at E11.5 (A,B), E12.5 (C-F) and E13.5 (G,H) in WT (A,C,E,G) and $Shh^{MFCS4/-}$ (B,D,F,H) embryos. *Scx* expression in the future aponeurosis and lingual septum is indicated by arrows and the dotted line in C and G, which is not consecutive in $Shh^{MFCS4/-}$ (arrows in D,F,H). Arrowheads in G and H indicate the short genioglossus (gg) tendon origins at the paired superior mental spines. Scale bar for A-D is in B; for E,F is in F; for G,H is in H. (I,J) Expression of *Sox9* at E12.5 on coronal sections of the developing tongue in WT (I) and $Shh^{MFCS4/-}$ (J) embryos (arrow in I indicates expression in the future lingual septum). (K-N) Expression of *Col1a1* (K,L) and *Fn1* (M,N) at E13.5 on coronal sections of the developing tongue in WT (K,M) and $Shh^{MFCS4/-}$ (L,N) embryos. Scale bar for I,J is in J and for K-N is in N. (O) Lingual CNCC-derived and mesoderm-derived mesenchymal cell proliferation index at E11.5 and E12.5 in WT (wt) and $Shh^{MFCS4/-}$ (mut) embryos. *P*-values for each pair (left to right) were 0.14, 0.051, 0.26 and 0.25. The experiment was performed three times. Data are shown as mean±s.d. and compared by two-tailed *t*-test.

In accordance with the *Scx* expression pattern, type I collagen (*Col1a1*) and fibronectin (*Fn1*), which mark the extracellular matrices of tendon tissues, were clearly transcribed in WT tendon, including the aponeurosis and septum (Fig. 2K,M), whereas their expression in the $Shh^{MFCS4/-}$ tongue was weak and not well patterned (Fig. 2L,N). We next studied cell proliferation in the tongue mesenchyme at E11.5 and E12.5, during which tenocyte specification occurs in CNCCs. However, there were no significant differences in proliferative activity of CNCC-derived mesenchyme or myoblasts during this period (Fig. 2O) and no differences in patterns of cell death (assayed through active caspase 3 detection; data not shown) in WT or mutant embryos.

SHH is required for appropriate arrangement of the intrinsic musculature after tongue primordium formation

The analysis of $Shh^{MFCS4/-}$ mutant mice demonstrated an essential role for SHH signalling in the tongue primordium; however, *Shh* expression is present in the oropharyngeal epithelium before early tongue primordium formation and continues until a prototype intrinsic lingual muscle arrangement is established (see Fig. S1) (Sagai et al., 2009). Furthermore, some SHH activity is still present in $Shh^{MFCS4/-}$ embryos, mediated through *Shh* transcription independently of *MFCS4* activity (Fig. 1B,D,F). Therefore, we investigated the temporal requirements of SHH during tongue formation using stage-specific abrogation of gene function. Specifically, we crossed $pCag\text{-}CreER^{TM}$ mice (Hayashi and McMahon, 2002) with a line harbouring a conditional (floxed) Shh^{c} allele (Dassule et al., 2000). Single maternal tamoxifen administration was carried out at E10.5, E11.5 and E12.5, respectively, with the tongue phenotype analysed at E14.5. In comparison with WT littermates (Fig. 3A,E), significant disorganisation of the intrinsic musculature was present within the tongue of mutant mice, which was grossly dependent upon the timing of signal loss (Fig. 3B-D,F-H). In mice treated with tamoxifen at E10.5, in comparison with WT the normal striated architecture of the intrinsic muscles was lost and tongue size reduced (Fig. 3A,B,E,F), whereas deletion at later stages resulted in a progressively less severe disruption of myogenic pattern (Fig. 3C, D,G,H; Fig. S5). *In situ* hybridisation for *Myod1* suggested that myoblast differentiation had occurred in both the intrinsic and the extrinsic musculature, even in the most severely affected mutants (E10.5 treatment, Fig. 3J), but that organisation of the myotubes was defective (Fig. 3I-L). In comparison with WT, *Scx* expression was lost in the midline septum tendon and significantly downregulated in aponeurosis linguae-forming regions of the dorsum, whereas expression remained in the developing genioglossus and geniohyoid tendons of the mutant (Fig. 3M,N). *Scx* expression in the midline dorsum was progressively increased in foetuses exposed to later injections at E11.5 and E12.5 (Fig. 3O,P). Collectively, these data demonstrate an important timing-dependent role for SHH signalling after E10.5 in mediating normal tendon architecture and organisation of the intrinsic musculature of the tongue, with low levels of SHH signalling present in $Shh^{MFCS4/-}$ mice sufficient to support gross growth of the tongue. Interestingly, examination of K14-*Shh* transgenic embryos, which overexpress *Shh* in the developing tongue epithelium from around E11.5 (Cobourne et al., 2009) revealed a gross distortion of tongue architecture at E15.5 but a seemingly normal arrangement of the intrinsic muscles within the body of the tongue (data not shown). Therefore, myoblast organisation and differentiation is able to occur in the presence of excess SHH signal from the tongue epithelium during development.

SHH signalling in the developing tongue targets CNCCs through the primary cilium

Given that myoblast migration and differentiation was not affected by decreased SHH signalling in the tongue after E11.0, but that

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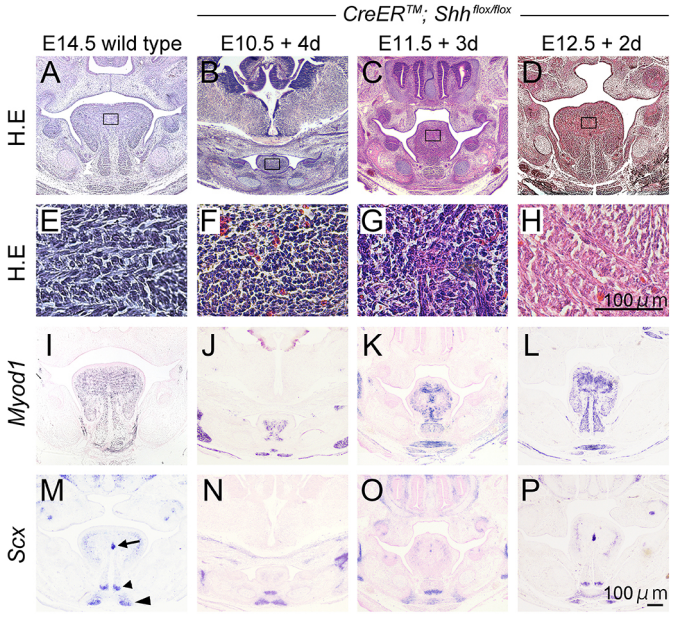

Fig. 3. Temporal loss of SHH produces a graded effect on tongue development. (A-D) H&E-stained coronal histological analysis of the developing tongue in E14.5 WT (A) and *pCag-CreER*^{TM}*; Shh*$^{flox/flox}$ (B-D) embryos injected with tamoxifen at E10.5, E11.5, E12.5 and harvested at E14.5, respectively. (E-H) Intrinsic muscle organisation in the tongue dorsum identified within the black rectangles in A-D. (I-P) Expression of *Myod1* (I-L) and *Scx* (M-P) on WT (I,M) and *pCag-CreER*^{TM}*; Shh*$^{flox/flox}$ embryos that received tamoxifen at E10.5, E11.5, E12.5 and were harvested at E14.5 (J-L,N-P). *Scx* expression in WT embryos at E14.5 highlights the lingual septum (M, arrow), genioglossus (small arrowhead) and geniohyoid muscles (large arrowhead). Scale bar in P is for A-D,I-P; in H for E-H.

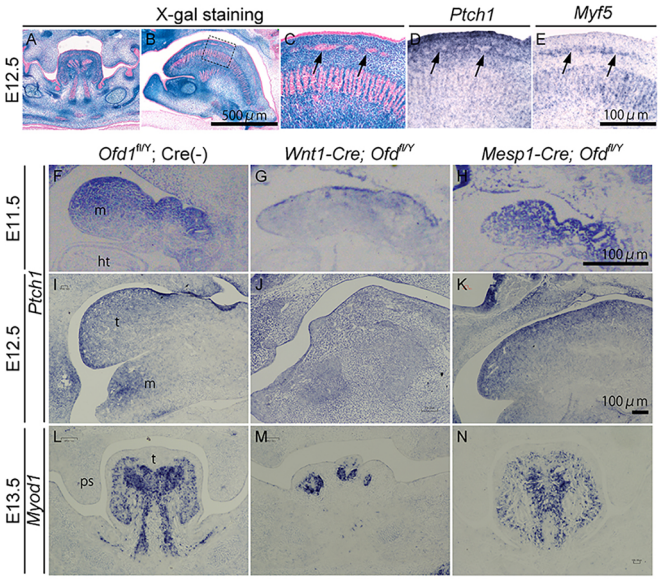

Fig. 4. SHH signalling is received by CNCCs during tendon formation and myotube arrangement. (A-C) CNCC-derived mesenchyme visualised in the developing tongue by X-gal staining for β-galactosidase activity on coronal (A) and sagittal (B,C) sections of E12.5 *Wnt1-Cre; R26R* embryos (C is a magnified view of the boxed area in B). (D,E) SHH-responsive cells and mesodermal cells were determined by *in situ* hybridisation on adjacent sections to B (and corresponding to the region shown in C) for *Ptch1* (D) and *Myf5* (E), respectively (arrows indicate superior longitudinal muscles in C-E). Scale bar in B is for A,B; and in E for C-E. (F-K) SHH signal activity was studied by *Ptch1* expression on sagittal sections of E11.5 (F-H) and E12.5 (I-K) WT (F,I), *Wnt1-Cre; Ofd*$^{fl/Y}$ (G,J) and *Mesp1-Cre; Ofd*$^{fl/Y}$ (H,K) embryos. (L-N) Lingual myotube arrangement was examined by *Myod1* expression on coronal sections of E13.5 WT (L), *Wnt1-Cre; Ofd*$^{fl/Y}$ (M), and *Mesp1-Cre; Ofd*$^{fl/Y}$ (N). Scale bar in H is for F-H, in K for I-N. ht, heart; m, mandibular process; ps, palatal shelf; t, tongue.

tendon formation and myotube arrangement specifically were, we hypothesised that CNCCs were the direct recipients of SHH signals. We therefore investigated the spatial relationship between SHH-responding cells, CNCCs and myoblasts in the developing tongue (Fig. 4A-E). Specifically, we examined the tongue of *Wnt1-Cre; R26R* embryos, which constitutively express β-galactosidase in CNCCs (and their progeny) from their point of exit at the neural tube (Chai et al., 2000). At E12.5, X-gal staining for β-galactosidase revealed the distribution of CNCCs in the developing tongue (Fig. 4A-C), and the expression of *Ptch1* on adjacent sections indicated that CNCC-derived mesenchymal cells were the primary target of SHH signal transduction during tendon formation and myotube arrangement (Fig. 4D). In contrast, *Myf5* was expressed in a broadly complementary pattern, suggesting that mesodermal cells are not the direct target of SHH in the tongue (Fig. 4E). Interestingly, *Gli1* showed a more uniform expression within the tongue, whereas the SHH co-receptor-encoding genes *Gas1*, *Cdon* and *Boc* were detected in the mesenchymal core, including myoblasts and more peripheral regions in domains consistent with a negative correlation between co-receptor expression and SHH signal levels (Martinelli and Fan, 2007) (Fig. S6).

The primary cilium is recognised as a key cellular organelle necessary for normal hedgehog reception and signal transduction within receiving cells (Huangfu and Anderson, 2005). Consistent with this, humans with ciliopathic loss-of-function orofaciodigital 1 (*OFD1*) mutations have multiple craniofacial anomalies, including cleft palate and tongue defects that involve clefting, cystic formation and hamartoma (Fenton and Watt-Smith, 1985). Animal models of *Ofd1* also show a ciliogenesis defect and loss of SHH signal reception (Adel Al-Lami et al., 2016; Ferrante et al., 2006) and mice lacking function of the ciliary protein Kif3a in CNCCs have aglossia (Millington et al., 2017). We generated CNCC-specific deletion in *Ofd1* using *Wnt1-Cre; Ofd*$^{fl/Y}$ mice and found decreased levels of *Ptch1* expression in CNCC-derived mesenchyme at E11.5 and 12.5 (Fig. 4F,G,I,J). In contrast, *Mesp1-Cre; Ofd*$^{fl/Y}$ embryos, which have *Ofd1* deletion in mesodermal cells from the onset of gastrulation (Saga et al., 1999) had relatively normal levels of *Ptch1* expression in the tongue (Fig. 4H,K). Significantly, there was a complete loss of normal myotube arrangement and hypoglossia in *Wnt1-Cre; Ofd*$^{fl/Y}$ embryos (Fig. 4L,M), whereas in *Mesp1-Cre; Ofd*$^{fl/Y}$ mutants myotube arrangement was largely unaffected (Fig. 4N). These observations further suggested that muscle patterning in the tongue requires continued SHH signalling from epithelium to CNCCs after tongue primordium establishment.

TGFβ and FGF signalling are influenced by SHH in the developing tongue

Transforming growth factor β (TGFβ) signalling has previously been shown to mediate interactions between CNCCs and myoblasts during cell proliferation, differentiation and tongue muscle organisation (Han et al., 2014; Hosokawa et al., 2010) and TGFβ receptors are transcribed in developing tongue mesenchyme (Hosokawa et al., 2010; Millington et al., 2017), which suggests potential cross-talk between TGFβ and SHH signalling. We therefore examined transcription of the ligands *Tgfb1*, *-2* and *-3* at E12.5 in WT and *Shh*$^{MFCS4/-}$ mice using section *in situ*

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hybridisation (Fig. 5A-F). *Tgfb1* did not show any defined expression within tongue mesenchyme of WT mice, but *Tgfb2* was mainly detected in the myoblasts and *Tgfb3* in the lingual septum tendon (Fig. 5A,C,E). Although *Tgfb1* did not show any expression in $Shh^{MFCS4/-}$ mutants either, *Tgfb2* and *-3* were both downregulated in the intrinsic musculature (Fig. 5B,D,F). However, these changes were subtle and, although qPCR analysis confirmed this trend, the changes were not significant (Fig. S7). Therefore, we further investigated TGFβ signalling in $pCag\text{-}CreER^{TM}; Shh^{flox/flox}$ mice using temporally controlled deletion of *Shh*. As SMAD2 and -3 are essential components of the TGFβ intracellular signal transduction pathway, their phosphorylation (pSMAD2/3) was examined by western blotting of tongues obtained from $pCag\text{-}CreER^{TM}; Shh^{flox/flox}$ mice treated with tamoxifen at E10.5 and analysed at E13.5. Interestingly, pSMAD2/3 was significantly reduced in all mutants compared with controls (Fig. 5G). It has also been demonstrated that fibroblast growth factor (FGF) signalling contributes to tenocyte differentiation in other parts of the body (Brent et al., 2005; Sukegawa et al., 2000; Du et al., 2016). We found that *Fgfr1* was expressed in the lingual septum tendon and aponeurosis linguae in WT mice; however, *Fgfr1* transcripts were significantly reduced in $Shh^{MFCS4/-}$ mice as revealed by both qRT-PCR comparison and *in situ* hybridisation (Fig. 5H) confirming the potential involvement of FGF signalling in tendon formation. Finally, we considered the possibility that WNT signalling could also be affected in our mutant models as previously reported (Zhu et al., 2017). However, neither RT-PCR analysis of the WNT target gene *Axin2* in $Shh^{MFCS4/-}$ mice at E12.5, nor mRNA *in situ* hybridisation for *Axin2* at E14.5 in $pCag\text{-}CreER^{TM}; Shh^{flox/flox}$ mice treated with tamoxifen at E10.5 showed any significant differences (Fig. S7).

Taken together, these data demonstrate that a loss of SHH or decreased SHH in the developing tongue epithelium from the time of tongue primordium establishment results in disrupted patterning of the lingual tendon and intrinsic tongue musculature, which is potentially mediated through TGFβ and FGF signalling.

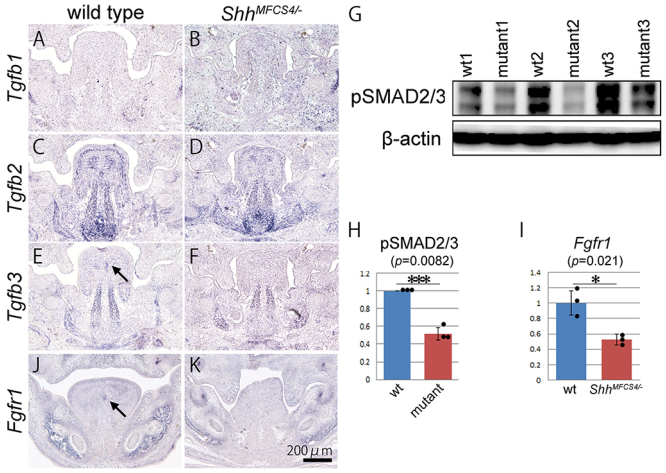

Fig. 5. TGFβ signalling is downstream of SHH in the developing tongue. (A-F,J,K) *Tgfb1*, *Tgfb2*, *Tgfb3* and *Fgfr1* expression on coronal sections through the developing tongue of E13.5 WT (A,C,E,I) and $Shh^{MFCS4/-}$ (B,D,F,J) embryos. *Tgfb3* and *Fgfr1* transcription in the lingual septum is indicated by arrows. Scale bar in J is for A-F,I,J. (G) Western blot analysis of pSMAD2/3 in the tongue of WT and $pCag\text{-}CreER^{TM}; Shh^{flox/flox}$ mutant embryos injected with tamoxifen at E10.5 and harvested 3 days later. (H) Western blot band intensity was analysed statistically (***P<0.01 significance; n=3 for each experimental group). Data are shown as mean±s.d. and compared by two-tailed *t*-test. (I) *Fgfr1* transcription in the developing tongue at E13.5 evaluated in WT and $Shh^{MFCS4/-}$ mice by semi-quantitative PCR. All experiments were performed three times, each one in triplicate. Data are shown as mean±s.d. and compared by two-tailed *t*-test.

DISCUSSION

Tongue anomalies are a common finding amongst the multiple defects that can affect the developing craniofacial region (Cobourne et al., 2019). In this study, we have investigated the temporospatial contribution of SHH signalling in the developing tongue through the analysis of multiple mouse mutants. During normal development, SHH signals from the early tongue primordium, pharyngeal and laryngeal epithelium to the underlying CNCCs and is required for normal formation of the lingual tendons and patterning of the intrinsic musculature. $MFCS4^{-/-}$ mice have downregulated SHH in the pharyngeal and laryngeal regions with soft palate truncation, deformation of the posterior tongue and loss of the epiglottis, and $Shh^{MFCS4/-}$ mice have an almost complete loss of SHH function in these regions and a corresponding lack of the lingual tendons and disorganised intrinsic muscle. In $pCag\text{-}Cre; Shh^{fl/fl}$ mice engineered with loss of SHH from E10.5, there is absence of the lingual tendons, disorganised intrinsic muscles and severe hypoglossia. Moreover, $Wnt1\text{-}Cre; Ofd^{fl/Y}$ mice with reduced SHH signal response in CNCCs have severe hypoglossia, clefting of the tongue and poorly organised muscular clumps within the dorsum. SHH from the tongue epithelium is therefore required for the specification and differentiation of CNCC-derived tenocytes, with formation of the lingual tendons providing a scaffold for myoblast cell populations and facilitating normal patterning of the intrinsic musculature. These findings are summarised in Fig. 6.

A local requirement for SHH signalling to CNCCs during formation of the lingual septum tendon and patterning of the intrinsic tongue musculature

Shh is expressed in multiple regions of the early pharyngeal epithelium; global *Shh* signal disruption prior to tongue anlage formation leads to aglossia (Billmyre and Klingensmith, 2015) and early transduction to migrating CNCCs is essential for normal development (Jeong et al., 2004; Millington et al., 2017; Xu et al., 2019). However, local *Shh* expression is maintained in epithelium of the early tongue primordium and the significance of this signalling has remained unclear.

Here, we have shown that loss of SHH from E10.5 onwards causes significant disruption to the intrinsic musculature with hypoglossia, but not aglossia, demonstrating an essential temporal role for local signalling. We also demonstrate that SHH directly induces formation of the CNCC-derived lingual septum tendon, which is required for normal myotube arrangement within the intrinsic musculature. The crucial time point appears to be from E11.0, as expression of *Shh* in the epithelium via the *MFCS4* enhancer is important for this process, with the removal of enhancer activity from one allele and *Shh* expression from the other resulting in severely decreased SHH signalling in CNCC-derived mesenchyme and defective tendon formation. Collectively, these results suggest that SHH from the oropharyngeal epithelium is required to induce tongue primordium formation before E10.5, with subsequent signalling from the oropharyngeal and tongue primordium epithelium required for differentiation of the lingual tendon during intrinsic tongue muscle patterning.

It is likely that some residual SHH signalling activity remains in $Shh^{MFCS4/-}$ mice because *Shh* and *Ptch1* expression was not completely lost (see Fig. 1A-D) and loss of *Scx* did not always occur. One possibility is that additional *Shh* enhancers or regulatory

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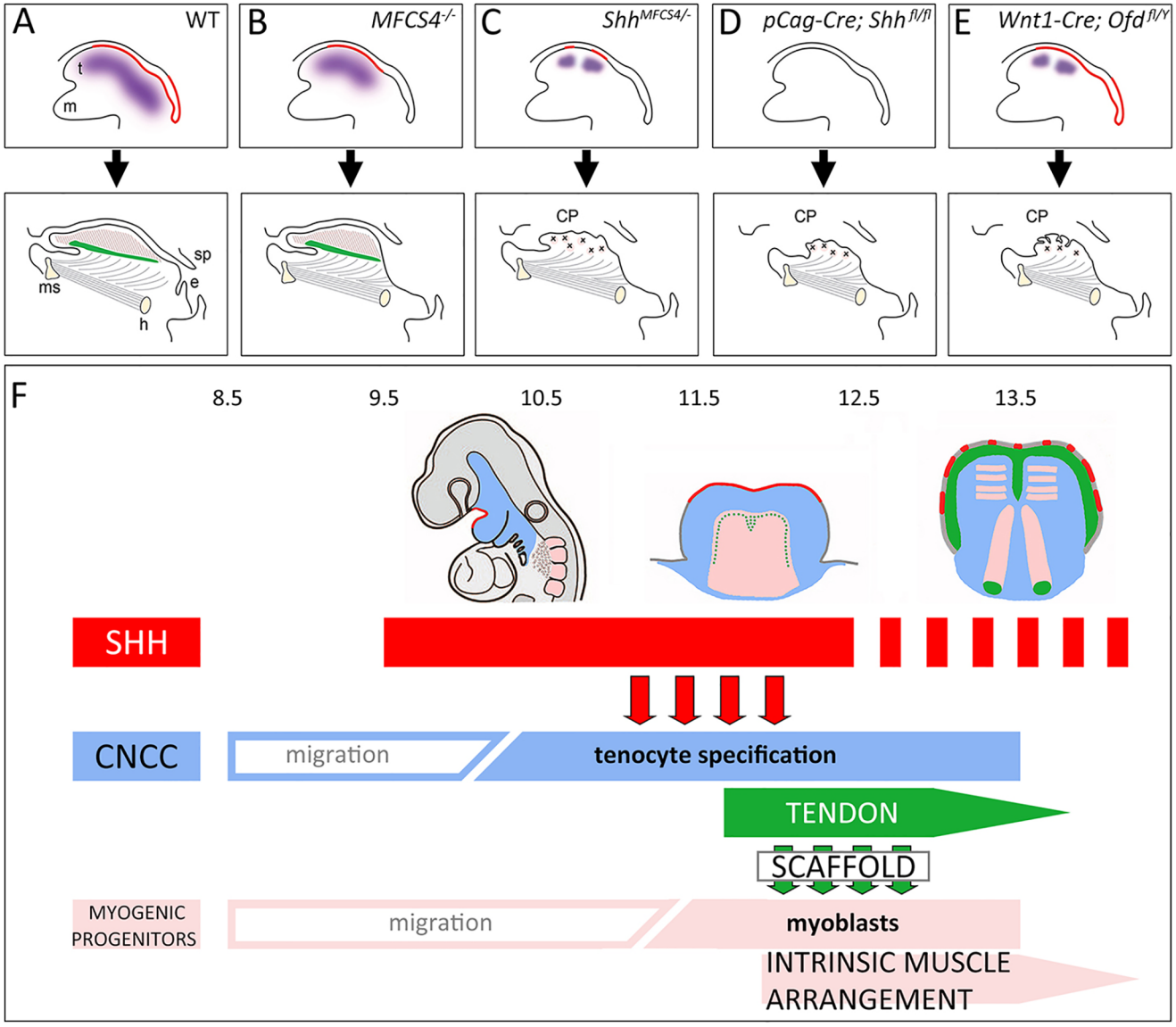

Fig. 6. Schematic of SHH function during murine tongue development. (A-E) SHH signalling during normal and abnormal tongue development. The upper panels represent SHH signal transduction in the early tongue at E11.5 in WT and mutant mice analysed in this investigation, with the lower panels representing subsequent tongue development at E14.5. D receives tamoxifen injection at E10.5. *Shh* expression is shown in red, *Ptch1* CNCC expression in purple, lingual septum tendon in green; extrinsic tongue muscles are curved grey lines, normal intrinsic muscles are vertical grey lines with pink background and disrupted intrinsic muscles are black crosses with pink background. CP, cleft palate; e, epiglottis; h, hyoid bone; m, mandibular process; ms, mandibular symphysis; sp, soft palate; t, tongue. (F) Temporal requirements for SHH function in tongue development. Key stages of early tongue development in the developing embryo at E9.5, E11.5 and E13.5 (SHH is in red, CNCCs in blue, myogenic progenitors and intrinsic/extrinsic tongue musculature in pink and lingual septum tendon in green). Gross development of the tongue therefore includes establishment of the primordium, formation of the lingual tendon from CNCC and muscle formation from myogenic progenitors. SHH from the tongue epithelium is required for the specification and differentiation of tenocytes from CNCC. Formation of the lingual tendon provides a scaffold for myoblast cell populations, which facilitates normal organization and patterning of the intrinsic musculature.

factors may compensate for loss of *MFCS4* function; however, the other known oropharyngeal epithelium enhancer *MRCS1* is unlikely to be responsible because it only activates *Shh* after E12.5 (Sagai et al., 2009) when expression of *Scx* has already been initiated in the tendon-forming area. Therefore, the precise regulatory mechanisms underlying *Shh* expression in the oropharyngeal epithelium remain to be fully elucidated (Anderson et al., 2014; Irimia et al., 2012). A slightly reduced tongue size was observed in *pCag-CreER*^{TM}*; Shh*$^{flox/flox}$ mice with loss of SHH from E11.5, suggesting that the remaining signalling in *Shh*$^{MFCS4/-}$ mice functions in tongue growth, which is supported by the observed cell proliferation assays (see Fig. 2O).

SHH signalling and tendon formation

The contribution of SHH signalling to tendon formation has been reported in a variety of anatomical systems, but not the tongue (Schweitzer et al., 2001; Subramanian and Schilling, 2015). Tendons are specialised connective tissues that assemble musculoskeletal tissues and anchor force-generating muscles to the skeleton, which facilitates locomotion and mobility in vertebrates. Consistent with our findings, a common feature of early tendon formation is the relationship between SHH and FGF signalling. During chick axis tendon formation, *Shh* expressed from ventral midline structures, such as the floor plate and notochord, inhibits induction of the syndetome, the dorsolateral compartment

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of the somite from which tendon cells are generated. However, SHH indirectly induces *Scx* expression through activation of FGF in the dermomyotome, which promotes *Scx* transcription in the somite with FGF signalling required for differentiation of tenocyte precursors in mice (Brent et al., 2005). In the chick digestive system, expression of *Scx* in two tendon domains that develop in close relation to visceral smooth muscles also depends upon FGF signalling (Le Guen et al., 2009) and *Shh* expressed from the endoderm is involved in regulation of smooth muscle cell and tenocyte differentiation (Sukegawa et al., 2000). A recent study has reported that some FGFs are increased during the period of lingual tendon formation (Du et al., 2016), suggesting involvement of FGF signalling in lingual tenocyte differentiation. In support of this, we detected diminished expression of *Fgfr1* in the lamina propria, future aponeurosis and lingual tendon of the $Shh^{MFCS4/-}$ tongue. Thus, we suggest that the differentiation of tenocytes derived from CNCCs is positively regulated by SHH via FGF signalling.

CNCC function, myogenic precursor cells and development of the tongue

It is established that normal CNCC function is required for tongue initiation through SHH signalling (Jeong et al., 2004; Millington et al., 2017). These investigations have shown that disrupting hedgehog responsiveness in CNCCs from their point of migration results in large-scale apoptosis within the first arch, with an accompanying failure of mesoderm-derived muscle precursor cell migration into the tongue anlage and aglossia. However, the role of SHH during regulation of subsequent developmental events, such as myoblast differentiation, is less clear. *Pax3*-positive muscle progenitors begin migration at E9.75 and reach the pharyngeal region subsequent to CNCCs by around E11.5 (Relaix et al., 2004). Our data point to a key step in tongue development whereby CNCCs receive epithelial SHH cues in the tongue primordium, which are essential for the normal organisation of intrinsic myogenic precursors, but not for myoblast differentiation. However, the expression of *Shh* and related pathway components (Fig. S6) suggests involvement in muscle formation and requires further investigation.

Molecular targets of SHH signalling in the embryonic tongue

We considered a number of molecular pathways known to regulate early tongue formation that may act downstream of SHH. Disruption of canonical TGFβ signalling in CNCCs through loss of *Tgfbr2* results in microglossia secondary to abnormal myogenic precursor cell proliferation and organisation via loss of Fgf10 (Hosokawa et al., 2010). The differentiation and proliferation of CNCCs is also disrupted (Hosokawa et al., 2010; Iwata et al., 2013), partially mediated by non-canonical TGFβ signalling acting through TGFβ-activated kinase (TAK1; Map3k7) (Song et al., 2013) and FGF/bone morphogenetic protein (BMP) signalling through ABL1 (Iwata et al., 2013). The disruption of Tgfβr1 (ALK5) in CNCCs also leads to severely disrupted tongue muscle formation via a lack of BMP4-mediated myogenic proliferation and Fgf4/6-mediated myogenic differentiation (Han et al., 2014). We found no significant change in *Tgfb2* and *-3* expression although non-significant changes in Tgfβr1 and -r2 have previously been reported in *Wnt1-Cre; Kif3a*$^{flox/flox}$ mice (Millington et al., 2017). A change in pSMAD2/3 was detected through western blot in the tongues of *pCag-CreER*^{TM}*; Shh*$^{flox/flox}$ embryos, which might conceivably be due to potential changes in BMP signalling rather than TGFβ. Indeed, hedgehog-SMO-GLI1 signalling is required for transcription of the Forkhead box genes *Foxf1*, *-f2*, *-d1* and *-d2* in CNCCs (Everson et al., 2017; Jeong et al., 2004; Millington et al., 2017) and negatively regulates BMP signalling in CNCCs through *Foxf1* and *Foxf2* during early patterning and survival of this cell population in the mandibular arch (Xu et al., 2019).

WNT signal transduction from the epithelium is also essential for tongue development (Lin et al., 2011). A loss of WNT secretion results in disrupted development of the epidermis, tenocytes and internal musculature, likely due to a requirement for signalling to underlying CNCC-derived connective tissue and muscle progenitor cells (Zhu et al., 2017). Interestingly, we did not observe altered *Axin2* expression in $Shh^{MFCS4/-}$ or *pCag-CreER*^{TM}*; Shh*$^{flox/flox}$ mice suggesting that WNT signalling was not altered in the mutants used in this study. However, further investigation of any association between SHH and WNT signalling in tongue development is required.

Tongue defects in the ciliopathies through disrupted SHH signalling

As our hypothesis was that CNCCs are key recipients of SHH signalling within the developing tongue, we considered models of human *OFD1* mutations that are predicted to result in neurocristopathies. *OFD1* mutations lead to a spectrum of craniofacial phenotypes, including gingival frenulae, lingual hamartomas, cleft palate and, significantly, cleft and/or lobulated tongue (Franco and Thauvin-Robinet, 2016). Moreover, ablation of the Kif3a ciliary protein in CNCCs is associated with aglossia (Millington et al., 2017). *Wnt1-Cre; Ofd*$^{fl/Y}$ embryos lacking *Ofd1* function in CNCCs had a severely hypoplastic tongue and complete disruption of myotube arrangement. The *Ofd1* gene is located on the X chromosome and encodes a component of the centrosome and basal body of primary cilia, a key mediator of SHH signalling (Satir et al., 2010). Impaired function of the cilium results in a variable effect on signal transduction, affecting not only SHH but also other pathways, such as WNT, depending upon the molecular context (Bangs et al., 2015). Again, although further investigation is required to clarify the cross-network interactions between SHH, FGF, TGFβ, BMP and WNT signalling during tongue formation, we note that the majority of phenotypes can be attributed to *Shh* expression from the epithelium. It is of further note that, in contrast to *Wnt1-Cre; Kif3a*$^{flox/flox}$ embryos (Millington et al., 2017), *Wnt1-Cre; Ofd*$^{fl/Y}$ embryos did not show aglossia but hypoglossia, which is suggestive that SHH signals can be transduced to some extent in these mice and may provide a useful tool for further analysis of SHH signalling in orofacial development.

Conclusions

Taken together, investigation of these temporospatial tissue-tissue interactions provides new insight into formation of the tongue, specifically the lingual septum tendon and intrinsic musculature, and reveal how localised signalling can influence gross structure and function of this highly adapted organ. This has relevance for further understanding of the molecular basis of multiple craniofacial disorders associated with tongue anomalies.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committees of Tokyo Medical and Dental University (0170238A), King's College London (PPL7007441 and PPL P8D5E2773, KJL), and National Institute of Genetics (28-7). *MFCS4*$^{+/-}$ (Sagai et al., 2009), *Shh*$^{-/+}$ (Amano et al., 2009), *pCag-CreER*^{TM} (Hayashi and McMahon, 2002), *Shh*$^{flox/+}$ (Dassule et al., 2000), *Wnt1-Cre* (Danielian et al., 1997), *Mesp1-*

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Cre (Saga et al., 1999) and *ROSA26* reporter (*R26R*) (Soriano, 1999) mice were maintained in a C57BL/6N background, whereas $Ofd^{fl/Y}$ (Ferrante et al., 2006) were maintained in a CD-1 background. In compound heterozygote $Shh^{-/+}$; $MFCS4^{+/-}$ mice (here referred to as $Shh^{MFCS4/-}$) the (*MFCS4*) long-distance *Shh* enhancer is deleted on one allele, whereas the (*Shh*) gene remains intact on the same allele. *pCag-CreER*^{TM}; $Shh^{flox/+}$ mice were mated with $Shh^{flox/+}$ mice, with pregnant mice receiving tamoxifen by intraperitoneal injection (75 mg/kg, equivalent to 3 mg per 40 g body weight) through the maternal body at the appropriate developmental stage. All comparisons were performed between the mutants above versus WT littermates.

Histological analysis

Specimens were fixed with Bouin's solution for Haematoxylin and Eosin (H&E) staining or fixed in 4% paraformaldehyde (PFA) in PBS for embedding in paraffin or OCT compound (Sakura Finetek) for other histological analyses. Sections were taken at 5 µm (paraffin) or 12 µm (frozen) thickness. All paired images for comparison were derived from littermates and were representative of at least three independent experiments.

Immunohistochemistry

For immunohistochemistry, anti-desmin antibody (clone D33, 413651, Nichirei Biosciences) at 1:1 dilution, anti-smooth muscle actin (SMA) antibody (RRID: AB_476701, clone 1A4, A2547-100UL, Sigma-Aldrich) at 1:1000 dilution, anti-bromodeoxyuridine (BrdU) antibody (clone BMC9318, 11 170 376 001, Roche Diagnostics) at 1:100 dilution, anti-MYF5 antibody (RRID: AB_10744494, polyclonal, SAB4501943, Sigma-Aldrich) at 1:100 dilution, anti-CD31 antibody (RRID: AB_726362, polyclonal, ab28364, Abcam) at 1:100 dilution, and anti-synaptophysin antibody (RRID: AB_2198854, clone SY38, ab8049, Abcam) at 1:100 dilution were used. For visualisation, corresponding secondary antibodies from the Vectastain ABC Kit (RRID: AB_2336827, AK-5000, Vectastain) and diaminobenzidine (DAB) were applied, or corresponding fluorescence-conjugated secondary antibody was applied [RRID: AB_2630356, goat anti-rabbit IgG H&L (Alexa Fluor 488), ab150077 and RRID: AB_2576208, goat anti-mouse IgG H&L (Alexa Fluor 488), ab150113, both Abcam]. Haematoxylin was used to counterstain DAB-labelled sections and Hoechst 33342 was used to stain DNA in fluorescent sections.

In situ hybridisation

For *in situ* hybridisation, specimens were hybridised in whole mounts or section using digoxygenin-labelled RNA probes specifically designed to be complementary to the partial mRNA of mouse *Shh*, *Ptch1*, *Gli1*, *Myf5*, *Myod1*, *Sox9*, *Scx*, *Col1a1*, *Fn1*, *Fgfr1*, *Ptch2*, *Cdon*, *Boc*, *Gas1*, *Hhip*, *Tgfb1*, *-2*, and *-3*, and *Axin2*, followed by incubation with anti-digoxygenin-AP conjugate. Nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) were used for visualisation. Section ^{35}S (Perkin Elmer) radioactive *in situ* hybridisation was carried out for *Shh* and *Ptch1* as previously described (Cobourne et al., 2004). Light- and dark-field images of sections were photographed using a Zeiss Axioskop microscope and merged in Adobe Photoshop CS6. All commercial reagents for *in situ* hybridisation were purchased from Roche Diagnostics. Probe sequences are listed in Table S1.

β-Galactosidase staining

For the detection of β-galactosidase activity using X-gal staining, sections were incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in phosphate buffer (pH 7.3) supplemented with 2 mM $MgCl_2$, 5 mM potassium ferrocyanide [$K_4Fe(CN)_6$-$3H_2O$] and 5 mM potassium ferricyanide [$K_3Fe(CN)_6$] at 30°C after fixation in 4% PFA. Nuclear Fast Red was used for counter staining.

Organ culture

The dissected maxilla (with vertically oriented palatal shelves) of E13.5 $Shh^{MFCS4/-}$ or WT littermates were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (Sigma-Aldrich) and BGJb Medium (Life Technologies) for 48 h with continuous supply of 95% O_2+5% CO_2 at 37°C using a rotary culture system.

Cell proliferation analysis

Sixty minutes before dissection, BrdU at 100 mg/ml was injected intraperitoneally into pregnant females at 10 mg/kg on the designated day. Every sixth of seven sections through the tongue primordium of a specimen were used for the cellular proliferation analysis. BrdU incorporation and MYF5 localisation were detected by immunohistochemistry. External cells scored as epithelial cells were confirmed by basement membrane staining. Myoblast cell lineage was determined as MYF5-positive cells, and MYF5-negative cells were considered to be CNCC derived. A proliferation index was calculated from the number of BrdU-positive cells divided by the total number of cells of each population and statistical significance was examined by two-tailed Student's *t*-test for three individual experiments for each genotype.

Real-time RT-PCR

RNA was extracted from the tongues of $Shh^{MFCS4/-}$ and WT littermate embryos at E12.5 using the Direct-zol RNA MiniPrep Kit (R2050S, Zymo Research) following the product protocol. mRNA (250 ng) was transcribed to cDNA by ReverTra Ace (TRT-101, Toyobo Life Science). Real-time PCR was performed with LightCycler 480 High Resolution Melting Master (04909631001, Roche Diagnostics). The expression was normalised to the β-actin gene and relative expression to the littermate WT was shown. Statistical significance was examined by two-tailed *t*-test for three individual experiments for each genotype. The primer sequences are listed in Table S2.

Western blotting

Whole tongue lysates were obtained from *pCag-CreER*^{TM}; $Shh^{flox/flox}$ and WT littermate embryos in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) (n=3 for each). Equivalent amounts of protein lysate were run on a 4-12% gradient Norex gel (Thermo Fisher Scientific) and separated proteins electro-transferred onto a nitrocellulose membrane. Phosphorylated SMAD2/3 (pSMAD2/3) and β-actin (β-actin) were detected using the corresponding antibodies [phospho-SMAD2 (Ser 465/467)/SMAD3 (Ser 423/425) (D27F4) rabbit monoclonal antibody; β-actin (13E5) rabbit monoclonal antibody; RRID: AB_2631089 and AB_10694076, respectively, Cell Signalling Technology] both at 1:1000 dilution. Bands were analysed using the Chemidoc MP imaging system (Bio-Rad) and intensity was measured using Image lab software (RRID:SCR_014210, version 5.2.1). Band intensities were normalised against β-actin and data plotted on a histogram. The integrated volume of pSMAD2 and -3 bands were divided by that of β-actin band and statistically examined for comparison.

Acknowledgements

We thank Zoe Webster and Jane Sealby (Embryonic Stem Cell Facility, MRC Clinical Sciences Centre, Imperial College London) and Paul Sharpe (King's College London) for generation of K14-*Shh* embryos through microinjection. We also thank William Barrell for expertise with schematics.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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Funding

This work was supported by Japan Society for the Promotion of Science KAKENHI grants (19890071, 22592254, 25463130, 16K11744 to S.O.; 20390510 to S.I.), and National Institute of Genetics NIG-JOINT grants (2008-A, 2009-B7, 2012-B4 to S.I.).

H.A.A.-L. is funded by the Higher Committee for Education Development in Iraq. K.J.L. received funding from the Biotechnology and Biological Sciences Research Council (BB/I021922/1) and the Medical Research Council (MR/L017237/1). M.T.C. received a Small Research Grant from the Royal College of Surgeons of Edinburgh for generation of K14-*Shh* transgenic mice. G.M.X. was funded by the programme ALBAN, the European Union programme of high-level scholarships for Latin Americans (E07D400355BR).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.180075.supplemental

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