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Expression of FGFs during early mouse tongue development

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Abstract

The fibroblast growth factors (FGFs) constitute one of the largest growth factor families, and several ligands and receptors in this family are known to play critical roles during tongue development. In order to provide a comprehensive foundation for research into the role of FGFs during the process of tongue formation, we measured the transcript levels by quantitative PCR and mapped the expression patterns by *in situ* hybridization of all 22 *Fgfs* during mouse tongue development between embryonic days (E) 11.5 and E14.5. During this period, *Fgf5, Fgf6, Fgf7, Fgf9, Fgf10, Fgf13, Fgf15, Fgf16* and *Fgf18* could all be detected with various intensities in the mesenchyme, whereas *Fgf1* and *Fgf2* were expressed in both the epithelium and the mesenchyme. Our results indicate that FGF signaling regulates tongue development at multiple stages.

Keywords

Tongue; FGF; expression; papilla

Introduction

The tongue is a highly flexible organ that is important for speaking, swallowing, mastication and degustation (Noden and Francis-West, 2006). Various malformations of the tongue such as macroglossia (Vogel et al., 1986), hypoglossia (Kuroda and Ohyama, 1981) and aglossia (Johnson and Robinow, 1978) have been described in clinical practice. Therefore, it is

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important to understand the factors that impact the development of the tongue during its development.

The mouse provides a useful model to study mammalian tongue development because of the availability of molecular and genetic tools. In mice, tongue formation begins at E11, when two lateral lingual swellings called the tongue buds arise from the first pharyngeal arch (Paulson et al., 1985). These buds merge to form the tongue primordium at E11.5, and cell proliferation followed by differentiation in both epithelium and mesenchyme ensue, resulting in the rapid enlargement of the tongue (Nagata and Yamane, 2004; Nie, 2005). Between E11.5 and E13, the mesenchymal cells give rise to muscle progenitor cells which proliferate, increase in number, and become myoblasts by E13 (Buckingham et al., 2003). These myoblasts then differentiate to form myotubes between E13 and E15 (Yamane et al., 2000). Differentiation of the tongue epithelium also begins around E13 with initiation of a single circumvallate papilla and numerous fungiform papillae, followed by the differentiation of filiform papillae and foliate papillae at E15 (Paulson et al., 1985).

The fibroblast growth factor (FGF) family is one of the largest growth factor families and consists of 22 members that share 13–71% sequence homology in mammals (Ornitz and Itoh, 2001). Most FGFs mediate their biological responses by means of binding to and activating cell surface tyrosine kinase receptors (FGFRs) (Itoh and Ornitz, 2004; Ornitz and Itoh, 2001). FGF signaling plays essential roles in regulating distinct biological processes and has been shown to be a critical regulator of the development of several craniofacial structures including the tooth (Wilkinson et al., 1989), the palate (Foreman et al., 1991), the brain (Caday et al., 1990) and the salivary gland (Amano et al., 1993).

In the case of tongue development, FGF signaling is known to play a number of important roles. For instance, partial ankylosis of the tongue has been described in both *Fgfr2b*$^{-/-}$ and *Fgf10*$^{-/-}$ mice, where there is defective epithelialization between the floor of the mouth and the tongue (Rice et al., 2004). At E13, *Fgfr2b*$^{-/-}$ tongue epithelium was thinner and disorganized when compared to the control, whereas *Fgf10*$^{-/-}$ mutants form occasional patches of thicker epithelium on the dorsum of the tongue (Rice et al., 2004). In addition, tongues of *Fgf10*$^{-/-}$ mice appeared to have larger fungiform papillae and did not have a circumvallate papilla (Petersen et al., 2011). Another example of the importance of FGFs during tongue development comes from studies showing that *Fgf6* acts downstream of Smad4-mediated TGFβ signaling to control myogenic differentiation and myoblast fusion during tongue development (Han et al., 2012). Furthermore, deletion of *Sprouty2* (*Spry2*), a negative regulator of FGF signaling, led to fewer fungiform papillae and duplicated circumavallate papillae. Consistent with this, *Spry1;Spry2* double knockout (KO) embryos exhibited multiple circumvallate papillae (Petersen et al., 2011). Thus, these studies demonstrate the involvement of several key FGFs during the process of tongue formation. However, a comprehensive analysis of the expression of FGFs during tongue development is currently missing. Here, we describe the expression patterns and levels of all 22 FGFs during tongue formation between E11.5 and E14.5, thus providing a basis for future research regarding the role of FGF signaling during the early stages of tongue development.

Results

Screening for expression of members of the FGF family during early tongue development

In order to determine which of the 22 FGF family members are expressed in the developing tongue between E11.5 to E14.5 and to quantitatively detect their changes in expression levels over time, we first performed quantitative PCR (qPCR) analysis (Fig. 1). We found that *Fgf10, Fgf9, Fgf18, Fgf13, Fgf11, Fgf14, Fgf8* and *Fgf21* (in order of relative expression level from high to low) were expressed at relatively constant levels during this period. In contrast, *Fgf2, Fgf6, Fgf7* and *Fgf12* were expressed at low levels at E11.5 and E12.5 but were upregulated at E13.5 and E14.5. This was especially pronounced with *Fgf6*, where a 7-fold increase was observed ($p<0.001$). Interestingly, the transcript level of *Fgf7* from E13.5 onwards was nearly identical to *Fgf10*, and it was one of the most expressed FGFs at those time points. Similar expression dynamics were also observed for *Fgf4* and *Fgf16*, although their fold changes occurred more gradually and their expression level in general was low.

In the case of *Fgf15*, the opposite expression profile to that described above was observed. *Fgf15* initially has high expression at E11.5 but drastically decreases at E12.5 ($p<0.05$) and eventually reaches a basal level at E14.5. A similar trend was also noted for *Fgf3*, although within a much smaller range.

Fgf1 and *Fgf5* each had distinct expression profiles. For instance, whereas *Fgf1* peaked at E13.5 with a 2-fold increase ($p<0.001$) and then was reduced to its basal level at E14.5($p<0.01$), *Fgf5* expression plunged 3-fold at E12.5 ($p<0.01$) before recovering at E13.5. Finally, the transcript levels of *Fgf17, Fgf20, Fgf22* and *Fgf23* were barely detectable, suggesting that they are either expressed at very low levels or not at all.

The expression domains of FGFs during tongue development

Although qPCR provides a quantitative measure of the expression level of each FGF ligand over time, it could not resolve the spatial distribution of these transcripts. Therefore, we performed whole mount *in situ* hybridization in order to discern the expression patterns of all 22 FGFs during early mouse tongue development. Vibratome sections along the coronal plane were subsequently generated from the whole mount samples to visualize the staining within the tissue. Between E11.5 and E14.5, the expression of Fgf3, Fgf4, Fgf8, Fgf11, Fgf12, Fgf14, Fgf17, Fgf20, Fgf21, Fgf22 and *Fgf23* was very low compared with other Fgfs by *in situ* hybridization and qPCR (Fig. 1). In addition, the representative sense probe controls for *Fgf8* (Fig. S1) and *Fgf22* (Fig. S2) showed the same intensity of non-specific background of the antisense probe. For those Fgf family members with very low levels of expression by qPCR (Fig. S1, S2), any signal observed by *in situ* hybridization is likely nonspecific. But interestingly, distinct expression patterns were observed for a number of FGFs at each stage, as detailed below.

At E11.5, *Fgf9* expression was present in both the lingual margin and posterior part of the tongue (Fig. 2U). In comparison, *Fgf10* and *Fgf18* were only expressed in the lingual margin of the tongue (Fig. 3A, Q), whereas *Fgf15* expression was restricted to the posterior part of the tongue (Fig. 3I). On coronal sections, *Fgf9, Fgf10* and *Fgf18* were expressed in the

lateral sub-epithelial mesenchyme, whereas *Fgf9* was also expressed in the inferior mesenchyme (Fig. 2U', 3A', Q'). *Fgf15* was weakly expressed in the central mesenchyme (Fig. 3I').

At E12.5, we observed expression of *Fgf1, Fgf2, Fgf9* and *Fgf10* in the anterior part of the tongue, except for the median sulcus (Fig. 2B, F, V, 3B). For *Fgf18*, strong expression was detected in the lingual margin of the anterior half of the tongue, with weaker expression in the posterior half (Fig. 3R). The coronal sections showed that *Fgf2* was expressed in the epithelium and the sub-epithelial mesenchyme, where we also found weak expression of *Fgf1* (Fig. 2B', F'). Interestingly, *Fgf9* was no longer expressed in the sub-epithelial mesenchyme at this stage, but rather in the deep mesenchyme in the body of the tongue (Fig. 2V'), where strong expression of *Fgf10* was also detected (Fig. 3B'). Finally, *Fgf18* was highly expressed in the lateral sub-epithelial mesenchyme (Fig. 3R').

At E13.5, we detected the expression of *Fgf1, Fgf2, Fgf5, Fgf6, Fgf7, Fgf9, Fgf10* and *Fgf18* with various intensities (Fig. 2C, G, K, O, S, W, 3C, S). The expression of *Fgf1, Fgf5, Fgf7, Fgf9, Fgf10*, and *Fgf18* was observed in the anterior part of the tongue (Fig. 2C, K, S, W, 3C, S), and *Fgf2* expression could be detected in the epithelium throughout the entire tongue (Fig. 2G). On the other hand, *Fgf6* expression was restricted to the mesenchyme along the anterior-posterior axis (Fig. 2O). On coronal sections, we found that *Fgf1* and *Fgf2* were expressed in the epithelium and the sub-epithelial mesenchyme (Fig. 2C', G'). *Fgf6* and *Fgf7* were expressed in restricted domains in the deep mesenchyme near the median sulcus (Fig. 2O', S'). *Fgf9* and *Fgf10* were highly expressed in the longitudinal muscles and the peripheral regions of the transverse and vertical muscles of the tongue, with very weak expression of *Fgf5* also detected in the same region (Fig. 2W', K', 3C'). Moreover, *Fgf18* expression was shifted closer to the center region at this stage (Fig. 3S').

At E14.5, we observed distinct expressions patterns of *Fgf1, Fgf2, Fgf5, Fgf6, Fgf9, Fgf10, Fgf13, Fgf16* and *Fgf18. Fgf2, Fgf5, Fgf6, Fgf9, Fgf10, Fgf13, Fgf16* and *Fgf18* in the anterior lateral part of the tongue near the median sulcus (Fig. 2D, H, L, P, X, 3D, H, P, T). On coronal sections, *Fgf1* expression weakened, whereas *Fgf2* continued to be expressed in the epithelium and sub-epithelial mesenchyme (Fig. 2D', H'); these findings were consistent with our qPCR results. In addition, *Fgf6* was highly expressed in the deep mesenchyme around the midline (Fig. 2P'), and *Fgf10* was expressed highly in the longitudinal muscles of anterior tongue (Fig. 3D'). In the transverse and vertical muscle region, we also detected expression of *Fgf5, Fgf9, Fgf13* and *Fgf16* bilaterally (Fig. 2L', X', 3H', P'), whereas *Fgf18* was only expressed in the inferior margin of this area (Fig. 3T'). Thus, several FGFs were expressed in specific domains within the tongue at different time points, suggesting that they may have functional roles in regulating distinct developmental processes during formation of the mouse tongue.

Discussion

During early tongue development, expression of *Fgf5, Fgf6, Fgf7, Fgf9, Fgf10, Fgf13, Fgf15, Fgf16* and *Fgf18* were detected at various intensities in the mesenchyme, while *Fgf1*

and *Fgf2* were expressed in both the epithelium and the mesenchyme between E11.5 and E14.5.

Although no distinct expression patterns were observed at E11.5 for *Fgf1* and *Fgf2*, which belong to the same *Fgf* subfamily, qPCR results suggested that their transcripts were present (Fig. 1, 2A, A', E, E'). Thus, it is likely that they are expressed throughout the entire mesenchyme, as opposed to in discrete domains. As FGF1 and FGF2 both act as mitogens (Seed and Hauschka, 1988; Suh et al., 2014), they may play a role in promoting cell proliferation in the muscle tissue. Interestingly, their expression became localized to the epithelium and the sub-epithelial mesenchyme at E12.5 and the transcript level further increased at E13.5 (Fig. 1, 2B', C', F', G'). As *Fgf1* expression was much weaker than that of *Fgf2* and it was downregulated at E14.5 (Fig. 1, 2D, D', H, H'), we hypothesize that *Fgf2* is the main driver for differentiation of the epithelium and the mesenchyme, consistent with previous reports (Nie, 2005).

Fgf5 has previously been shown to inhibit the terminal differentiation of myotomal myoblasts when they migrate through the trunk (Haub and Goldfarb, 1991). This suggests that *Fgf5* might act to maintain the process of proliferation and differentiation in other muscles as well. As we observed *Fgf5* expressionin the anterior part of the tongue within the muscle tissue at E14.5 (Fig. 2L, L'), *Fgf5* may be involved in the regulation of differentiation of the tongue muscles.

While *Fgf6* is expressed at a low level at E11.5 and E12.5, its expression is upregulated significantly from E13.5 onwards, suggesting that *Fgf6* functions in the differentiation period of the tongue (Fig. 1). This result is consistent with earlier work showing that during tongue myogenesis FGF6 acts downstream of Smad4-mediated TGFβ signaling to control myogenic differentiation and myoblast fusion(Han et al., 2012).

Although the transcript level of *Fgf7* was very high, its expression in the mesenchyme based on *in situ* hybridization at E13.5 in the anterior part of the tongue was rather weak, and there was no distinct expression pattern at E11.5, E12.5 and E14.5(Fig. 1, 2Q-T, Q'-T'). One explanation for this might be that *Fgf7* is expressed in a diffuse manner throughout the entire mesenchyme and epithelium. Given its high expression level by means of qPCR, it is still likely that that *Fgf7* has a functional role in regulating cell proliferation and differentiation during early tongue development.

Fgf10, another FGF belonging to the *Fgf7* subfamily, is the most abundant family member expressed during early tongue development (Fig. 1). *Fgf10* and *Fgf7* have previously been shown to exert similar functions during development. For example, both $Fgf7^{-/-}$ and $Fgf10^{-/-}$ mice develop smaller kidneys (Ohuchi et al., 2000). Specifically, *Fgf10/Fgfr2b* signaling is critical for epithelial-mesenchymal interactions in several developing organs, including the limb, the tooth and the palate, where signals from the developing mesenchyme induce the overlying epithelium to thicken. The thickened epithelium in turn signals back to the underlying mesenchyme to regulate growth and patterning (Kettunen et al., 2000; Revest et al., 2001). Given that FGF7, which is also a ligand of FGFR2b, may play a role in mesenchymal stimulation of normal epithelial cell proliferation (Rubin et al., 1989), it is

possible that *Fgf10* and *Fgf7* have similar functions in the tongue as in other organs to regulate shape and size by controlling proliferation and/or differentiation of tongue epithelium.

Fgf9 is another highly expressed FGF between E11.5 and E14.5 (Fig. 1). At E11.5, as its expression is restricted to the lateral sub-epithelial and the inferior mesenchyme (Fig. 2U'), it is possible that *Fgf9* acts on peripheral mesenchyme to induce proliferation and may play a role in epithelial-mesenchymal interactions. This would be consistent with what has been described in the mouse embryonic lung, where FGF9 is able to promote proliferation in both the epithelium and the mesenchyme, while inhibiting differentiation in the mesenchyme without affecting distal epithelial differentiation (del Moral et al., 2006). From E12.5 onwards, *Fgf9* is widely expressed in the mesenchyme in the anterior part of the tongue, and its expression more or less coincides with that of *Fgf10* (Fig. 2V-X, V'-X', 3B-D, B'-D'). As it has been reported that *Fgf9* is a main up-regulator of *Fgf10* expression during early lung development (del Moral et al., 2006), this raises the possibility that it may have similar functions in the forming tongue.

Fgf16 is another member of the *Fgf9* subfamily that is initially expressed at a low level and gradually increases. While no distinct expression patterns were observed prior to E14.5, it was expressed in restricted domains of the deep mesenchyme at E14.5 (Fig. 3M-P, M'-P'). Previously, FGF16 was shown to induce the proliferation of hepatocytes (Danilenko et al., 1999), embryonic brown adipocytes (Konishi et al., 2000) and possibly cardiomyoblasts (Lavine et al., 2005). However, given the low transcript level of *Fgf16*, it may play a minor role in driving proliferation in the tongue.

Fgf13, an FGF in the *Fgf11* subfamily, has prominent and widespread expression throughout the embryonic central and peripheral nervous systems (Hartung et al., 1997). In our study, *Fgf13* was detected throughout the period between E11.5 andE14.5 by qPCR (Fig. 1), and by *in situ* hybridization in the mesenchyme adjacent to the midline from E11.5 to E13.5 (Fig. 3E-G, E'-G'). At E14.5, it was expressed in bilateral regions of transverse and vertical muscles in the anterior tongue (Fig. 3H, H'). *Fgf13* may be involved in mesenchymal proliferation and differentiation, but the function of *Fgf13* remains largely unexplored.

The expression of *Fgf15* could be observed in the central region of the tongue mesenchyme at E11.5, after which its expression was downregulated and no apparent expression could be found (Fig. 3I-L, I'-L'). Based on earlier reports that *Fgf15* may play important roles in controlling liver cell proliferation (Padrissa-Altes et al., 2014), we predict that *Fgf15* mainly acts as a mitogen in the tongue without affecting differentiation of the tongue mesenchyme.

Fgf18 is a member of the *Fgf8* subfamily and has been shown to play a key role in skeletal growth and development (Marie, 2003; Moore et al., 2005). In our study, *Fgf18* was expressed in the lateral sub-epithelial mesenchyme at E11.5, and this expression intensified at E12.5 (Fig 3Q, Q', R, R'), consistent with our qPCR results. FGF18 is a pleiotropic growth factor that can stimulate proliferation in a wide variety of mesenchymal and epithelial cells and tissues, such as lungs, kidneys, hearts, testes, spleens, skeletal muscles

and brains (Haque et al., 2007). As a result, it is likely that *Fgf18* functions similarly during tongue development.

Finally, although no specific staining for Fgf3, Fgf4, Fgf8, Fgf11, Fgf12, Fgf14, Fgf17, Fgf20, Fgf21, Fgf22 and *Fgf23* was found in mouse tongues between E11.5 and E14.5 (Fig. S1, S2), weak expression of *Fgf3, Fgf4, Fgf8, Fgf11, Fgf12, Fgf14* and *Fgf21* by qPCR (Fig. 1) suggests that these 7 genes might have limited roles during these stages or might be restricted to extremely small cell populations or single cells, which are beyond the detection limit by *in situ* hybridization.

Experimental Procedures

Animals

Embryos were harvested on a mixed background of C57BL/6 and CD-1, which was used to increase litter size and thus efficiency of embryo harvest. Mice were mated overnight, and the presence of a vaginal plug indicated E0.5. The embryos were harvested at E11.5, E12.5, E13.5 and E14.5. Pregnant mice were euthanized by CO_2 followed by cervical dislocation, and embryos were removed from the uterus.

In situ hybridization (ISH)

Whole mount ISH was performed according to standard protocols (Wilkinson and Nieto, 1993). Briefly, mandibles with tongues were dissected, washed in RNAse free PBS, and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. A 45 minute 6% H_2O_2 treatment was performed on tissues followed by digestion with Proteinase K at room temperature for 5 min, and overnight hybridization temperature was set at 69°C. Antisense RNA probes labeled with digoxigenin (DIG) were generated from plasmids described elsewhere: *Fgf2* (Hebert et al., 1990), *Fgf3* (Mansour and Martin, 1988), *Fgf4* (Hebert et al., 1990), *Fgf6* (Han and Martin, 1993), *Fgf8* (Storm et al., 2003), *Fgf9* (Colvin et al., 1999), *Fgf10* (Bellusci et al., 1997), *Fgf11* (Smallwood et al., 1996), *Fgf15* (McWhirter et al., 1997), *Fgf16* (Miyake et al., 1998), *Fgf17* (Hoshikawa et al., 1998), *Fgf18* (Maruoka et al., 1998), *Fgf21* (kind gift from Gail Martin), *Fgf22* (Nakatake et al., 2001) and *Fgf23* (Yamashita et al., 2000). Other probes were made from PCR fragments amplified with primers (Table 1) designed by us and cloned in pGEM-T® Easy vector (Promega). Three samples were used for each *Fgf* probe per stage. Representative sense probes of *Fgf8* and *Fgf22* were used as controls for non-specific expression; if the same intensity of staining as the antisense probe was found in the context of overall low expression, the staining was deemed to be non-specific. Images were taken using a Leica MZ16F stereomicroscope equipped with Leica DFC310 FX digital color camera (Leica Microsystems GmbH). After imaging, all samples were processed for vibratome sections.

Vibratome sections

The hybridized samples were embedded in 15% gelatin (300 Bloom)/PBS. The samples were cut in a series of 100μm coronal sections with speed 4.5 and vibration frequency 10 using the Leica VT1000 S vibrating blade microtome (Leica Microsystems GmbH). Images

were obtained using a Carl Zeiss Oberkochen Universal (West Germany) equipped with Nikon DS-Fi2 camera (Nikon).

qPCR

The qPCR reactions were performed in a 12-μl PCR total reaction mixture containing iTaq Universal SYBR Green Supermix (Bio-Rad) in a Mastercycler Realplex (Eppendorf). All primers were purchased from IDT (Integrated DNA Technologies); the sequences are indicated in Table 2. The RNAs were extracted with RNeasy® Plus Mini Kit (QIAGEN) from the mouse tongues including both epithelium and mesenchyme at E11.5, E12.5, E13.5 and E14.5. Single-stranded cDNA at each stage was synthesized from 100 ng RNA with the SensiFast™ cDNA Synthesis Kit (Bioline) according to the manufacturer's recommendations. RNA from 3 embryos was used for the biological replicates. For each biological sample, 3 technical replicates were used per PCR reaction. The amplification condition was set at: 95°C, 2 minutes; 40 cycles at 95°C, 15 seconds; 60°C, 15 seconds; 68°C, 20 seconds; followed by a melting curve analysis in all cases. Expression levels for the genes of interest were normalized to levels of *L19*. Single factor ANOVA was performed to analyze the statistical difference in the expression levels among different embryonic stages for every *Fgf*, then followed by independent sample t-tests to compare between adjacent stages if statistical significance among groups was detected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The expression patterns of all members of the fibroblast growth factor family during early mouse tongue formation were examined.
2. The transcript level of each FGF was analyzed by qRT-PCR and the expression was localized by *in situ* hybridization.
3. Fgf1, Fgf2, Fgf5, Fgf6, Fgf7, Fgf9, Fgf10, Fgf13, Fgf15, Fgf16 and *Fgf18* were expressed in tongue mesenchyme, whereas *Fgf1* and *Fgf2* were detected in tongue epithelium during the period between E11.5 and E14.5.

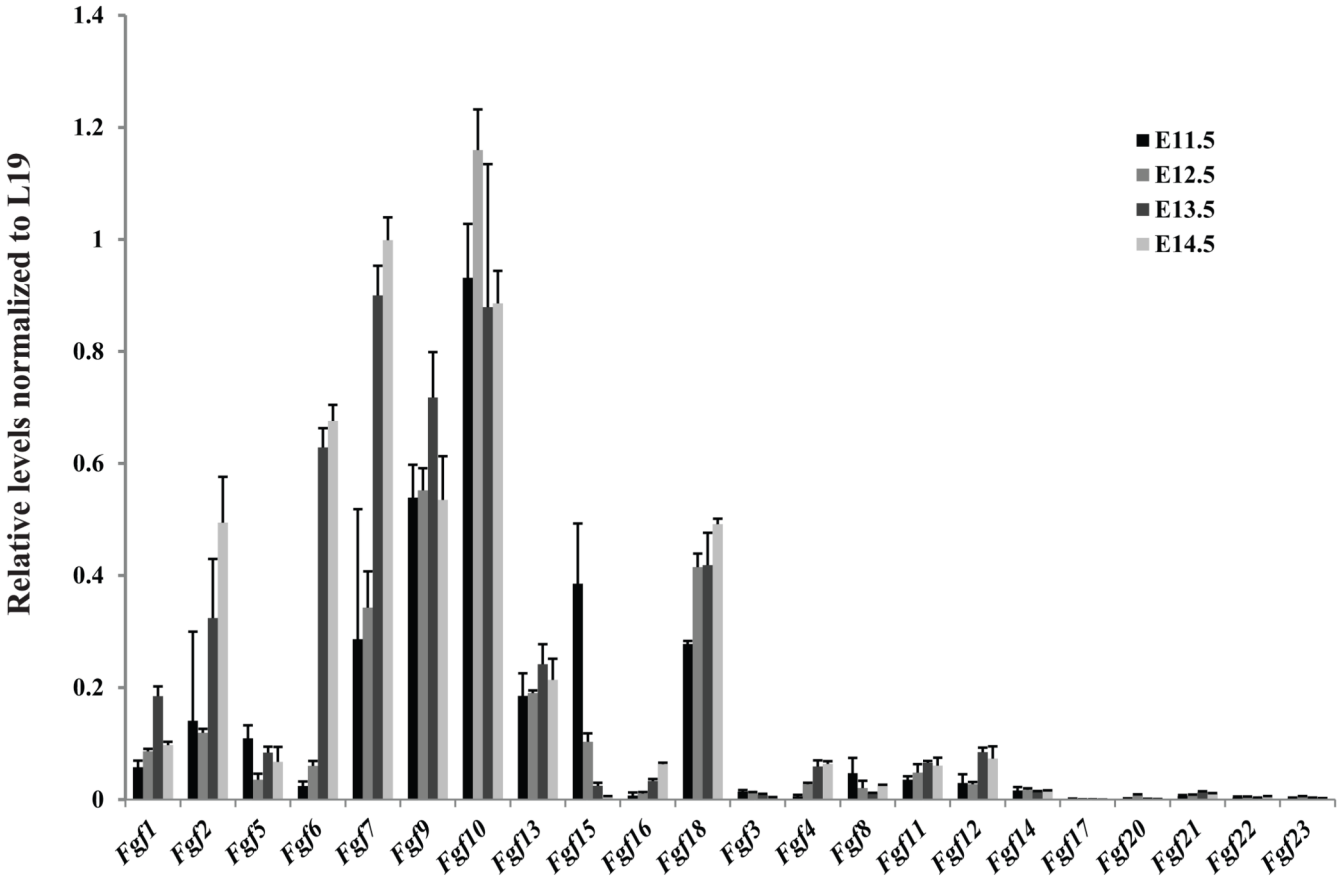

Figure 1.

qPCR analysis of the expression profiles of all *Fgf* genes during early mouse tongue development. The error bars represent standard deviation among the biological replicates. Statistical significance is indicated in Table S1.

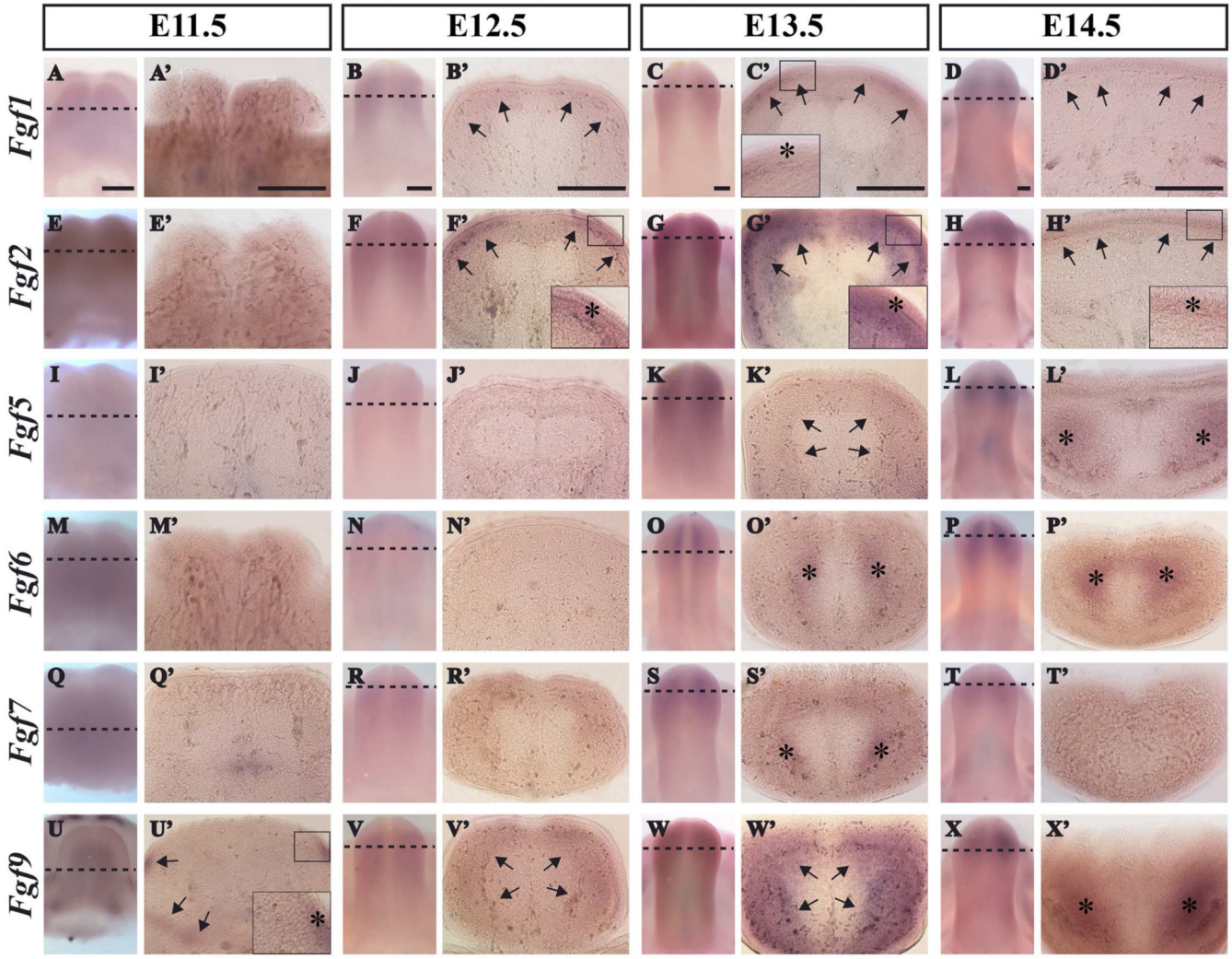

Figure 2.

Fgf5, Fgf6, Fgf7 and *Fgf9* are expressed in the mesenchyme, whereas *Fgf1* and *Fgf2* are expressed in both epithelium and mesenchyme in early mouse tongue development. (A-X) Whole mount *in situ* hybridization of *Fgf1, Fgf2, Fgf5, Fgf6, Fgf7* and *Fgf9* at E11.5, E12.5, E13.5 and E14.5. Dashed line indicates coronal plane of section for A'-X'. (A'-X') Coronal sections of A-X. Frame areas in C', F', H' and U' are magnified 2 times and superimposed onto the images. Arrows and asterisks indicate mRNA expression. Scale bar, 250 μm.

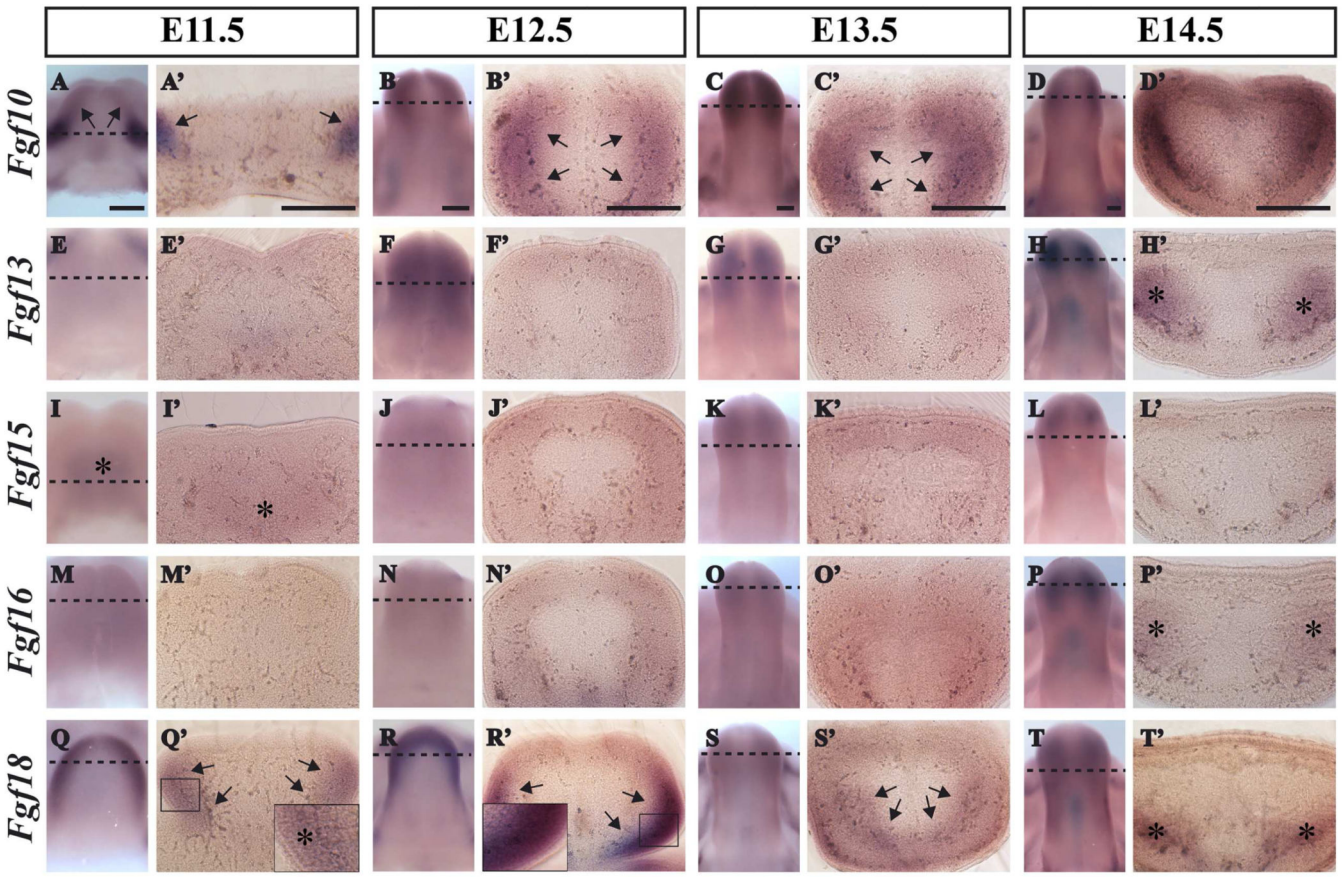

Figure 3.

Fgf10, Fgf13, Fgf15, Fgf16 and *Fgf18* are expressed in the mesenchyme in early mouse tongue development. (A-T) Whole mount *in situ* hybridization of *Fgf10, Fgf13, Fgf15, Fgf16* and *Fgf18* at E11.5, E12.5, E13.5 and E14.5. Dashed line indicates coronal plane of section for A'-T'. (A'-T') Coronal sections of A-T. Framed areas in Q' and R' are magnified 2 times and superimposed onto the images. Arrows and asterisks indicate expression. Scale bar, 250 μm.

Table 1

Primers for the target *Fgf* cDNA amplification

Transcript	Exon	Forward primer	Reverse primer
Fgf1	Exon4	5′-GTTGACCCTACCATGTTCCCTTG-3′	5′-GCCAGCAGCATCTATGGGAC-3′
Fgf5	Exon3	5′-AAGTTCAGGGAGAGATTCCAAG-3′	5′-TTGAGCCCAGCCACTAATATG-3′
Fgf7	Exon2-4	5′-CTGCCAACTCTGCTCTACAGATC-3′	5′-AGCATCCCAGCAGCCTTTAG-3′
Fgf12	Exon3-5	5′-TTCCTGTAGGACTGCGTGTG-3′	5′-CCCTGGAAACAGGATGTAATTGC-3′
Fgf13	Exon2-5	5′-GCAGGCAGATGGAACCATTG-3′	5′-GCCAATTCAGATTCAACCCTGC-3′
Fgf14	Exon2-5	5′-ATGGAACCAAGGATGACAGCAC-3′	5′-TCTCCTAAGCAAACCAACTGCAAC-3′
Fgf20	Exon2-3	5′-GGACTGGTCAGTATCAGAGGTG-3′	5′-CTCAGTGTGGTGTGGTTTGTAG-3′

Table 2

qPCR primers

Transcript	Forward primer	Reverse primer
Fgf1	5′-GTAGTTTCCTAGAGGCAGGTTG-3′	5′-TGATAAAGTGGAGTGAAGAGAGC-3′
Fgf2	5′-GAAACACTCTTCTGTAACACACTT-3′	5′-GTCAAACTACAACTCCAAGCAG-3′
Fgf3	5′-TCCACAAACTCACACTCTGC-3′	5′-GAACAGCGCCTATAGCATCC-3′
Fgf4	5′-ACTCGTCGGTAAAGAAAGGC-3′	5′-GACACGAGGGACAGTCTTC-3′
Fgf5	5′-AACTCCTCGTATTCCTACAATCC-3′	5′-CGGATGGCAAAGTCAATGG-3′
Fgf6	5′-ATGGCAATGAAGAGAGCACT-3′	5′-CATTGGCTTCCACCTCCAG-3′
Fgf7	5′-ATAGAAACAGGTCGTGACAAGG-3′	5′-CAGACAGCAGACACGGAAC-3′
Fgf8	5′-CATGGCAGAAGACGGAGAC-3′	5′-CATGCAGATGTAGAGACCTGTC-3′
Fgf9	5′-GTAGAGTCCACTGTCCACAC-3′	5 ′-CAACGGTACTATCCAGGGAAC-3′
Fgf10	5′-CAACTCCGATTTCCACTGATGT-3′	5′-GCTGTTCTCCTTCACCAAGT-3′
Fgf11	5′-ACGATGCCTTTGAGCTGAG-3′	5′-GTACCAAGTCACTTTGCCAGA-3′
Fgf12	5′-ATCTGAGCTGTAGAGATAGCC-3′T	5′-CAAGGACGAAAACAGCGACTA-3′
Fgf13	5′-TTGTCGGCTGTATAGTTTGGT-3′	5′-GCGACAAAAACAAGTTAAACGTC-3′
Fgf14	5′-ACAGACTCTTTAAACTTGCATTCAG-3′	5′-CACTGTTCAACCTCATCCA-3′
Fgf15	5′-AGCCTAAACAGTCCATTTCCTC-3′	5′-TCTGAAGACGATTGCATCAAG-3′
Fgf16	5′-CACCTTGAGATCTTCCCCAAC-3′	5′-TTCCTAGGTACAGGCCAGAG-3′
Fgf17	5′-GTGATTCTCCCCCTGTGT-3′	5′-CAAGAAGTCTCTCCAGCGATG-3′
Fgf18	5′-ACGTGGATGCGGAAGTC-3′	5′-CCTGCACTTGCCTGTGTT-3′
Fgf20	5′-AGATGCATTCAGAAGTCAAATTTCT-3′	5′-AGGATCACAGTCTCTTCGGTAT-3′
Fgf21	5′-GGGATGGGTCAGGTTCAGA-3′	5′-CAGCCTTAGTGTCTTCTCAGC-3′
Fgf22	5′-GAGTACACAGCTTTGATCACCA-3′	5′-CCTCTTCTCCTCCACTCACTT-3′
Fgf23	5′-TCATGGCTCCTGTTATCACC-3′	5′-GGACCAGCTATCACCTACAGA-3′