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Epithelial-Specific Requirement of FGFR2 Signaling During Tooth and Palate Development

RYOICHI HOSOKAWA¹, XUEMEI DENG¹, KAZUNORI TAKAMORI², XUN XU¹, MARK URATA¹, PABLO BRINGAS, JR¹, and YANG CHAI^{1,*}

¹Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, Los Angeles, California

²Division of Pediatric Dentistry, Department of Human Development and Fostering, Meikai University School of Dentistry, Sakado, Saitama, Japan

Abstract

Reciprocal interactions between epithelium and mesenchyme are crucial for embryonic development. Fibroblast growth factors (FGFs) are a growth factor family that play an important role in epithelial–mesenchymal tissue interaction. We have generated epithelial-specific conditional knockout mice targeting Fibroblast growth factor receptor 2 (*Fgfr2*) to investigate the function of FGF signaling during craniofacial development. *K14-Cre;Fgfr2^{fl/fl}* mice have skin defects, retarded tooth formation, and cleft palate. During the formation of the tooth primordium and palatal processes, cell proliferation in the epithelial cells of *K14-Cre;Fgfr2^{fl/fl}* mice is reduced. Thus, FGF signaling via FGFR2 in the epithelium is crucial for cell proliferation activity during tooth and palate development.

Fibroblast growth factor (FGF) is one of the growth factors that are involved in intercellular signaling during embryogenesis (Thesleff and Sharpe, '97; Hogan, '99). To date, 22 FGF ligands and four FGF receptors have been identified (Ornitz and Itoh, 2001; Itoh and Ornitz, 2004). FGFR1, 2, and 3 contain three extracellular, immunoglobulin-like (Ig-like) domains, an acid box (between Ig-like I and II), a single transmembrane domain, and paired intracellular tyrosine kinase domains. Alternative mRNA splicing of the *Fgfr* gene specifies the sequence of the carboxyl-terminal half of Ig-domain III, called either IIIb or IIIc (Miki et al., '92; Chellaiah et al., '94; Naski and Ornitz, '98). Exon IIIb is expressed in epithelial lineages, whereas exon IIIc tends to be expressed in mesenchymal lineages (Avivi et al., '93; Scotet and Houssaint, '95).

FGF signaling is involved in craniofacial development such as that of the craniofacial bone, palate, salivary gland, tooth, and craniofacial muscle (Nie et al., 2006). During tooth development, *Fgfr2b* (IIIb) is mainly expressed in the dental and palatal epithelia and *Fgfr2c* (IIIc) is expressed in the dental mesenchyme (Kettunen et al., '98). FGF10, which is the ligand for FGFR2b, is expressed in the dental and palatal mesenchyme (Harada et al., 2002; Rice et al., 2004). Moreover, the phenotypes of *Fgf10* conventional knockout mice are consistent with those of *Fgfr2b* mice including retardation of limbs, lungs, salivary glands, and thymus (Sekine et al., '99; De Moerloose et al., 2000; Ohuchi et al., 2000). These observations imply that reciprocal interactions between mesenchymal and epithelial tissue play a role in this process.

There are exceptions to these patterns of receptor expression that occur in tooth development. For instance, *Fgfr2b* is expressed in both ameloblasts and odontoblasts at the newborn stage, and *Fgfr2c* is expressed in the inner dental epithelium at the bell stage (E16-17) (Kettunen et al., '98). Furthermore, in the absence of *Fgfr2b*, Fgf10 can bind to other isoforms of the FGF receptor, such as FGFR1b (Beer et al., 2000). To examine these discrepancies, we created mice lacking all isoforms of *Fgfr2* specifically in the epithelium (*K14-Cre;Fgfr2^{fl/fl}*). *K14-Cre;Fgfr2^{fl/fl}* mice have defects in skin, teeth, plate, and hindlimbs, but not forelimbs. During tooth and palatal development, epithelial-specific loss of *Fgfr2* causes defects in cell proliferation in epithelial of tooth and palate. Organization of the rugae of the palate, which is considered a landmark of palatal development (Cui et al., 2005), is also compromised in *K14-Cre;Fgfr2^{fl/fl}* mouse.

MATERIALS AND METHODS

Generation of *K14-Cre;Fgfr2^{fl/fl}* mutant mice

All animal studies were performed according to IACUC guidelines. *K14-Cre* transgenic mice have been described previously (Indra et al., 2000; Andl et al., 2004). We crossed *K14-Cre;Fgfr2^{fl/+}* with *Fgfr2^{fl/fl}* mice to generate *K14-Cre;Fgfr2^{fl/fl}* null alleles that were genotyped using PCR primers as previously described (Yu et al., 2003).

Two-component genetic system for marking the progeny of epithelial cells

The *R26R* conditional reporter allele has been described previously (Soriano, '99). We mated *K14-Cre* and *R26R* mice to generate *K14-Cre;R26R* embryos. Detection of β -galactosidase activity in whole embryos (E10.5) was carried out as previously described (Chai et al., 2000).

Staining of whole skeleton

Whole skeletal preparations of newborn mice were prepared and stained with Alizarin Red and Alcian Blue as previously described (McLeod, '80).

Histological analysis

Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline, decalcified in Decalcifying solution (Richard-Allan Scientific, Kalamazoo, MI), paraffin embedded, sectioned at 5 mm, and stained with hematoxylin/eosin.

BrdU labeling/histology

A 10 mg/mL stock of bromodeoxyuridine (BrdU; Sigma, St Louis, MO) was injected intraperitoneally into mice at E13.5 at a dose of 100 μ g BrdU per gram of body weight. Mice were sacrificed 1 hr after injection and bones were harvested. BrdU staining was carried out on paraffin sections by using a BrdU staining kit according to the manufacturer's directions (Zymed, South San Francisco, CA).

In situ hybridization

Whole-mount and sectioned in situ hybridizations were performed according to standard procedure (Wilkinson, '98). Several negative controls (sense probe and no probe) were run in parallel with the experimental reaction. RNA probes were generated as reported previously: *Fgfr2b* (Sasaki et al., 2006) and *Shh* (Hui and Joyner, '93).

RESULTS

K14-Cre;Fgfr2^{fl/fl} mice have skin and skeletal defects

We generated an epithelial-specific gene deletion of *Fgfr2* in mice using Cre recombination driven by the epithelial-specific *K14* promoter (Andl et al., 2004). *K14-Cre;Fgfr2^{fl/fl}* mice were indistinguishable from their wild-type littermates (Fig. 1A and C). We crossed *K14-Cre;Fgfr2^{fl/+}* mice with *Fgfr2^{fl/fl}* mice and recovered approximately 25% *K14-Cre;Fgfr2^{fl/fl}* mice, implying that no significant lethality occurred before birth. *K14-Cre;Fgfr2^{fl/fl}* mice died within 24 hr of birth without feeding, likely owing to their cleft palate (Fig. 2). The eyes of *K14-Cre;Fgfr2^{fl/fl}* mice were open owing to the absence of eyelids, and they had shiny skin (Fig. 1B). We detected limb dysgenesis in the hindlimb but not in the forelimb (Fig. 1A–D). Bone staining of *K14-Cre;Fgfr2^{fl/fl}* mice revealed no bones in the lower limb except the pelvic girdle (Fig. 1E and F).

To confirm the tissue-specific deletion of *Fgfr2* gene expression, we performed in situ hybridization. The epithelium and primordium of the hair follicle expressed *Fgfr2b* at E12.5 (Fig. 1G, arrow). In contrast, *Fgfr2b* expression was not detectable in *K14-Cre;Fgfr2^{fl/fl}* mice (Fig. 1H), consistent with successful epithelial-specific loss of *Fgfr2b*. Interestingly, *K14-Cre;Fgfr2^{fl/fl}* mice had limb defects only in the hindlimb, although *Fgfr2b* and *Fgf10* conventional knockout mice form neither fore- nor hindlimbs. To confirm the timing of Cre recombination under the control of the *K14* promoter, we crossed *K14-Cre* mice with *Rosa26R* mice (Soriano, '99) and performed X-gal staining of the embryo. E10.5 samples clearly indicated that Cre recombination occurred earlier in the hindlimb than in the forelimb (Fig. 1I). At later stages, Cre recombination was detectable in all epithelial cells (Fig. 1J). This time gap might be a critical reason that the forelimb escaped having defects in formation in *K14-Cre;Fgfr2^{fl/fl}* mice. We also confirmed the expression pattern in the oral epithelium of the *K14* promoter of our construct. *K14-Cre;R26R* mice exhibited mosaic X-gal staining of the first branchial arch at E10.5 (Fig. 1I, arrowhead) (Chai and Maxson, 2006). At E11.5, we detected strong X-gal staining in the oral epithelium (Fig. 1J). At E12.5, the dental and palatal epithelium are strongly positive for X-gal staining (Fig. 1K) (Xu et al., 2006,2008).

Tooth formation is compromised in K14-Cre; Fgfr2^{fl/fl} mice owing to reduced cell proliferation

Newborn *K14-Cre;Fgfr2^{fl/fl}* mice lacked teeth (data not shown). At E12.5, the lamina stage, we detected thickened epithelium in the presumptive tooth development area of *K14-Cre;Fgfr2^{fl/fl}* mice, which was indistinguishable from that of control mice (Fig. 2A and B). At E13.5, the teeth of control mice had reached the bud stage, but the dental epithelium of *K14-Cre;Fgfr2^{fl/fl}* mice still appeared to be in the lamina stage (Fig. 2C and D). At E14.5, control mice had reached the cap stage (Fig. 2E), but tooth development remained retarded in *K14-Cre;Fgfr2^{fl/fl}* mice (Fig. 2F).

To investigate possible mechanisms of this tooth retardation, we analyzed gene expression of candidates expressed in the dental epithelium. *Fgf8* is one of the molecules indicating the onset of tooth development (Zhang et al., 2003). *Fgf8* was expressed in the presumptive tooth region in control mice at E11.5, whereas it was dramatically reduced in *K14-Cre;Fgfr2^{fl/fl}* mice, even though *Fgf8* expression in the nasal pit area was indistinguishable in *K14-Cre;Fgfr2^{fl/fl}* and control mice (Fig. 2G and H, open arrowheads). *Pitx2*, another marker for tooth initiation (Chai and Maxson, 2006), was unchanged in *K14-Cre;Fgfr2^{fl/fl}* mice relative to control (data not shown).

Next, we analyzed cell proliferation activity in the lamina and bud stages. At E12.5 (lamina stage), cell proliferation activity of *K14-Cre;Fgfr2^{fl/fl}* mice was indistinguishable from that of control mice in both epithelial and mesenchymal cells (data not shown). At E13.5 (bud stage),

we detected a reduction of cell proliferation activity in dental epithelial cells of *K14-Cre;Fgfr2^{fl/fl}* mice compared with those of control mice, although there was no change in proliferation in dental mesenchymal cells (Fig. 2I–K).

K14-Cre; Fgfr2^{fl/fl} mice develop cleft palate with compromised rugae formation

We observed complete cleft palate in 75% of *K14-Cre;Fgfr2^{fl/fl}* mice (Fig. 3B), and the remaining 25% displayed submucous cleft palate at the newborn stage. We were unable to detect rugae formation in *K14-Cre;Fgfr2^{fl/fl}* mice, although rugae were clearly visible on the hard palate of newborn mice (Fig. 3A, black arrows, and 3B). To confirm the presence or absence of rugae, we examined *Shh* expression, which is a marker for rugae formation (Sasaki et al., 2007), using whole mount in situ hybridization. We detected seven rugae on the secondary palate of control mice at E15.5, based on *Shh* expression (Fig. 3C). In contrast, *Shh* expression appeared dotted on the secondary palate of *K14-Cre;Fgfr2^{fl/fl}* mice, implying that the rugae were not well organized (Fig. 3D). Interestingly, hair follicle formation in the nose in *K14-Cre;Fgfr2^{fl/fl}* mice was well organized when compared with control mice (Fig. 3C and D). Histological analysis demonstrated that the palatal shelves of *K14-Cre;Fgfr2^{fl/fl}* mice transformed from vertical to horizontal positions, but the left and right sides of the palatal shelves did not contact each other (Fig. 3F). We hypothesized that cleft palate was owing to insufficient palatal shelf growth, which might be the result of a reduction of mesenchymal cell proliferation. We did detect a reduction in cell proliferation in *K14-Cre;Fgfr2^{fl/fl}* palatal epithelium at E13.5. We conclude that the reduction of epithelial cell caused the cleft palate in *K14-Cre;Fgfr2^{fl/fl}* mice.

Furthermore, we investigated whether morphological changes of the palatal shelf occurred before shelf elevation owing to the reduction of epithelial cell proliferation. At E13.5, the size of the palatal shelves of *K14-Cre;Fgfr2^{fl/fl}* mice was reduced from anterior to posterior when compared with that of control mice (Fig. 4A–D). The shape of the palatal shelves in *K14-Cre;Fgfr2^{fl/fl}* mice was also compromised. The palatal shelves of *K14-Cre;Fgfr2^{fl/+}* (control) mice formed an asymmetric, fingerlike shape (Fig. 4A and C), whereas this asymmetry was largely lost in *K14-Cre;Fgfr2^{fl/fl}* mice (Fig. 4B and D).

DISCUSSION

In this study, we examined the biological significance of FGF signaling through *Fgfr2* in epithelial cells using a tissue-specific deletion of the *Fgfr2* gene. Through the analysis of conditional knockout (*K14-Cre;Fgfr2^{fl/fl}*) mice, we conclude that the FGF signal pathway is crucial for cell proliferation activity in epithelial cells and is involved in the organization of the rugae, the thickened lines on the secondary palate.

Previous studies have reported the expression pattern of FGF ligands and their receptors during embryonic development. There are multiple FGF receptor isoforms owing to RNA splicing, and these isoforms have tissue-specific expression patterns. For instance, *Fgfr2* IIIb is expressed in the epithelium and *Fgfr2* IIIc is expressed in mesenchymal cells, although exceptions exist at different developmental stages (Kettunen et al., '98; Rice et al., 2004). These observations suggest that *Fgfr2b* conventional knockout mice may be highly similar to epithelial tissue-specific conditional knockout mice. Surprisingly, *K14-Cre;Fgfr2^{fl/fl}* and *Fgfr2b^{-/-}* mice exhibit some phenotypes that differ as well as common defects in the eye and skin. First of all, the severity of the limb formation defect is significantly different. *Fgfr2b^{-/-}* mice exhibited failure of limb initiation (De Moerloose et al., 2000), whereas *K14-Cre;Fgfr2^{fl/fl}* mice showed normal forelimb development and compromised hindlimb development. X-gal staining of *K14-Cre;R26R* mice demonstrates that Cre recombinase derived from the *K14* promoter is active around E10.5 in the hindlimb but not in the forelimb, implying that epithelial cells in the forelimb escape the deletion of *Fgfr2* at early developmental

stages. This observation in *K14-Cre;Fgfr2^{fl/fl}* mice is consistent with the previous finding that *Fgf8* plays a critical role in the apical endodermal ridge (AER) during limb formation (Revest et al., 2001).

Fgfr2b null mice exhibited tooth defects and cleft palate (De Moerloose et al., 2000), but the mechanism of the tooth defect has remained unknown. In this study, we conclude that the tooth defect in the absence of *Fgfr2* is likely the result of a cell proliferation defect in the dental epithelium. Although *Fgf8* is not the ligand for *Fgfr2* (Ornitz et al., '96), *Fgf8* expression is reduced in *K14-Cre;Fgfr2^{fl/fl}* mice in the presumptive tooth region, implying that *Fgf8* is a downstream target gene of FGF signaling through FGFR2. We have recently shown that the antagonistic interaction between BMP4 and *Fgf8* and the setup of proximal vs. distal domains of the first branchial arch epithelium is dependent upon Smad4-mediated BMP signaling in the neural crest-derived mesenchyme (Ko et al., 2007). Interestingly, however, the induction of *Fgf8* expression in the first branchial arch epithelium is not dependent on the neural crest cells. For example, *Fgf8* expression is unaffected in *Msx1* null or *Msx1/Msx2* double null mice (Bei and Maas, '98; Ishii et al., 2005). This study provides crucial evidence that FGF signaling through *Fgfr2* is required for the induction of *Fgf8* in the first branchial arch epithelium during craniofacial development. In spite of the reduction of *Fgf8* expression, tooth development is normal until E12.5, implying there is likely compensation by other molecules. *Pitx2* expression in *K14-Cre;Fgfr2^{fl/fl}* mice is indistinguishable from control mice. Recently, it has been reported that *Pitx2* cooperates with *Lef-1* for activation of β -catenin during tooth initiation (Amen et al., 2007). These results suggest that members of the Wnt canonical pathway with *Pitx2* might compensate for *Fgf8* function during tooth initiation. A previous study of *Fgfr2b* null mice has reported that *Fgf8* is not downstream of *Fgfr2* IIIb isoform during limb development (Revest et al., 2001). Recently Lu and coworkers report that AER-specific knockout of all isoforms of *Fgfr2* causes the reduction of *Fgf8* during limb development, which is consistent with our observations (Lu et al., 2008). Furthermore, mice with epithelial-specific *Fgfr2b* gene deletion do not exhibit tooth retardation and cleft palate (Grose et al., 2007). Taken together, these observations imply that another isoform of *Fgfr2* such as IIIc not only is involved in the induction of *Fgf8* but also compensates for the function of *Fgfr2* IIIb.

In regards to cleft palate, *Fgf2b* conventional knockout mice exhibit reduced cell proliferation defect in both epithelial and mesenchymal cells (Rice et al., 2004). Furthermore, the palatal shelves of *Fgfr2b* conventional knockout mice were shorter and did not show palatal projection lateral with the tongue. In contrast, we detect a cell proliferation defect only in the palatal epithelium in *K14-Cre;Fgfr2^{fl/fl}* mice. Although the size of the palatal shelves in *K14-Cre;Fgfr2^{fl/fl}* mice are smaller than control mice, palatal projection lateral to the tongue does occur. Taken together, these observations imply that there is an intrinsic requirement for FGF signaling in the epithelium and *Fgfr2b* expressed in the palatal mesenchyme is likely to be responsible for mediating palatal mesenchymal cell proliferation.

In summary, our epithelial-specific gene deletion of *Fgfr2b* shows that FGF signaling functions through FGFR2. Future exploration of the difference in the phenotypes of conventional and conditional knockout mice may provide insights into the reciprocal interactions of epithelial and mesenchymal cells during tooth and palate development.

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Fig. 1.

K14-Cre;Fgfr2^{fl/fl} mice show skin and skeletal defects. (A–D) Macroscopic appearance of newborn control (A, C; *K14-Cre;Fgfr2^{fl/+}*) and *K14-Cre;Fgfr2^{fl/fl}* (B, D) mice. Note the shiny skin, open eye, and retarded hindlimb of *K14-Cre;Fgfr2^{fl/fl}* mice. (E, F) Alizarin red/Alcian blue-stained control (E; *K14-Cre;Fgfr2^{fl/+}*) and *K14-Cre;Fgfr2^{fl/fl}* (F) skeletons. *K14-Cre;Fgfr2^{fl/fl}* mice possess the pelvic girdle but not limb skeleton. (G, H) In situ hybridization of *Fgfr2b* expression (arrow) in hair follicle of control (G; *K14-Cre;Fgfr2^{fl/+}*). There is no detectable *Fgfr2* expression in *K14-Cre;Fgfr2^{fl/fl}* mice (H) at E12.5. (I, J) Whole mount X-gal staining of *K14-Cre;R26R* mice at E10.5 (I) and E11.5 (J). (I) The hindlimb, but not the forelimb, of *K14-Cre;R26R* mice exhibit strong X-gal staining at E10.5. (J) Oral epithelium exhibit strong X-gal staining (arrow). (K) Section X-gal staining at E12.5. X-gal staining is detectable in dental (arrowhead) and palatal (arrow) epithelium. Scale bars 5 mm in A–F, 200 μ m in G, H, K and 1 mm in I.

Fig. 2.

Tooth development is retarded in *K14-Cre;Fgfr2^{fl/fl}* mice. (A–F) Hematoxylin and eosin staining at 12.5 (A, B), at 13.5 (C, D), and at 14.5 (E, F). (G, H) Whole mount in situ of *Fgf8* at E11.5 maxillary process. (I, J) BrdU staining of mandibula primordium at E13.5. (A, C, E, G, I) *K14-Cre;Fgfr2^{fl/+}* (control) mice, (B, D, F, H, J) *K14-Cre;Fgfr2^{fl/fl}* mice. (A, B) The dental lamina formation of *K14-Cre;Fgfr2^{fl/fl}* mice is indistinguishable for control mice. Dotted lines indicate the basal lamina. (C, D) The dental epithelium of control mice is bud stage but is still lamina stage in *K14-Cre;Fgfr2^{fl/fl}* mice (D, arrow). (E, F) Control mice show the cap stage of epithelium but *K14-Cre;Fgfr2^{fl/fl}* mice indicate the retardation of dental epithelium (F, arrow). (G) The presumptive dental epithelium express *Fgf8* in *K14-Cre;Fgfr2^{fl/+}* mice (black arrow). (H) *Fgf8* expression was reduced in the process of *K14-Cre;Fgfr2^{fl/fl}* mice (black arrow). (I, J) Dental epithelium exhibit the BrdU positive cells in *K14-Cre;Fgfr2^{fl/+}* mice but not in *K14-Cre;Fgfr2^{fl/fl}* mice. Dotted line indicates the basal lamina. (K) BrdU labeling index of E13.5 sections from *K14-Cre; Fgfr2^{lox/+}* (Control) and *K14-Cre; Fgfr2^{lox/lox}* (conditional knock out; C.K.O.) mice. Five randomly selected, nonoverlapping samples were used from each experimental group. Student *t*-tests were used for statistical analysis. *: $P < 0.05$. ($N = 5$). Scale bars 100 μ m in A–F, I, J, and 2 mm in (G, H).

Fig. 3.

The cleft palate of *K14-Cre;Fgfr2^{fl/fl}* mice. (A, B) Macroscopic appearance of hard palate at newborn stage. (C, D) Whole mount in situ of *Shh* of the secondary palate at E15.5. (E, F) Hematoxylin and eosin staining at E15.5. (G, H) BrdU staining of palatal shelf at E13.5. (A) *K14-Cre;Fgfr2^{fl/+}* mice exhibit the rugae formation on the secondary palate (black arrows). (B) *K14-Cre;Fgfr2^{fl/fl}* mice display the cleft palate without rugae formation (white arrows). (C) *K14-Cre;Fgfr2^{fl/+}* mice exhibit well organized *Shh* expression consisted with the rugae formation (Black arrows). (D) *Shh* is compromised in *K14-Cre;Fgfr2^{fl/fl}* mice (white arrows). (E, F) *K14-Cre;Fgfr2^{fl/fl}* mice exhibit the cleft palate although palatal shelf is horizontal position. (G) The palatal epithelium of *K14-Cre;Fgfr2^{fl/+}* mice exhibit BrdU positive cells (black arrows). (H, I) The BrdU positive cells is reduced in *K14-Cre;Fgfr2^{fl/fl}* mice (white arrows). (I) BrdU labeling index of E13.5 sections from *K14-Cre; Fgfr2^{fl/+}* (Control) and *K14-Cre; Fgfr2^{fl/fl}* (conditional knock out; C.K.O.) mice. Five randomly selected, nonoverlapping samples were used from each experimental group. Student *t*-tests were used for statistical analysis. *: $P < 0.05$. ($N = 5$). Scale bars 2 mm in A–D, I, J, and 400 μ m in E, F, and 50 μ m in G, H.

Fig. 4. Morphological changes of the palatal shelves before elevation in *K14-Cre;Fgfr2^{fl/fl}* mice. (A–D) Hematoxylin and eosin staining in *K14-Cre;Fgfr2^{fl/+}* (control; A, C) and *K14-Cre;Fgfr2^{fl/fl}* (B, D) mice at E13.5. A and B are sections from the anterior and C and D are sections from the posterior regions of the palatal shelves. Scale bars 200 μm in A–D.