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Exogenous FGF8 rescues development of mouse diastemal vestigial tooth ex vivo

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Abstract

Regression of vestigial tooth buds results in the formation of the toothless diastema, a unique feature of the mouse dentition. Revitalization of the diastemal vestigial tooth bud provides an excellent model for studying tooth regeneration and replacement. It was shown previously that suppression of FGF signaling in the diastema is a causative of vestigial tooth bud regression. In this study, we report that application of exogenous FGF8 to the mouse embryonic diastemal region rescues diastemal tooth development. However, this rescue of diastemal tooth development occurs only in isolated diastemal region, but not in mandibular quadrant containing incisor and molar germs. FGF8 promotes cell proliferation and inhibits apoptosis in diastemal tooth epithelium, and revitalizes tooth developmental program, evidenced by the expression of genes critical for normal tooth development. Our results support the idea that the adjacent tooth germs contribute to the suppression of diastemal vestigial tooth buds via multiple signals.

Keywords

FGF8; diastemal tooth; tooth development; ex vivo culture

INTRODUCTION

Unlike many other mammals including humans, the mouse has only one incisor and three molars in each jaw quadrant. Existed between the incisor and molar is a toothless region called diastema, which is resulted from the arrested development and repression of vestigial tooth buds (Peterkova et al., 2006). Histological analyses reveal similar but distinct developmental processes of the lower (mandibular) diastemal rudimentary tooth as compared to the molar germs (Yamamoto et al., 2005; Yuan et al., 2008). At E11.5, the oral epithelium becomes thickened at the lower diastemal region similar to its adjacent first lower molar. At E12.5 and E13.5, the lower diastemal rudimentary tooth germ reaches to the bud stage as a maximum, but lacks mesenchymal condensation. Subsequently, the diastemal tooth bud exhibits retarded growth and eventually regresses via apoptosis, forming the

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toothless gap between the incisor and the first molar (Viriot et al., 2000; Peterková et al., 2003, 2006; Yamamoto et al., 2005). In the lower diastemal region, there exist two vestigial tooth buds in the posterior region of the diastema: the mesial segment (MS) and the wide R2 bud. After E13.5, the MS regresses, but the R2 becomes incorporated into the mesial end of the first molar (Peterkova et al., 1995; Peterkova et al., 1996; Viriot et al., 2000).

While the underlying molecular mechanisms for the lack of tooth in the diastemal region remain elusive, the formation of supernumerary tooth in the diastema in a number of mutant or transgenic mouse lines suggests the potential tooth-forming capability in this region (Sofaer, 1969; Zhang et al., 2003; Kangas et al., 2004; Tucker et al., 2004; Kassai et al., 2005; Klein et al., 2006; Ohazama et al., 2009; Ahn et al., 2010; reviewed in Cobourne and Sharpe, 2010). Mutations in *Wise* (*Ectodin*) that encodes a Wnt signaling inhibitor, or in *Spry2* and *Spry4*, both encoding negative feedback regulators of FGF signaling, lead to a relief of suppression and revitalization of the rudimentary diastemal tooth buds through increased cell proliferation and decreased apoptosis (Peterkova et al., 2009; Ahn et al., 2010). The supernumerary tooth in these mutants appears to be resulted from a continuous development of R2 (Peterkova et al., 2009; Ahn et al., 2010). These results indicate that these factors function to inhibit tooth development in the diastema. Tissue recombination experiments have shown that the lower diastemal mesenchyme is unable to maintain the vestigial tooth development, suggesting that the defective diastemal mesenchyme is responsible for tooth regression in the mouse lower diastema (Yamamoto et al., 2005; Yuan et al., 2008).

The formation of supernumerary tooth in *Spry2* or *Spry4* null mice demonstrates a requirement for FGF signaling in maintaining the development of diastemal vestigial tooth, consistent with the essential role of FGF signaling in normal tooth development (Tummers and Thesleff, 2009). Like many other developing organs, tooth development is regulated by signaling molecule mediated epithelial-mesenchymal interactions. Members of the FGF family have been implicated in tooth development at several distinct steps, including the determination of tooth forming sites, the initiation of tooth development, tooth growth, and cusp patterning (Peters and Balling, 1999; Thesleff and Mikkola, 2002). Among those FGF ligands that are expressed in the developing mouse tooth, FGF8 is initially expressed in the presumptive dental epithelium and persists there until the bud stage, being responsible for the determination of tooth forming sites, induction of a number of tooth developmental genes, and initiation of molar development (Neubüser et al., 1997; Grigoriou et al., 1998; Tucker et al., 1998; Trumpp et al., 1999; St Amand et al., 2000). In addition, FGF8 is likely responsible for the induction of *Fgf3* expression in the dental mesenchyme (Bei and Maas, 1998; Kettunen et al., 2000); the latter represents one of FGF feedback signals from the dental mesenchyme to act on the dental epithelium. Given the importance of FGF8 in tooth development, in the present study, we tested if application of exogenous FGF8 to the lower diastemal region would revitalize the vestigial tooth bud and resume diastemal tooth formation, resembling premolar in other mammals.

RESULTS AND DISCUSSION

Formation of lower diastemal tooth induced by FGF8

Using tissue recombination approach, we have shown previously that defective diastemal mesenchyme is responsible for tooth regression in the mouse lower diastema (Yuan et al., 2008). Based on the report that *Spry2* and *Spry4* are expressed in the diastemal mesenchyme and supernumerary teeth form in the diastemal region in *Spry2* or *Spry4* null mice (Klein et al., 2006), we asked if application of exogenous FGF could override the repressive effects of *Spry* proteins and revitalize the diastemal tooth. Given the importance of FGF8 in tooth development, we set to test if exogenous FGF8 could rescue the development of diastemal

tooth germ beyond the bud stage. Mandibular quadrants were isolated from E13.5 wild type embryos and were further dissected into the diastemal region, the molar, and the incisor germ, respectively. The identification and excision of the mandibular diastemal region have been described in details in our previous publication (Yuan et al., 2008). Tissues from each quadrant were processed in parallel and counted as one experimental group. Two to three protein-soaked beads were inserted to the mesenchyme of diastemal tissues in culture. After 24 hours in culture in a semisolid medium, the diastemal tissue, the incisor germ, and the molar germ were transplanted underneath the adult mouse kidney capsule, respectively. Samples were harvested 4-week after subrenal culture. Successful tooth formation in the diastemal grafts was counted only when the incisor germ and molar germ from the same quadrant developed into well-defined teeth simultaneously, which precluded potential contamination from either incisor or molar germ. Our results showed that tooth failed to form in 38 diastemal grafts (0/38) implanted with BSA control beads. In contrast, among 51 grafting groups carrying FGF8 beads, diastemal tooth was found in 33 groups. However, only in 20 groups where one incisor, one diastemal tooth, and one or two molars were found at the same time, giving an about 40% successful rate (20/51). In contrast to FGF8, we failed to find any tooth formation in diastema grafts carrying FGF2 (0/15) and BMP4 (0/23) beads. We also tested FGF3, which indeed induced diastemal tooth formation, but at a very low successful rate (10%), suggesting a specific effect of FGF8. The data are summarized in Table 1. We then determined the size of grafted teeth by measuring the maximal length in diameter. The diastemal teeth induced by FGF8 appeared slightly smaller in diameter as compared to the first molar grafts (Fig., but is larger than the second molar grafts (Fig. 1A – 1D'; Table 2). These diastemal teeth had relatively blunt cusps, with the number of cusps varying from 0 to 3 (Fig. 1A), resembling premolar in other mammals. Histological analysis showed that these FGF8-induced diastemal teeth formed well-developed dentin and enamel (Fig. 1A'), which was further confirmed by the expression of *Dspp* in the odontoblasts and *Amelogenin* in the ameloblasts of FGF8-induced diastemal teeth after two weeks in subrenal culture (Fig. 2A, 2B).

We further tested if FGF8-soaked beads were able to rescue diastemal tooth development in a whole mandibular quadrant graft. In this experiment, FGF8-soaked beads were inserted into the diastemal mesenchyme from the aboral side of mandibular quadrant containing both incisor and molar germs. Surprisingly, while incisor and molar formed in every quadrant graft after 4-week in subrenal culture, we did not find a diastemal tooth in all 14 quadrant grafts (data not shown). These results indicate that FGF8 alone is not enough to rescue diastemal tooth formation in the presence of incisor and molar germs, suggesting the existence of additional suppressing factors from the adjacent developing tooth germs. Indeed, Wise (Ectodin), a secreted protein capable of modulating the Wnt canonical signaling, from the developing tooth germs is known to suppress survival of the diastemal vestigial buds (Ahn et al., 2010). Isolated diastemal vestigial buds appeared to escape the suppressing effects of factors from the adjacent developing tooth germs and in the presence of FGF8, became revitalized and continued to development. In order to determine if the additional repressive signal derives the molar or the incisor, we grafted FGF8-implanted diastemal region with either incisor or molar germ (6 cases for each group), and failed to find a diastemal tooth, though incisor or molar formed in all cases, respectively (data not shown). Thus both incisor and molar appear to contribute to the repressive effect on diastemal tooth development.

FGF8 prevents regression of the diastemal tooth bud by stimulating cell proliferation and inhibiting cell apoptosis

To determine the early morphological and cellular changes in the FGF8-induced diastemal tooth, we first compared the histology of the diastemal tooth germ implanted with FGF8 to

control samples carrying BSA beads after 12, 24 and 48 hrs in organ culture. Isolated molar germs were also cultured in parallel as controls. As shown in Fig. 3, E13.5 diastemal explants with BSA beads showed that the vestigial tooth bud had begun to regress after 12 hours in organ culture; however, explants carrying FGF8 beads retained the tooth structure at the bud stage (Fig. 3A, 3B). After 24 hours in culture, the diastemal vestigial tooth bud in the control explants had disappeared completely, while the diastemal tooth germ in FGF8-implanted explants continued to grow, reaching to the late bud stage (Fig. 3D, 3E). The diastemal tooth germ in FGF8-implanted explants further developed to the cap stage after 48 hours in organ culture (Fig. 3G). As controls, cultured molar germs appeared to develop faster than FGF8-implanted diastemal tooth, reaching to the late bud stage at 12 hours in culture (Fig. 3C), to the cap stage at 24 hours in culture (Fig. 3F), and to the late cap stage after 48 hours in culture (Fig. 3I). These results indicate that exogenously applied FGF8 prevented regression and subsequently led to continuous development of the diastemal tooth in vitro.

We next performed BrdU labeling and TUNEL assays to examine cell proliferation and apoptosis in explanted diastema with or without FGF8 beads. After 12 hours in organ culture, the control explants with BSA beads exhibited rare BrdU-positive cells in the regressing diastemal tooth bud; in contrast, FGF8-implanted diastemal explants showed a significant amount of BrdU-positive cells, particularly in the diastemal tooth epithelium (Fig. 4A, 4B). Elevated level of cell proliferation was also observed in the diastemal tooth epithelium and surrounding mesenchyme carrying FGF8 beads after 24 hours in organ culture (Fig. 4C). These results demonstrate that exogenously applied FGF8 stimulates cell proliferation in the diastemal tooth bud. It has been shown previously that the diastemal tooth bud regresses via apoptosis (Viriot et al., 2000; Peterková et al., 2003, 2006; Yamamoto et al., 2005). Using TUNEL assays, we indeed detected apoptotic cells in the regressing diastemal tooth epithelium in the control explants after 12 hours in organ culture (Fig. 4F). Interestingly, we also detected apoptotic cells in the diastemal tooth epithelium in FGF8-implanted explants; however, these apoptotic cells were mainly restricted in the stellate reticulum region, but not the enamel epithelium (Fig. 4E). In contrast, after 24 hours in culture, apoptotic cells were rarely detected in the diastemal tooth epithelium in FGF8-implanted explants (Fig. 4G). Taken together, these observations indicate that exogenous FGF8 prevents regression of diastemal tooth germ by stimulating cell proliferation and by protecting cells from undergoing apoptosis.

Activation of tooth developmental program in the diastemal tooth by FGF8

To investigate whether FGF8 revitalizes the diastemal tooth by activating tooth developmental program, we examined the expression of several genes that have been known to be critical for normal tooth development. Among these genes, *Pax9* is induced in the dental mesenchyme by the dental epithelium-derived FGF8 and is required for tooth development to progress beyond the bud stage (Neubüser et al., 1997; Peters et al., 1998). Similarly, *Msx1* is also expressed in the dental mesenchyme and inactivation of *Msx1* leads to an arrested tooth development at the bud stage (Satokata and Maas, 1994; Chen et al., 1996). *Msx1* and *Pax9* activate *Bmp4* expression synergistically in the dental mesenchyme from where BMP4 acts on the dental epithelium to promote progression of the bud stage to the cap stage and induces enamel knot formation (Chen et al., 1996; Jernvall et al., 1998; Peters et al., 1998; Zhang et al., 2000; Ogawa et al., 2006; Makatomi et al., 2010). *Pitx2*, one of the earliest molecular markers in the dental epithelium, is known to be induced by FGF8 and its inactivation causes an arrest of tooth development at the bud stage as well (Lin et al., 1999; Lu et al., 1999; St. Amand et al., 2000). Our results showed that application of exogenous FGF8 to explanted E13.5 diastema induced the expression of *Pax9* and *Msx1* in the mesenchyme of diastemal tooth bud after 24 hours in culture (Fig. 5A, 5D); however, the

expression level appeared to be relatively low. As controls, BSA-beads failed to induce the expression of these two genes (Fig. 5C, 5F). After 48 hours in culture, strong *Pax9* and *Msx1* expression was observed in the diastemal tooth mesenchyme (Fig. 5B, 5E). In line with the expression of *Pax9* and *Msx1* is the activation of *Bmp4* in the mesenchyme of FGF8-induced diastemal teeth but not in the BSA-bead implanted sample (Fig. 5G–I). We also observed a strong *Pitx2* expression in the diastemal tooth epithelium in the same period time of culture (Fig. 5J, 5K), but detected residual *Pitx2* expression in the control (Fig. 5L).

Shh is a signaling molecule important for cell proliferation, survival, and tooth patterning during odontogenesis. *Shh* is weakly expressed in the diastemal vestigial tooth bud, but its expression is strongly enhanced in *Spry2* or *Spry4* mutants (Klein et al., 2006; Peterkova et al., 2009). In addition, *Fgf3*, which is induced by FGF8 in the dental mesenchyme at the bud stage and represents a feedback FGF signal from the dental mesenchyme to the dental epithelium (Bei and Maas, 1998; Kettunen et al., 2000), is also activated in the diastemal tooth mesenchyme in *Spry4* mutants (Klein et al., 2006). Similar to what was reported in *Spry2* or *Spry4* mutants, we detected strong *Shh* expression in the enamel knot and *Fgf3* transcripts in the mesenchyme of diastemal tooth implanted with FGF8-soaked beads after 24 hours and 48 hours in culture, respectively (Fig. 5M, 5N, 5P, 5Q). These results demonstrate that FGF8 indeed activates tooth developmental program in the diastemal tooth, allowing its continuous development in vitro.

The essential role for FGF8 in tooth development has been well documented, especially for the molar development (Neubüser et al., 1997; Trumpp et al., 1999). *Fgf8* expression appears in the presumptive dental epithelium before tooth initiation, and persists in the dental epithelium throughout the bud stage, with a restriction to the distal part of the tooth epithelial bud (Kettunen and Thesleff, 1998; Kettunen et al., 2000; Lin et al., 2007). FGF8 is known to induce *Pax9* expression in the dental mesenchyme, and is also responsible for the induction of *Fgf3* expression in the dental mesenchyme (Neubüser et al., 1997; Bei and Maas, 1998; Kettunen et al., 2000). In the present study, we demonstrate that FGF8 is capable of rescuing the development of diastemal tooth under isolated condition. FGF8 alone can stimulate cell proliferation and prevent apoptosis in the diastemal tooth germ. Meanwhile, FGF8 revitalizes tooth developmental program, allowing continuous development of diastemal tooth. The negative regulators of FGF signaling, *Spry2* and *Spry4*, are strongly expressed in the diastemal tooth, and prevent the development of the diastemal tooth (Klein et al., 2006). Apparently, exogenously applied FGF8 overcomes the repressive effects of *Spry2* and *Spry4*. It was demonstrated previously that the amount of bead-carried growth factors drops below a critical level 24 hours after implantation (Fallon et al., 1994). In the present studies, we observed activation of genes critical for tooth development in the diastemal tooth in 24 hours after application of FGF8-soaked beads. It appears that once the tooth developmental program is activated, it could self-sustain and take over to direct subsequent developmental processes. This is evidenced by the enhanced expression of *Pax9*, *Msx1*, and *Fgf3* in the mesenchyme of the diastemal tooth in 48 hours after FGF8 application. FGF signals are known to regulate *Shh* expression in the dental epithelium (Tummers and Thesleff, 2009). Since *Shh* and other signaling molecules are expressed in the diastemal tooth epithelium (Prochazka et al., 2010), therefore, the continuous expression of *Shh* in the FGF8-implanted diastemal tooth is likely initially maintained/enhanced by the exogenously applied FGF8. Activation of *Fgf3* in the mesenchyme of the diastemal tooth could take over to maintain *Shh* expression afterwards. Furthermore, the expression of *Bmp4* in the mesenchyme could also strengthen *Shh* expression (Zhang et al., 2000). Given the fact that *Shh* is associated with stimulation of cell proliferation and with cell survival in developing tooth germ (Cobourne and Sharpe, 2010), the effects exerted by bead-carried FGF8 on cell proliferation and apoptosis are therefore likely mediated by the sustained *Shh* expression in the explanted diastemal tooth. Our studies also show that exogenously applied

FGF3 is capable of rescuing diastemal tooth development; however, it is much less potent as compared to FGF8. While the underlying mechanism warrants further investigation, it is likely due to the fact that FGF8 but not FGF3 is able to induce constant endogenous *Fgf3* expression (Bei and Maas, 1998). Most interestingly, in our studies, we found that FGF8 failed to rescue the development of the diastemal vestigial tooth in the presence of incisor and/or molar germs. These observations suggest the presence of additional suppressing factors emanated from the adjacent incisor or molar germs.

The pressing demand for replacement teeth has become a major challenge in regenerative dental medicine. While tooth tissue engineering using appropriate stem cells to generate fully functional replacement teeth is considered a powerful approach, an ideal alternative would be to reactivate tooth development program to allow new tooth formation in the site where a tooth had been lost. Revitalization of the diastemal vestigial tooth bud provides an excellent model for studying tooth regeneration and underlying molecular mechanisms (D'Souza and Klein, 2007). The evidence we provide in this study that application of a single growth factor is able to reactivate tooth developmental program in the mouse diastemal vestigial tooth bud raises the hope for future dental replacement and regenerative therapy in humans using similar approach. Together with our previous report that FGF8 is expressed throughout the development and differentiation stages in the human tooth, and application of exogenous FGF8 is able to induce the differentiation of human keratinocytes into enamel-secreting ameloblasts in a human-mouse chimeric tooth crown (Lin et al., 2007; Wang et al., 2010), our present studies support the idea that FGF8 represents a major player to regulate tooth development and differentiation in both mice and humans, and will have great potential for future tooth regenerative therapy in humans.

EXPERIMENTAL PROCEDURES

Bead implantation and mouse subrenal culture

Embryonic day 13.5 (E13.5) embryos from timed pregnant CD-1 females were collected, and mandibles were isolated in ice-cold PBS. Mandibular quadrants were carefully dissected out, containing only the diastema, incisor and molar germs. Each quadrant was either used as a tissue transplant, or was further dissected into the diastema, the incisor, and molar tissues, as described previously (Yuan et al., 2008). Affi-gel blue agarose beads (100 – 200 μm in diameter) were prepared and soaked with growth factor proteins as described previously (Zhang et al., 2000). BSA at the concentration of 1mg/ml was used as control as well as carrier for growth factor. Growth factors (all from R&D) used and their concentrations were: BMP4 (100ng/ μl), FGF2 (250ng/ μl), FGF3 (250ng/ μl), and FGF8 (250ng/ μl). For whole quadrant transplant or the diastemal region with either incisor or molar, 3–4 protein soaked beads were inserted into the diastemal mesenchyme from the aboral side, and then beads-containing samples were transferred to a semisolid culture medium according to the protocol reported previously (Hu et al., 2006). For isolated diastema transplant, 2–3 protein soaked beads were firmly stuck to the mesenchyme of each diastemal tissue and the tissues were cultured in the semisolid medium. The incisor and molar germs isolated from the same quadrant were also cultured in parallel. After 24 hours in culture, samples were subjected to subrenal culture using adult male CD1 mice as hosts, as described previously (Zhang et al., 2003). Grafts were cultured underneath the kidney capsule for either 2 or 4 weeks prior to being harvested for further analyses. To ensure that tooth formation in isolated diastema region did not result from tissue contamination from either incisor or molar germs, we only counted result from each diastema graft when teeth were found in the incisor and molar grafts isolated from the same quadrant. To examine early morphological change, cell proliferation, and apoptosis in the diastemal tooth induced by FGF8, isolated diastema with either BSA or FGF8 beads were cultured in the semisolid medium for 12, 24, and 48 hrs before samples were harvested.

Histology, in situ hybridization, BrdU Labeling and TUNEL assays

Samples were fixed in 4% paraformaldehyde (PFA) at 4°C for overnight, embedded in paraffin, and sectioned at 10-µm. Sections were subjected to standard hematoxylin-eosin (H&E) staining for histological analysis, and non-radioactive in situ hybridization for gene expression assay, as described previously (St. Amand et al., 2000). For in situ hybridization, at least three samples were used for each probe. Cell proliferation assay was performed by using the bromodeoxyuridine (BrdU) labeling and Detection Kit (Roche Diagnostics Corporation, Indianapolis). BrdU labeling was conducted by adding 70-µl culture medium containing 0.15% BrdU labeling solution onto samples in semisolid culture medium. Samples were pulsed for 1 hr, fixed in the Carnoy fixative, and then processed into paraffin sections at 5-µm for immunostaining according to manufacturer's instruction. To detect cell apoptosis, 5-µm paraffin sections were subjected to TUNEL assay, as described previously (Alappat et al., 2005).

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Figure 1.

Tooth formation in tissue grafts. Panels A–E show tooth formation in grafts of diastema, incisor, and molar from E13.5 mandibular quadrants; panels A'–E' show histology sections of corresponding grafts. (A, A') FGF8-induced diastemal tooth with blunt cusp (arrow) is shown. (B, B') An incisor formed. (C, C') A first molar with multiple cusps (arrows) formed in graft from the same quadrant. (D, D') A second molar with multiple cusps (arrows) was found in the molar graft. (E, E') Keratinized cyst and intramembranous bone formed in a control diastemal graft. Scale bar: 1-mm.

Figure 2.

Expression of tooth differentiation markers. (A) *Amelogenin* expression (arrow) is detected in an FGF8-induced diastemal tooth. (B) *Dspp* expression (arrow) is observed in an FGF8-induced diastemal tooth. (D, C) Expression of *Amelogenin* (C) and *Dspp* (D) is seen in a grafted molar. D, dentin.

Figure 3.

FGF8 prevents regression of diastemal vestigial tooth bud and allows continuous development of diastemal tooth. (A) A tooth bud is visible in a diastemal explant containing FGF8-soaked beads after 12 hours in culture. (B) The vestigial tooth bud (arrow) has begun to regress in a control diastemal explant after 12 hours in culture. (C) An E13.5 molar germ developed to the late bud stage after 12 hours in organ culture as control. (D) The diastemal tooth bud in a graft carrying FGF8-beads began to grow after 24 hours in culture. (E, H) The vestigial tooth bud in control diastemal explants has regressed completely after 24-hour (E) and 48-hour (H) in organ culture. (F) After 24 hour in organ culture, an explanted molar germ developed to the early cap stage. (G) The diastemal tooth in FGF8-implanted explants has developed to the cap stage after 48 hours in organ culture. (I) An explanted molar germ reached to the late cap stage. de, dental epithelium.

Figure 4.

FGF8 increases cell proliferation and prevents apoptosis in diastemal tooth bud. (A, B) BrdU labeling shows significantly increased cell proliferation in the epithelium of diastemal tooth bud in FGF8-implanted diastemal explants after 12 hours in culture (A), as compared to the BSA-implanted diastemal explants (B). (C, D) After 24 hours in culture, large amount of BrdU-positive cells could be observed in both epithelium and mesenchyme of the diastemal tooth explants carrying FGF8-beads (C), while the vestigial tooth bud had disappeared in the control explants (D). (E) TUNEL assay detected apoptotic cells in the stellate reticulum region (*), but not the enamel epithelium of a diastemal tooth implanted with FGF8-beads after 12 hours in organ culture. (F) Apoptotic cells (arrows) were found in

the epithelium of diastemal vestigial tooth in control explants after 12 hours in culture. (G) No apoptotic cells were seen in the explanted diastemal tooth carrying FGF8-beads after 24 hours in culture. (H) After 24 hours in culture, diastemal tooth bud had regressed completely. Apoptotic cells could be found in the oral epithelium.

Figure 5.

Induction of genes in diastemal tooth by FGF8. (A, B) *Pax9* expression was initially induced by FGF8 at a relatively low level in the mesenchyme of diastemal tooth explants after 24 hours in culture (A), but became stronger and localized to the dental papilla after 48 hours in culture (B). (D, E) Weak *Msx1* expression induced by FGF8 is seen in the mesenchyme of diastemal tooth explants after 24 hours in culture (D). The expression became much stronger after 48 hours in culture (E). (G, H) Expression of *Bmp4* is observed in the mesenchyme of diastemal tooth 24-hour (G) and 48-hour (H) after FGF8 induction. (J, K) *Pitx2* expression is seen in the epithelium of diastemal teeth in FGF8-implanted diastemal explants after 24 hours (J) and 48 hours (K) in culture. (M, N) *Shh* expression was detected in the enamel

knot of diastemal teeth after 24 hours (M) and 48 hours (N) in culture with FGF8. (P, Q) Weak expression of *Fgf3* was detected in the mesenchyme of diastemal tooth after 24 hours induction by FGF8 (P). However, the expression was strengthened after 48 hours (Q). (C, F, I, L, O, R) In situ hybridization shows no expression of corresponding genes in explanted diastema with BSA beads, respectively, except that weak *Pitx2* expression was detected (L). b, bead; de, dental epithelium.

Table 1

Success rate of diastema tooth formation*

Growth factor	Diastema tooth formation
FGF8	40% (20/51)
FGF2	0% (0/15)
FGF3	10% (1/10)
BMP4	0% (0/23)
BSA	0% (0/38)

* Diastemal tooth formation was counted only when incisor, molar, and diastema teeth were formed in grafts from the same quadrant.

Table 2

Number of tooth cusp and tooth size in tooth grafts

	Diastema Teeth	First Molar	Second Molar
Cusp Number	0–3	5–7	2–3
Tooth Size (um)	710.9	848.75	640.01