Regulating the Role of Bone Morphogenetic Protein 4 in Tooth Bioengineering

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Purpose: Culture of the whole organ and regulation of its development using biologic and engineering principles can be used to produce structures and organs for reconstructing defects. The application of these bioengineering approaches in artificial tooth development may be the alternative way to replace missing dentition.

Materials and Methods: For the artificial bioengineering of a mouse tooth, tooth buds were dissected and transplanted into the diastema of the developing mandible. The mandibular primordia containing transplanted tooth buds were culture in vitro and in vivo using a bioengineering method. In addition, to regulate the development of tooth germs, bone morphogenetic protein 4 (BMP4) or its antagonist, Noggin was administered.

Results: After the period of in vitro and in vivo culture, the transplanted tooth germ in the diastema showed tooth development with supportive structure formation. In the BMP-treated group, the bioengineered tooth was observed with increased maturation of cusp and enamel matrix. However, in the Noggin-treated tooth germs, the developing molar had a crater-like appearance with the immature development of the cusp and suppressed formation of the enamel matrix.

Conclusions: This study confirmed that tooth germ transplantation in the diastema and culture with administration of BMP4 could lead to the mature development of the dental structures. In addition, these results suggest the possibility of bioengineering the tooth in morphogenesis and differentiation even in the toothless area.

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Based on developmental principles, the culturing of a whole organ and regulation of its development using biological and engineering principles can be used to produce structures and organs for reconstructing defects. Using such bioengineering methods, a structural relationship and cellular association within the developing organ can be maintained, and its development can be regulated in the artificial environment.

The application of bioengineering to artificial tooth development may provide an alternative approach to replacing missing dentition. Tooth development occurs within developing embryos through sequential and reciprocal interactions between different cellular layers. For artificial development of the tooth, biological principles of the developmental process must be maintained in the artificial environment. In addition, creation of a replacement tooth critically relies on the regulation of its development according to engineering principles.

Various transcription factors and signaling molecules participate in epithelial–mesenchymal signaling, including bone morphogenetic proteins (BMPs), fibroblast growth factors, and hedgehog and Wnt family molecules. Among these molecules, BMPs have been extensively evaluated for odontogenic initiation and morphogenesis. BMP4 and BMP2 are believed to be key signals participating in the epithelial–mesenchymal interactions during tooth development. In addition, the BMP4 expression sites in the maxillary and mandibular processes suggest a role in early tooth development. At the initial stage of murine tooth formation, both BMP2 and BMP4 are expressed in the dental lamina, whereas BMP4 alone is expressed in the underlying mesenchyme. As tooth development proceeds to the bud stage, BMP4 expression shifts to the mesenchyme, whereas BMP2 and BMP4 are expressed in the dental epithelium. The region forming the enamel knot, an organizing center for establishing the tooth form, exhibits elevated BMP2 and BMP7 expression. At the cap stage, BMP4 is also expressed in the enamel knot and the dental mesenchyme, and localized BMP2 expression occurs in the mesenchymal cells deep in the dental papilla. At the bell stage, the presumptive ameloblasts express BMP4 and the odontoblasts express BMP2, BMP4, and BMP7. The application of BMP4-soaked beads to isolated dental mesenchymal tissue induces gene expression patterns that also have been observed in condensing mesenchyme in vivo and in vitro recombination studies. Moreover, the induction potential of tooth development shifts from the epithelium to the mesenchyme at embryonic day (E) 11.5, coinciding with the timing of the shift of BMP4 expression from the tooth germ epithelium to the dental papilla. In addition, BMP4 expression in the odontoblasts and ameloblasts at the bell stage and later has been reported. These findings suggest that BMP4 is involved in the differentiation of these cells and in the deposition of the matrices of dental hard tissue, dentin, and enamel.

In this study, artificial development of the tooth and the role of BMP4 on tooth morphogenesis and cell differentiation were examined using bioengineering methods. Toward this end, dissected tooth germs were transplanted into the diastema of mandibles and cultured with beads soaked with either BMP4 or its antagonist, noggin. The developing teeth were examined after the organ cultures were obtained.

**Materials and Methods**

**PREPARATION OF EMBRYOS, MANDIBLES, AND TOOTH GERMS**

Mice from the CD-1 strain and their embryos were used. At E11.5 and E13.5, the embryos were removed from the fetal membranes. The heads were removed, and the primordial mandibles of the first brachial arch were dissected. The molar tooth germs were then dissected.

**TRANSPLANTATION OF THE TOOTH GERMS INTO THE DIASTEMA AND IN VITRO ORGAN CULTURE**

The dissected tooth germs of the E11.5 and E13.5 mice were transplanted into the diastema of a mandible at the same developmental stage (Fig 1), and the BMP4 or noggin-soaked beads were then implanted around the transplanted tooth germs. For bead implantation, the Affi-Gel blue agarose beads (75 to 150 μm in diameter; Bio-Rad, Hercules, CA) were incubated in either 50 μg/mL of recombinant human BMP4 (Genetics Institute, Cambridge, MA) or 50 μg/mL of noggin (R&D systems, Minneapolis, MN) at 37°C for 30 minutes. In addition, the Affi-Gel blue agarose beads were incubated with bovine serum albumin (BSA; 100 μg/mL) as a negative control under similar conditions.

The primordial mandibles containing the transplanted tooth germs were cultured according to the Trowell method. Culture medium was created using Dulbecco’s Modified Eagle Medium with glutamax-1 (Gibco-BRL, Detroit, MI) supplemented with 10% heat-inactivated fetal calf serum and 20 IU/mL of penicillin-streptomycin. The tissues were cultured in a standard incubator at 37°C in a humidified atmosphere of 5% CO₂ for 2 days.

**KIDNEY TRANSPLANTATION AND IN VIVO ORGAN CULTURE**

For the study of tooth development under a physiological environment, the diastema area containing the transplanted tooth germs and some of the sur-
rounding mesenchymal tissues were dissected from the in vitro cultured primordial mandibles and transferred to an adult mouse kidney under the inner capsule. The explants were then left to develop for 2 to 3 weeks in vivo. After the in vivo culture period, the mice were sacrificed, and the developing mandibular explants were removed from the kidney.

HISTOLOGICAL EXAMINATION

Subsequently, developing mandibular explants were fixed in 4% paraformaldehyde, dehydrated, and then embedded in paraffin. Then 5-μm-thick sections were prepared and stained with hematoxylin and eosin and Masson trichrome. The development and formation of the dental tissues were evaluated by optical microscopy. In some cases, the developing teeth were dissected from the cultured diastema and examined. All experiments involving animals were performed in accordance with the regulations and with the approval of the Dental Research Institute of Seoul National University Dental College.

Results

Tooth germs cultured in the diastema showed proliferation and differentiation that progressed to the late bell stage. The crown pattern of the developing tooth was established by a folding of the inner dental epithelium. Dentin and enamel formation were observed at the crest of the folded inner dental epithelium, the cusp-developing site. In addition, the development of supporting structures and the alveolar bone, and formation of the neurovascular bundles, were observed around the developing tooth (Fig 2). The tooth germs cultured with BSA-soaked beads developed into the late bell stage. After cusp development, dentin and enamel formation were observed in both E11.5 and E13.5 tooth germs. In some E13.5 tooth germ cases, second molar formation was observed along with first molar formation (Fig 3).

In the BMP4-soaked beads implant group, the tooth germs developed into the late bell crown stage, which is characterized by hard tissue formation. The E11.5 tooth germs showed crown development with the formation of enamel and dentin matrix. Formation and development of the first and second molars were frequently observed in this group (Fig 4A). The tooth germs, which were prepared from the E13.5 mice and cultured with BMP4-soaked beads, also reached the crown stage. The developing teeth were the first and second molars. In 46 of the 56 cases (82%), the BMP4-treated tooth buds showed the development of second molars along with that of first molars. The first molars showed mature crown formation with cusp development along the folded internal dental epithelium. In the developing cusp, there was an obvious ameloblast and odontoblast differentiation along with deposition of enamel and dentin matrix. In 49 of the 56 cases (88%), the BMP4-soaked beads implanted tooth buds showed mature cusp development with the deposition of the enamel and dentin matrix, as described earlier. The developing second molar was smaller than the first molar; however, its cusp development was evident at the postnatal development stage. At the cervical loop, Hertwig’s epithelial root sheath grew around the dental papilla between the dental papilla and the dental follicle. After this growth, the dental root developed with formation of the dentin matrix (Fig 4B).

The tooth germs transplanted into the diastema that were cultured with noggin-soaked beads exhibited tooth development. In the E11.5 tooth germs, crown development to the bell stage was observed.
after the proliferation and development of the transplanted tooth germs. Odontoblast and ameloblast differentiation occurred, and the dentin and enamel matrix formed in the entire region of the crown. However, the developing cusp exhibited flat occlusal surfaces, and the first molar developed without formation of the second molar (Fig 5A). The E13.5 tooth buds cultured with noggin-soaked beads showed tooth development. However, the crown of the developing tooth had an immature, crater-like appearance. The occlusal region of the crown was relatively flat with immature cusps, and there was no differentiation of the inner dental epithelial cells into ameloblasts. In contrast, those on the lateral surface of the region proximal to the tooth crown differentiated normally. Odontoblast differentiation was observed, and the dentin matrix was formed in the whole region of the crown. At the cervical loop, the dental root developed after Hertwig’s epithelial root sheath. In 37 of the 52 cases (72%), the noggin-treated tooth buds showed immature cusp development, as described earlier. In addition, the developing tooth originating from the noggin-treated tooth buds was the first molar, without formation of the second molar. Formation of the first molar without the second molar was observed in 43 of the 52 cases (84%) (Fig 5B). Although formation of the second molar was observed in 9 cases of the noggin-soaked beads implant group, the developing second molars were immature and smaller than those of the BMP4-treated group. The incidences of mature cusp development and second molar formation were sig-

**FIGURE 2.** Cultured diastema area containing the transplanted tooth germ. A, Tooth development (black arrow) and formation of the supporting structures were observed (E13.5 tooth germ; hematoxylin and eosin; original magnification X10) Alv b, alveolar bone. B, The transplanted tooth germs showed development to the late bell stage. The developing tooth showed formation of the dentin and enamel matrix, compatible with bell-stage development (E, enamel; D, dentin; E11.5 tooth germ; hematoxylin and eosin; original magnification X40).


**FIGURE 3.** Tooth germs cultured with BSA-soaked beads. A, E11.5 tooth germs showed enamel and dentin matrix formation following cusp development (E, enamel; D, dentin; Masson trichrome, original magnification X40). B, E13.5 tooth germs showed formation of the first and second molars. The developing molars showed enamel and dentin matrix formation.

Discussion

Odontogenesis is the process by which tooth fields are specified and developed into a mature dental structure. Tooth formation in mice usually begins after 11.5 days of intrauterine development, with a high proliferation of oral epithelium and neoformation of dental lamina. Subsequently, the dental lamina grows into the underlying mesenchyme of the first branchial arch, which forms the epithelial buds (bud stage: E13.5). After this stage, the epithelial component undergoes specific foldings during the cap stage (E14.5) and bell stage (E15.5), which eventually give rise to the ameloblasts that deposit enamel. This odontogenesis is regulated by a series of interactions between morphologically distinct tissues. Moreover, synergistic and antagonistic effects of signaling molecules are involved in the localized tissue responses at the different stages of tooth development.

For the artificial development of a tooth from a toothless area, developing tooth germs were transplanted into the diastema and cultured. In addition, to regulate the development of the tooth germs, BMP4 or noggin was administered during the culture period. The expression of BMP in the maxillary and mandibular processes and the BMP receptors ALK3 (BMPR 1A) and ALK6 (BMPR 1B) in the inner and outer dental epithelium are suggestive of its role in the tooth development. Two known antagonists of BMP are chordin and noggin. These proteins bind BMPs in the extracellular space to prevent the activation of BMP receptors. In tooth development, noggin binds BMP4 with a high affinity and can eliminate its activity by blocking its binding to cell surface receptors. This activity transformed the tooth identity from incisor to molar when the E11.5 tooth germs from the incisor development site were cultured with noggin. This suggests that BMP4 in the dental epithelium at the initiation stage is involved in determining a tooth’s identity.

As a BMP antagonist, noggin evidently affects the cusp formation in the tooth germs and results in a crater-like appearance of the dental crown. The transition from the bud to cap stage is a determining step in tooth morphogenesis. In cusp development, the primary enamel knot is a prerequisite for tooth development to proceed to the cap stage and acts as a signaling center. Among the numerous growth factors, BMP4 is intensively expressed on the enamel knot and acts as a signal to control the shape of the tooth. Based on these findings, the antagonistic effect of noggin on BMP also inhibited enamel knot function and caused immature cusp formation during tooth development. In addition, histological examination showed inhibited proliferation of the inner dental epithelium into ameloblasts in the occlusal region. In the tooth development stages, cusp formation ap-
pears to be caused by site-specific proliferation of the inner dental epithelial cells. Subsequently, dentin-forming odontoblasts and enamel-forming ameloblasts differentiate at the dental epithelium–mesenchyme interface. During the differentiation stage BMP2, BMP4, and BMP7 are expressed in the inner enamel epithelium as epithelial signals regulating the differentiation of the underlying mesenchymal cells into odontoblasts, and are expressed by the ameloblasts during their terminal differentiation. Of these, BMP4 is the most intensively expressed in the ameloblasts.8,23 The BMP4 expression level in the odontoblasts decreases with advancing development, whereas it persists in the ameloblasts.2 Therefore, BMP4s are present at the right time and location to act as signals for dental hard tissue formation. The binding of noggin to BMP4 receptors stops the differentiation of inner dental epithelial cells into ameloblasts and suppresses formation of the enamel matrix. These results are in agreement with a previous experimental study in which treatment of the antisense oligodeoxynucleotide against BMP4 suppressed formation of a molar cusp in vitro cultured tooth germs.23 In contrast, thick layers of dentin and enamel matrix separating ameloblasts from the odontoblasts were observed in the BMP4-treated tooth germs.

In mice, development of the second molar was discernible at E14.0,24 and the anterior extremities of the upper and lower second molars could be detected by E16.0.25 At E17.0, the dental epithelium of the

<table>
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<tr>
<th>Treatment</th>
<th>Number of Transplanted Tooth Buds</th>
<th>Cusp Development (%)</th>
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<tbody>
<tr>
<td>BMP4</td>
<td>56</td>
<td>49 (88.0) 7 (12.0)</td>
</tr>
<tr>
<td>Noggin</td>
<td>52</td>
<td>15 (28.0) 37 (72.0)</td>
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**Table 1. EFFECT OF BMP4 AND NOGGIN ON CUSP DEVELOPMENT**


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Transplanted Tooth Buds</th>
<th>Second Molar Formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP4</td>
<td>56</td>
<td>46 (82.0) 10 (18.0)</td>
</tr>
<tr>
<td>Noggin</td>
<td>52</td>
<td>9 (16.0) 43 (84.0)</td>
</tr>
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**Table 2. EFFECT OF BMP4 OR NOGGIN ON THE SECOND MOLAR FORMATION OF E13.5 TOOTH BUDS**

second molars was separated from those representing the first molar. In addition, anterior-posterior elongation and the development of the molar tooth germs were detected. The development of second molars was evident in the BMP4-treated group, which was compatible with the postnatal dentin and enamel deposition period. However, in most cases of the noggin-treated tooth germs, the first molars without formation of the second molars arose from the transplanted tooth germs. This suggests that noggin inhibits initiation and proliferation of the second molar from the transplanted tooth germs.

Our findings confirm that tooth germ transplantation in the diastema and culture with the administration of BMP4 can lead to the mature development of dental structures. In addition, these results suggest the possibility of bioengineering tooth morphogenesis and differentiation even in toothless areas.

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