
Feasibility of Gene Therapy for Tooth Regeneration by Stimulation of a Third Dentition

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1. Introduction

The tooth is a complex biological organ that consists of multiple tissues, including enamel, dentin, cementum, and pulp. Missing teeth is a common and frequently occurring problem in aging populations. To treat these defects, the current approach involves fixed or removable prostheses, autotransplantation, and dental implants. The exploration of new strategies for tooth replacement has become a hot topic. Using the foundations of experimental embryology, developmental and molecular biology, and the principles of biomimetics, tooth regeneration is becoming a realistic possibility. Several different methods have been proposed to achieve biological tooth replacement[1-8]. These include scaffold-based tooth regeneration, cell pellet engineering, chimeric tooth engineering, stimulation of the formation of a third dentition, and gene-manipulated tooth regeneration. The idea that a third dentition might be locally induced to replace missing teeth is an attractive concept[5,8,9]. This approach is generally presented in terms of adding molecules to induce *de novo* tooth initiation in the mouth. It might be combined with gene-manipulated tooth regeneration; that is, endogenous dental cells *in situ* can be activated or repressed by a gene-delivery technique to produce a tooth. Tooth development is the result of reciprocal and reiterative signaling between oral ectoderm-derived dental epithelium and cranial neural crest cell-derived dental mesenchyme under genetic control [10-12]. More than 200 genes are known to be expressed during tooth development (<http://bite-it.helsinki.fi/>). A number of mouse mutants are now starting to provide some insights into the mechanisms of supernumerary tooth formation. Multiple supernumerary teeth may have genetic components in their etiology and partially represent the third dentition in humans. Such candidate molecules or genes might be those

that are involved in embryonic tooth induction, in successional tooth formation, or in the control of the number of teeth. This means that it may be possible to induce *de novo* tooth formation by the in situ repression or activation of a single candidate gene. In this review, we present an overview of the collective knowledge of tooth regeneration, especially regarding the control of the number of teeth for gene therapy by the stimulation of a third dentition.

2. The third dentition

It has been suggested that, in humans, a “third dentition” with one or more supernumerary teeth can occur in addition to the permanent dentition, and supernumerary teeth are sometimes thought to represent a partial post-permanent dentition [13-15]. The basic dentition pattern observed in mammals is diphyodont, and consists of three incisors, one canine, four premolars, and three molars, while Human teeth are diphyodont excepting the permanent molars [16]. The deciduous teeth are, ontogenetically, the first generation of teeth. The permanent teeth (except molar) belong to the second dentition. The term “third dentition” refers to the opinion that one more set of teeth can occur in addition to the permanent teeth (Figure 1). Human teeth are diphyodont excepting the permanent molars. The normal mouse dentition is monophyodont and composed of one incisor and three molars in each quadrant. The number of teeth is usually strictly determined. It was initially reported that there is an anlage of the third dentition in some mammals [17]. The presence of an epithelial anlage of the third dentition was also noticed in humans [18,19]. The teeth and anlagen that appear in third dentition in serial sections of infant jaws and some fetuses have been analyzed. The epithelium which is considered as the anlagen of the third dentition develops lingual to all permanent tooth germs [15]. Furthermore, when it appears, the predecessor (permanent tooth germ) is in the bell-shaped stage [15]. The timing of appearance of the third dentition seems to be after birth (Table 1). This means that we have a chance to access the formation of the third dentition in the mouth.

Teeth	The time of appearance of the third dentition	
	Maxilla	Mandible
central incisors	~ 3 months after birth	2 ~ 3 months after birth
lateral incisors	8 ~ 9 months after birth	2 ~ 3 months after birth
canines	2 ~ 7 months after birth	2 ~ 3 months after birth
the first premolar	1 year 1 month ~ 5 years 4 months after birth	1 year 1 month ~ 5 years 4 months after birth
the second premolar	1 year 1 month ~ 5 years 4 months after birth	2 years ~ 5 years 4 months after birth

Table 1. Timing of appearance of the third dentition

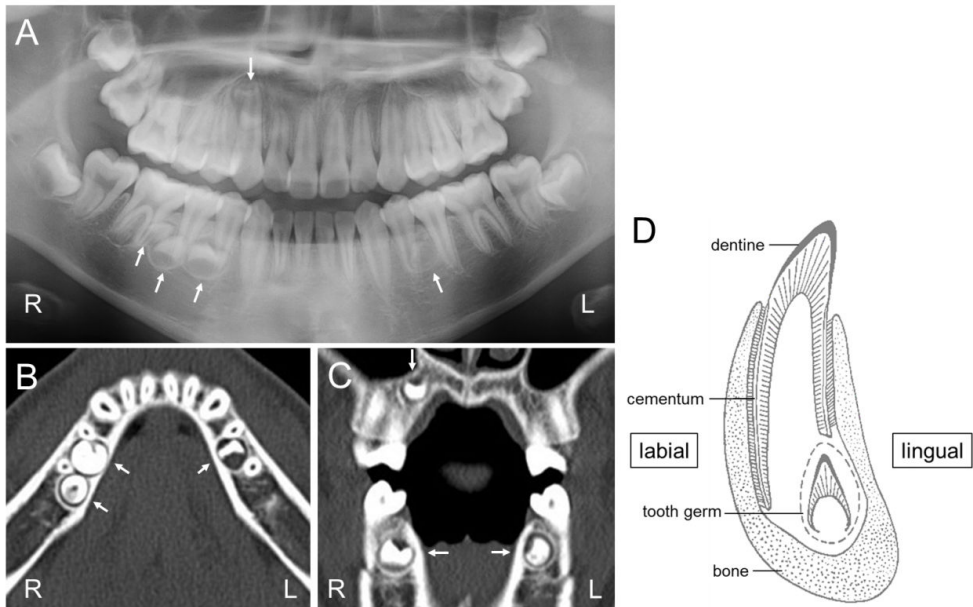


Figure 1. Multiple impacted supernumerary teeth in a 13-year-old non-syndromic patient. The third dentition develops lingual to the permanent tooth germ (D). All impacted supernumerary teeth in this patient are located to the lingual side of the permanent teeth (white arrow) (A-C). These multiple supernumerary teeth seem to be post-permanent dentition (“third dentition”).

Analysis of other model systems with continuous tooth replacement or secondary tooth formation, such as in the fish, snake, lizard, and ferret, is providing insights into the molecular and cellular mechanisms underlying successional tooth development, and will assist in studies on supernumerary tooth formation in humans. While some nonmammalian species have multi rowed dentition and replace their teeth regularly throughout life, mammalian vertebrates have one row of teeth and only renew their teeth once, or, in some rodents, show no replacement [20-23]. Detailed histological analysis of the tooth replacement in these models indicates that the successional teeth are initiated from the dental lamina epithelium, which grows from the lingual side of the deciduous tooth enamel organ, and it later elongates and buds into the jaw mesenchyme, forming successional teeth. Jarvien et al. showed that, in the ferret, *Sostdc1* (also known as USAG-1, ectodin, and Wise) is expressed in the elongating successional dental lamina at the interface between the lamina and deciduous tooth, as well as the buccal side of the dental lamina, suggesting that *Sostdc1* plays a role in defining the identity of the dental lamina [20]. Handrigan et al. analyzed successional tooth formation in the snake and in lizard, and proposed that dental epithelium stem cells are responsible for the formation of successional lamina, and Wnt signaling may regulate the stem cell fate in these cells [24]. Maintenance or reactivation of component dental lamina is thus pivotal for the replacement tooth and supernumerary formation.

3. Human syndromes associated with supernumerary teeth

Supernumerary teeth can be associated with a syndrome (Table 2) or they can be found in non-syndromic patients [25-28]. Only 1% of non-syndromic cases have multiple supernumerary teeth, which occur most frequently in the mandibular premolar area, followed by the molar and anterior regions, respectively [29-34]. There are special cases exhibiting permanent supernumerary teeth developing as supplementary teeth forming after the permanent teeth. These are thought to represent a third dentition, best known as manifestations of cleidocranial dysplasia (CCD).

Syndrome	Gene	Genetics	References
Cleidocranial Dysplasia; CCD (Dental anomalies, isolated dental phenotype) (MIM 119600)	Runx2 (MIM 600211)	Chromosome 6p21, autosomal dominant	Lee et al., 1997; Mundlos et al., 1997.
Familial adenomatous polyposis 1; FAP1 (including Gardner syndrome) (MIM 175100)	APC (MIM 611731)	Chromosome 5q21-22, autosomal dominant	Fader et al., 1962; Ida et al., 1981; Shafer et al., 1983; Jensen and Kreiborg, 1990.
Nance–Horan syndrome (Cataract-Dental syndrome) (MIM 302350)	NHS (MIM 300457)	Chromosome Xp22.13, X-linked dominant	Bixler et al., 1984; Van Dorp and Delleman, 1979; Walpole et al., 1990; Burdon et al., 2003.
Trichorhinophalangeal syndrome, Type III (TRPS3) (MIM 190351)	TRPS1 (MIM 604386)	Chromosome 8q23.3, autosomal dominant	Giedion., 1966; Momeni et al., 2000; Kantaputra et al., 2008.
Robinow syndrome (MIM 180700)	WNT5A (MIM 164975)	Chromosome 3p14.3, autosomal dominant	Mazzeu et al., 2007.
Hallermann-Streif syndrome; HSS (MIM 234100)	GJA1 (MIM 121014)	Chromosome 6q22.31, Isolated cases	da Fonseca and Mueller, 1994; Robotta and Schafer, 2011.
Rothmund–Thomson Syndrome; RTS (MIM 268400)	RECQL4 (MIM 603780)	Chromosome 8q24.3, autosomal recessive	Kitao et al., 1999.
Orofaciodigital Syndrome I ; OFD1 (Papillon-Leage and Psaupe syndrome) (MIM 311200)	OFD1 (MIM 300170)	Chromosome Xp22.2, X-linked dominant	Ferrante et al., 2001.
Uncombable Hair, Retinal Pigmentary Dystrophy, Dental Anomalies, and Brachydactyly (Bork Syndrome) (MIM 191482)	Unknown	autosomal dominant	Silengo et al., 1993.

Table 2. Human syndromes associated with supernumerary teeth

Genetic mutations have been associated with the presence or absence of individual types of teeth. Supernumerary teeth are associated with more than 20 syndromes and developmental abnormalities like CCD, and Gardner syndrome [35]. The percentage occurrence in CCD is 22% in the maxillary incisor region and 5% in the molar region [36-38]. CCD is a dominantly inherited skeletal dysplasia caused by mutations in *Runx2* [39-40]. It is characterized by persistently open sutures or the delayed closure of sutures, hypoplastic or aplastic clavicles, a short stature, delayed eruption of permanent dentition, supernumerary teeth, and other skeletal anomalies. There is a wide spectrum of phenotypic variability ranging from the full-blown phenotype to an isolated dental phenotype characterized by supernumerary tooth formation and/or the delayed eruption of permanent teeth in CCD (Figure 1) [41-44]. A dose-related effect seems to be present, as the milder case of CCD, and those exhibiting primary dental anomalies, are related to mutations that reduce, but do not abolish, protein stability, DNA binding, and transactivation [41,43-45]. *Runx2*-deficient mice were found to exhibit lingualbuds in front of the upper molars, and these were much more prominent than in wild-type mice [46,47]. These buds presumably represent the mouse secondary dentition, and it is likely that *Runx2* acts to prevent the formation of these buds. *Runx2* usually functions as a cell growth inhibitor [43]. *Runx2* reg-

ulates the proliferation of cells and may exert specific control on the dental lamina and formation of successive dentitions. Runx2 heterozygous mutant mice mostly phenocopied the skeletal defects of CCD in humans, but with no supernumerary tooth formation [48] (Otto, 1997). Notably, in Runx2 homozygous and heterozygous mouse upper molars, a prominent epithelial bud regularly presents. This epithelial bud protrudes lingually with active Shh signaling, and it may represent the extension of the dental lamina for successional tooth formation in mice. Hence, although Runx2 is required for primary tooth development, it prevents the growth of the dental lamina and successional tooth formation [47].

Familial adenomatous polyposis (FAP), also named adenomatous polyposis of the colon (APC), is an autosomal dominant hereditary disorder characterized by the development of many precancerous colorectal adenomatous polyps, some of which will inevitably develop into cancer. In addition to colorectal neoplasia, individuals can develop variable extracolonic lesions, including upper gastrointestinal polyposis, osteomas, congenital hypertrophy of the retinal pigment epithelium, soft tissue tumors, desmoid tumors, and dental anomalies [49-53]. Dental abnormalities include impacted teeth, congenital absence of one or more teeth, supernumerary teeth, dentigerous cysts associated with the crown of an unerupted tooth, and odontomas [50,52]. Gardner syndrome is a variant of FAP characterized by multiple adenomas of the colon and rectum typical of FAP together with osteomas and soft tissue tumors [49,51]. Supernumerary teeth and osteomas were originally described as a part of Gardner syndrome, but they can also occur in FAP patients with or without other extracolonic lesions [51,52]. FAP and Gardner syndrome are caused by a large number of germinal mutations in the *APC* gene [52,53]. *APC* is a tumor suppressor gene involved in the down-regulation of free intracellular β -catenin, the major signal transducer of the canonical Wnt signaling pathway, as well as a central component of the E-cadherin adhesion complex [54,55]. In addition, the *APC* protein may also play roles in chromosomal stability, the regulation of cell migration up the colonic crypt and cell adhesion through association with GSK3 β , and other functions associated with microtubule bundles [55,56]. Inactivation of *APC* would lead to the stabilization and accumulation of the proto-oncogene β -catenin, dysregulation of the cell cycle, and chromosomal instability [52]. Approximately 11-27% of patients have supernumerary teeth, but, so far, no specific codon mutation of the *APC* gene has been found to correlate with supernumerary teeth. Correlations seem to exist between dental abnormalities and the number and type of osteomas, with the highest incidence of supernumerary teeth and odontomas being found in FAP patients with three or more osteomas [52]. Conditional knockout of the *Apc*-gene resulted in supernumerary teeth in mice [57-59]. Notably, adult oral tissues, especially young adult tissues, are still responsive to the loss of *Apc* [60]. In old adult mice, supernumerary teeth can be induced on both labial and lingual sides of the incisors, which contain adult stem cells supporting the continuous growth of mouse incisors [60,61]. In young mice, supernumerary tooth germs were induced in multiple regions of the jaw in both incisor and molar regions. They can form directly from the oral epithelium, in the dental lamina connecting the developing molar or incisor tooth germ to the oral epithelium, in the crown region, as well as in the elongating and furcation area of the developing root [60].

The identification of mutations in *RUNX2* causing an isolated dental phenotype in CCD and in *APC* causing FAP has attracted attention as a possible route towards inducing *de novo* tooth formation.

4. Supernumerary tooth formation in a mouse model

The number of teeth is usually strictly determined. Whereas evidence supporting a genetic etiology for tooth agenesis is well established, the etiology of supernumerary tooth formation is only partially understood in the mouse model (Table 3). Unlike humans, mice have only molars and incisors separated by a toothless region called the diastema. In addition, mice only have a single primary dentition and their teeth are not replaced. Therefore, mice may not be an optimal model for studying tooth replacement and supernumerary tooth formation [62]. Most of the reported mouse supernumerary teeth are located in the diastema region. This is not a *de novo* tooth formation but the rescue of vestigial tooth rudiments. During the early stages of tooth development, many transient vestigial dental buds develop in the diastema area. Some of them can develop into the bud stage, but later regress and disappear by apoptosis, or merge with the mesial crown of the first molar tooth [63-68]. Major signaling pathways regulating tooth development are also expressed in these vestigial dental buds. Modulation of these signals can rescue these vestigial tooth rudiments to develop into supernumerary diastema teeth [23]. A number of mutant mouse strains have been reported exhibiting supernumerary diastema teeth. Although the rudimentary tooth buds form in the embryonic diastema, they regress apoptotically [69]. Transgenic mice in which the keratin 14 promoter directs Ectodysplasin (Eda), a member of the tumor necrosis factor (TNF) family of signaling molecules, or Eda receptor expression to the epithelium had supernumerary teeth mesial to the first molar as a result of diastema tooth development [70-72]. It has also been reported that Sprouty2 (Spry2) or Spry4 (which encode negative feedback regulators of fibroblast growth factor (FGF)) deficient mice showed supernumerary tooth formation as a result of diastema tooth development [73]. Hypomorphic Polaris mice and *Wnt-Cre* (Polaris conditional mutant mice with affected Shh signaling) [73-74], Pax6 mutant mice [75] and Gas1 null mutants [73] were also included. Uterine sensitization associated gene-1 (USAG-1) is a BMP antagonist, and also modulates Wnt signaling. We reported that USAG-1-deficient mice have supernumerary teeth (Figure 2).

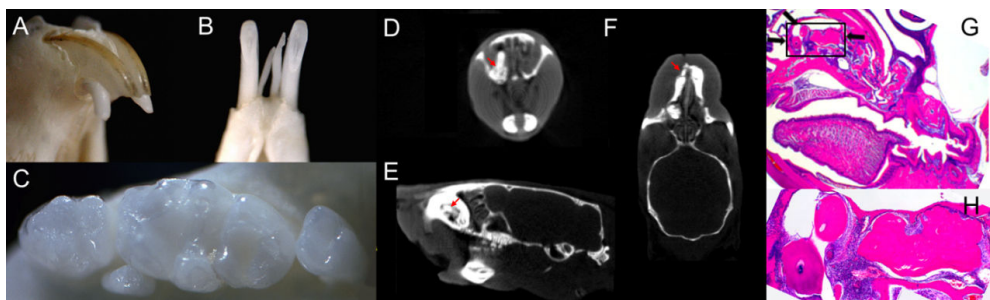


Figure 2. Supernumerary teeth formation in *Sostdc 1* (USAG-1) (A-C) and *CEBPB* (D-H) adult mutant mice. A: Oblique view of the maxillary incisors. B: Occlusal view of the mandibular incisors. C: Occlusal view of the mandibular molars. Micro-CT images (D-F) and HE-staining (G,H) of the murine head. A frontal view (D), a sagittal view (E) and a horizontal view (F) showed supernumerary tooth (red arrow). Two supernumerary teeth and an odontoma were seen in a low (G) and a high (H) magnification.

Mutant mouse	Tooth phenotype	references
Sostdc1 ^{-/-} (USAG-1, ectodin, Wise)	Supernumerary incisors in the maxilla and mandible Premolar mesial to first molar, peg-shaped tooth lingual to first molar	Munne et al., 2009; Ohazama et al., 2008; Yanagita et al., 2006; Murashima-Suginami et al., 2007, 2008.
CEBPB ^{-/-}	Supernumerary teeth and/or odontomas in the diastema between the incisor and the first molar	Huang et al., 2012.
Gas1 ^{-/-}	Premolar mesial to first molar, both jaws (100% penetrance)	Ohazama et al., 2009.
Gas1 ^{-/-} ; Shh ^{+/-}	Mandibular molar (associated with jaw duplication)	Seppala et al., 2007.
Tg737 ^{97pk} hypomorph	Premolar mesial to first molar, both jaws (100% penetrance)	Zhang et al., 2003; Ohazama et al., 2009.
Wnt1-Cre; Polaris ^{fllox/flox}	Premolar mesial to first molar, both jaws (100% penetrance)	Ohazama et al., 2009.
Spry2 ^{-/-}	Premolar mesial to first molar; maxilla (>5%), mandible (97%: 92% bilateral; 5% unilateral)	Klein et al., 2006; Peterkova et al., 2009.
Spry4 ^{-/-}	Both jaws? 16% penetrance (most unilateral)	Klein et al., 2006.
Lrp4 ^{-/-} (Megf7) hypomorph	Supernumerary incisors in the maxilla and mandible Premolar mesial to first molar (varying penetrance in both jaws) Lingual peg-shaped tooth (maxilla, variable penetrance)	Ohazama et al., 2008.
Osr2 ^{-/-}	Lingual molars	Zhang et al., 2009.
Epiprofin ^{-/-}	Multiple incisors and molars in both jaws	Nakamura et al., 2008.
K14-Cre; Apc ^{cko/cko}	Multiple incisor and molar tooth buds	Kuraguchi et al., 2006.
K14-Cre ^{EBM1} ; Apc ^{cko/cko}	Numerous labial and lingual incisor and molar teeth (↑ with age)	Wang et al., 2009.
K14-Cre ^{1Amc} ; Apc ^{cko/cko}	Numerous epithelial buds from E14.5	Wang et al., 2009.
K14-CreER TM ; Apc ^{cko/cko}	Numerous labial and lingual incisors (age P5–10/12)	Wang et al., 2009.
K14-CreER TM ; Ctnb1 ^{(ex3)fl/+}	Numerous labial and lingual incisors (age P5–6/12) P5 molar supernumeraries	Wang et al., 2009.
K14-Cre/+; β-catenin ^{ex3fl/+}	Multiple incisor and molar epithelial invaginations in both jaws	Jarvinen et al., 2006.
K14-Cre; Ctnnb1 ^{(ex3)fl/+}	Multiple molar epithelial invaginations	Liu et al., 2008.
K14-Lef1	Rudimentary teeth at inappropriate sites	Zhou et al., 1995.
Pax6 ^{Sey}	Incisor supernumeraries: 35% unilateral; 45% bilateral incisors	Kaufman et al., 1995.
K14-Eda	Premolar mesial to first molar; incomplete penetrance	Kangas et al., 2004; Mustonen et al., 2003.
K14-Edar	Premolar mesial to first molar; incomplete penetrance	Tucker et al., 2004.
Tabby ^{+/-}	Molar (2.5%; mandible > maxilla)	Gruneberg et al., 1966; Sofaer et al., 1969.
B6CBACa-A ^{w-JA} -Eda ^{T19/0}	Molar (1%; mandible > maxilla)	Peterkova et al., 2005.
di	Mandibular incisors (right > left)	Danforth et al., 1958.
β-cat ^{Δex3K14/+}	Supernumerary molars in the maxilla and mandible	Järvinen, E., 2006

Table 3. Mutant mouse associated with supernumerary teeth

The supernumerary maxillary incisor appears to form as a result of the successive development of the rudimentary upper incisor. USAG-1 abrogation rescued apoptotic elimination of odontogenic mesenchymal cells [14]. BMP signaling in the rudimentary maxillary incisor, assessed by expressions of Msx1 and Dlx2 and the phosphorylation of Smad protein, was significantly enhanced. Wnt signaling, as demonstrated by the nuclear localization of β-catenin, was also up-regulated. The inhibition of BMP signaling rescues supernumerary tooth formation in E15 incisor explant culture. Based upon these results, we conclude that enhanced BMP signaling results in supernumerary teeth and BMP signaling was modulated by Wnt signaling in the USAG-1-deficient mouse model (Figure 3) [76]. Canonical Wnt/β-catenin signaling and its down-stream molecule Lef-1 are essential for tooth development [77].

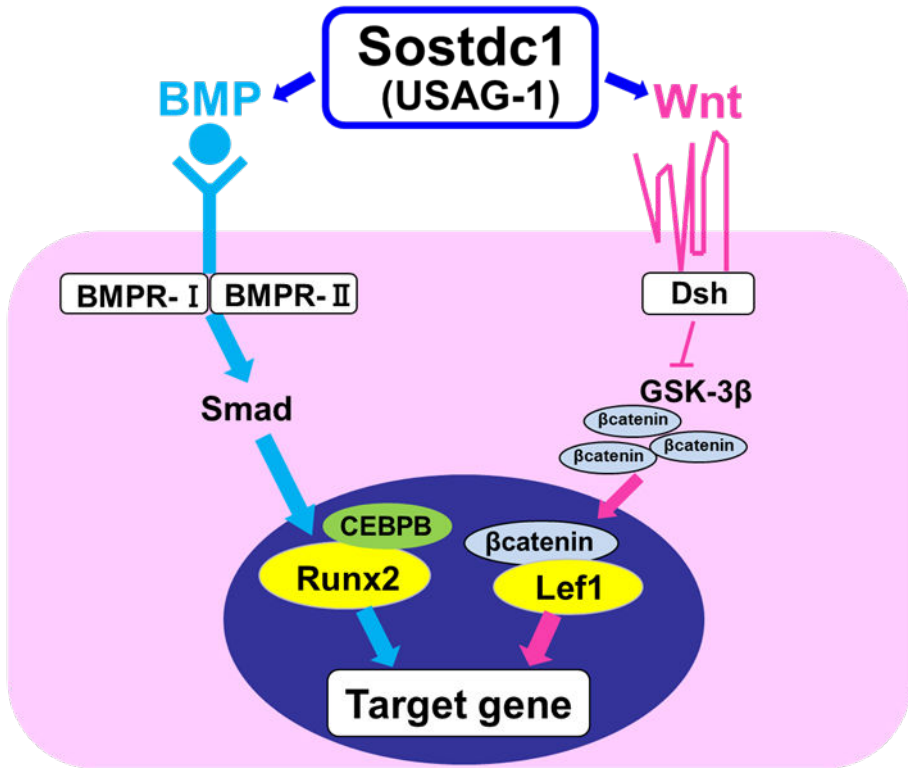


Figure 3. Diagrammatic representation of the Sostdc (USAG-1) pathway during development

Overexpression of Lef-1 under the control of the K14 promoter in transgenic mice leads to the development abnormal invaginations of the dental epithelium in the mesenchyme and formation of a tooth-like structure [78]. *De novo* supernumerary teeth arising directly from the primary tooth germ or dental lamina have been reported in *Apc* loss-of-function (as discussed in the previous section) or β -catenin gain-of-function mice, and *Sp6* (*Epiproofin*)-deficient mice. It was demonstrated that mouse tooth buds expressing stabilized β -catenin give rise to extra teeth[58] (Jarvinen et al., 2006). More recently, *Epiproofin* (*Epfn*) (a zinc finger transcription factor belonging to the Sp transcription factor superfamily)-deficient mice developed an excess number of teeth[79]. Mammals only have one row of teeth in each jaw. Interestingly, in the *Osr2* null mutant mouse embryo, supernumerary tooth germs were found developing directly from the oral epithelium lingual to their molar tooth germs [80]. More recently, we also demonstrated that CEBPB deficiency was related to the formation of supernumerary teeth[81]. A total of 66.7% of CEBPB^{-/-} 12-month-olds sustained supernumerary teeth and/or odontomas in the diastema between the incisor and the first molar. Two supernumerary teeth accompanied with a complex odontoma near the root of the upper right incisor were identified in a CEBPB^{-/-} adult (Figure 2), whilst two other CEBPB^{-/-} mice simply

showed a supernumerary tooth in the upper left quadrant. Another *CEBPB*^{-/-} adult mouse did not display any supernumerary teeth in either jaw, but an odontoma in the lower-right quadrant. All of the *CEBPB*^{-/-} adults appeared with a normal number of erupted incisors and molars. Nevertheless, 20% of the *CEBPB*^{+/-} 12-month-olds had a missing lower third molar. Dental anomalies such as supernumerary teeth, odontomas, or hypodontia were not found in mice of any other genotypes and/or age[81].

These mouse models clearly demonstrated that it was possible to induce *de novo* tooth formation by the in situ repression or activation of single candidate gene such as USAG-1.

5. Gene therapy approaches

Gene therapy provides a unique tool for the delivery of previously identified signaling molecules in both time and space that may significantly augment our progress toward clinical tooth regeneration. Stimulation of the formation of a third dentition and gene-manipulated tooth regeneration comprise an attractive concept (Figure 4). This approach is generally presented in terms of adding molecules to induce *de novo* tooth initiation in the mouth. It might be combined with gene-manipulated tooth regeneration; that is, endogenous dental cells *in situ* can be activated or repressed by a gene-delivery technique to make a tooth. We have a chance to access the formation of the third dentition in the mouth, because the time of appearance of the third dentition seems to be after birth. As the half-life of targeted proteins *in vivo* is transient, tooth regeneration is not a common outcome following conventional therapy. Typically, high concentrations are required to promote regeneration [82]). Therefore, supplemental local production via gene transfer could be superior to bolus delivery methods.

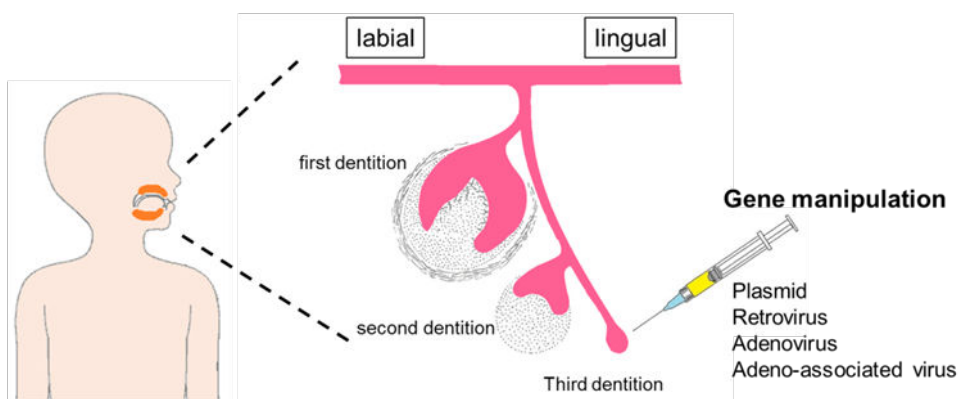


Figure 4. *In vivo* gene delivery approach for the tooth regeneration by stimulation of a third dentition.

Simply stated, gene therapy consists of the insertion of genes into an individual's cells directly or indirectly with a matrix to promote a specific biological effect. Gene therapy can be used to induce a more favorable host response. Targeting cells for gene therapy requires the use of vectors or direct delivery methods to transfect them. To overcome the short half-lives of peptides *in vivo*, gene therapy that uses a vector that encodes the candidate genes is utilized to stimulate the formation of the third dentition. The two main strategies of gene vector delivery have been applied. Gene vectors can be introduced directly to the target site (*in vivo* gene delivery) [83] or selected cells can be harvested, expanded, genetically transduced, and then reimplanted (*ex vivo* gene delivery). *In vivo* gene transfer involves the insertion of the gene of interest directly into the body, anticipating the genetic modification of the target cells. *Ex vivo* gene transfer includes the incorporation of genetic material into cells exposed from a tissue biopsy with subsequent reimplantation into the recipient. So far, *in vivo* gene delivery has been a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition, but *ex vivo* gene delivery is not realistic because of the poor availability of ideal cells.

Gene transfer is accomplished through the use of viral and nonviral vectors. The three main classes of virus used for gene therapy are the retrovirus, adenovirus, and adenoassociated viruses. Retroviruses are ideal for long-term gene therapy since, once introduced, their DNA integrates and becomes part of the genome of the host cells. Indeed, the current human genome contains up to 5 to 8% of endogenous retroviral sequences that have been acquired over the course of evolution [84]. Adenoviruses are more commonly suited for short-term gene delivery and are highly targeted for tissue engineering strategies that desire protein production over the course of several weeks. Efficient adenovirus-directed gene delivery to odontogenic mesenchymal cells derived from cranial neural crest cells was reported [85,86]. In addition, because the adenovirus is well-known as the "virus of the common cold," infection is generally nontoxic and self-limiting. However, determination of the genotoxicity for each specific application is necessary to keep the safety profile within acceptable parameters. Adenoassociated viruses have become the focus of much research in recent years because of their complete inability to replicate without a helper virus, potential for tissue-specific targeting, and gene expression in the order of months to years. The ability to specifically target one tissue type without adverse effects on neighboring tissues is highly desired in fields such as tooth regeneration. On the other hand, nonviral methods are safe and do not require immunosuppression for successful gene delivery, but suffer from lower transfection efficiencies. DNA injection followed by application of electric fields (electroporation) has been more effective for introducing DNA than the use of simple DNA injection [87]. However, this method involves the concern that the electric pulse causes tissue damage. Recently, we reported that gene transfer using an ultra-fine needle [88], in addition to microbubbles enhanced transcuteaneous sonoporation [87].

In vivo gene delivery seems to be a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition.

6. Conclusion

We have a chance to access the formation of the third dentition in the mouth, because the timing of the appearance of the third dentition seems to be after birth. The identification of mutations in *RUNX2* causing an isolated dental phenotype in CCD and supernumerary tooth formation in the mouse model clearly demonstrated that it was possible to induce *de novo* tooth formation by the *in situ* repression or activation of a single candidate gene. These results support the idea that the *de novo* repression or activation of candidate genes such as *RUNX2* or *USAG-1* might be used to stimulate the third dentition in order to induce new tooth formation in the mouse (Figure 4). *In vivo* gene delivery seems to be a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition.

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