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REVIEW

The application of bone morphogenetic proteins to dental tissue engineering

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Progress in understanding the role of bone morphogenetic proteins (BMPs) in craniofacial and tooth development, the demonstration of stem cells in dental pulp and accumulating knowledge on biomaterial scaffolds have set the stage for tissue engineering and regenerative therapy of the craniofacial complex. Furthermore, the recent approval by the US Food and Drug Administration (FDA; Rockville, MD, USA) of recombinant human BMPs for accelerating bone fusion in slow-healing fractures indicates that this protein family may prove useful in designing regenerative treatments in dental applications. In the near term, these advances are likely to be applied to endodontics and periodontal surgery; ultimately, they may facilitate approaches to regenerating whole teeth for use in tooth replacement.

The craniofacial complex includes the teeth, periodontium, bones, salivary glands and the temporomandibular joint (see **Box 1**, for glossary). The homeostasis and maintenance of teeth, together with the periodontal structures that anchor them into the alveolar bones of the jaws, deteriorate progressively both with age and with metabolic maladies such as diabetes and osteoporosis. According to the US National Institute of Dental and Craniofacial Research (Bethesda, MD, USA), 86% of adults over 70 years have at least moderate periodontitis and over a quarter have lost their teeth. In the aged, therefore, the decline in tooth function has serious repercussions for health and quality of life.

Tissue engineering offers a new option to supplement existing treatment regimens for periodontal disease. Moreover, compared with other parts of the body, the oral cavity offers distinct advantages to the tissue engineer, such as ease of observation and accessibility. Realizing the potential of regenerative treatments for the craniofacial complex will require integration of three key elements: inductive morphogenetic signals (morphogens), responding progenitor/stem cells and the extracellular matrix scaffold. To induce morphogenesis of the teeth and periodontium, these elements must be combined to facilitate a developmental cascade of pattern formation, craniofacial plan establishment and the creation of the mirror-image bilateral symmetry of teeth in maxilla and mandible. Regeneration of craniofacial tissues will also require a recapitulation of some of the mechanisms of embryonic development and morphogenesis.

Morphogens are extracellularly secreted signals governing morphogenesis during epithelial-mesenchymal interactions. They comprise four evolutionarily conserved protein families—BMPs, fibroblast growth factors (FGFs), hedgehog proteins (Hhs) and wingless- and int-related proteins (Wnts). These protein families exhibit redundant and reiterative signaling, each with distinct temporal and spatial expression during initiation, pattern formation, morphogenesis and cytodifferentiation. In the craniofacial complex, they govern patterning and morphogenesis of teeth and associated periodontal structures, including alveolar bone, cementum, periodontal ligament (PDL) and gingiva. As yet, it is unclear how the same set of morphogen families that specify the shape of teeth also confer the discrete morphologies of incisors, canines, premolars and molars. However, the promise of oral tissue engineering is that applications of recombinant morphogens, regenerative progenitor/stem cells in appropriate scaffolds and ultimately gene therapies (**Box 2**) will enable new dental treatments for caries, endodontic, periodontal and oral-maxillofacial surgery, alveolar ridge augmentation and cartilage repair in the temporomandibular joint.

Although four distinct families of morphogens are involved in embryonic craniofacial morphogenesis, BMPs alone appear to be sufficient for regeneration of dental tissues in adults as discussed in this article. For this reason, we limit the focus of the present review to the potential use of BMPs and progenitor/stem cells in dental tissue engineering. In the following sections, we describe what is known about tooth morphogenesis and then discuss the mechanisms by which BMPs modulate stem cell biology, how the extracellular environment influences morphogen action, and the integration of these components into tissue engineering approaches.

Tooth morphogenesis

Tooth morphogenesis is initiated by dynamic, reciprocal interactions between ectodermally derived stomodeal epithelium and mesenchyme derived from the neural crest (see **Fig. 1**). This process commences with the thickening of the oral epithelium and the formation of the dental lamina, which then buds into the mesenchyme. The initiation of tooth formation begins with invagination of the epithelium into the neural crest–derived ectomesenchyme. In the subsequent 'cap' and 'bell' stages, the tooth crown is established by folding of the epithelium. The mesenchyme, which is surrounded by dental epithelium, forms the dental papilla, which gives rise to tooth pulp and odontoblasts, which form dentin. The peripheral cells of the dental mes-

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Box 1 Glossary

Alveolar bone: The bone in the upper and lower jaws.
Ameloblast: An epithelial cell involved in enamel biosynthesis.
Canine tooth: The two teeth in each jaw placed bilaterally lateral to second incisor.
Cementum: The bone-like mineralized tissue surrounding the root of teeth.
Debridement: Removal of contaminating foreign tissue adjacent to normal tissue in an inflamed or infected wound.
Dental papilla: Condensed mesenchyme that gives rise to the tooth.
Dentin: Dense mineralized extracellular matrix of teeth produced by odontoblasts.
Enamel: A dense white mineralized matrix composite covering the dentin of the crown of teeth.
Enamel knot: The signaling center for tooth morphogenesis in the enamel organ epithelium.
Endodontics: The branch of dentistry dealing with diseases and injury to tooth pulp, root and periapical tissues.
Exfoliated/deciduous: Natural shedding of teeth often referred to as 'baby' or 'milk' teeth.
Furcation: The division of roots in a multiroot tooth.
Gingiva: The gum of the mouth, consisting of connective tissue that overlies the crowns of unerupted teeth and surrounds the necks of erupted teeth.
Incisor teeth: The four front teeth on each jaw consisting of two on either side of the midline.
Mandible: The bone of the lower jaw.
Maxilla: The bone of the upper jaw.
Molar teeth: Six teeth placed bilaterally on either side of the upper and lower jaw lateral to premolars.
Occlusal: The masticating surfaces of premolars and molars and the hypothetical plane between and contacting teeth in upper and lower jaws.
Odontoblast: Cell involved in dentin matrix deposition.
Osteodentin: Rapidly formed secondary dentin with entrapped cells resembling bone and lacking tubules characteristic of dentin.
Periodontium: The connective tissues surrounding and supporting the teeth in the jaws, including cementum, PDL, alveolar bone and gingiva.
Periosteum: Connective tissue surrounding all the bones, including the jaws.
Premolar teeth: Four teeth placed bilaterally on either side of the upper and lower jaws lateral to canines.
Reparative dentin: Dentin that lacks dentinal tubules, produced by dental pulp in response to injury. Also called tertiary dentin.
Rests of Malassez: An epithelial remnant of the sheath in periodontal membrane that sometimes develops into a dental cyst.
Sharpey's fibers: Principal fibers inserting into the cementum in periodontium.
Stomodeal: Pertaining to stomodeum, an invagination of the ectoderm of embryos where future mouth is formed.
Tubular dentin: Native primary dentin with branching spiral tubules produced by odontoblastic cell processes.

enchyme comprise the dental follicle and extend around the dental epithelium forming the enamel organ. Dentin-forming odontoblasts and enamel-forming ameloblasts differentiate during the bell stage of development. Finally, the teeth erupt into the oral cavity after crown morphogenesis is complete[1] (**Fig. 2**).

The enamel knot, an organizing center for tooth morphogenesis, which regulates pattern formation and teeth shape, forms in the enamel organ epithelium, a zone devoid of cell proliferation that appears transiently at the bud stage. Before the bud stage, the presumptive dental epithelium regulates tooth development. During budding, the instructive cues for tooth morphogenesis originate in the mesenchyme, which also provides instructive cues for ameloblast differentiation and enamel secretion[2].

During the development and morphogenesis of the teeth, the periodontium orchestrates the anchoring of teeth in alveolar bone. As the name implies, the periodontium forms a sheath around the tooth and consists of the PDL, cementum and the alveolar bone. The PDL arises from the inner layer of the dental follicle after the initiation of root development[2]. The migration of dental follicle cells to dentin leads to cementoblast differentiation. Cell proliferation and the differentiation of the PDL leads to formation of collagen fibrils anchoring the cementum. Epithelial-mesenchymal interactions are critical for PDL formation[3] and are accompanied by expression of the genes encoding cell surface proteoglycans, extracellular matrix glycoproteins, transforming growth factor (TGF) β-1 and β-2, and Msx-like homeoproteins. Fibronectin has been implicated in the migration of mesenchymal cells into the spaces between epithelial cells and their differentiation into fibroblasts of the PDL, and in the precise anchoring of PDL fibrils to the root surface[3,4].

During dental epithelial-mesenchyme development, BMPs, FGFs, Hhs and Wnts are expressed in distinct intracellular signaling cascades. BMPs and FGFs upregulate the expression of many genes and induce proliferation in the mesenchyme. Wnt family members, of which, at last count, there were nearly 20 in humans, signal via the frizzled family of cell surface receptors. In the absence of Wnt signaling, glycogen synthase kinase 3 (GSK-3) phosphorylates β-catenin and targets it for degradation by proteosomes. When Wnt activates the frizzled receptor, GSK-3 activity is blocked and therefore β-catenin is stable and is translocated to the nucleus where it binds to the lymphoid enhancer factor (LEF)/T-cell transcription factor, activating the transcription of Wnt responsive target genes. Sonic hedgehog (Shh), which is critical for regulating growth and shaping teeth, binds to the Patched receptor, as well as the transmembrane protein, Smoothened. This signal activates transcription factors of the Gli family, which are critical for craniofacial and tooth morphogenesis. Members of the Wnt and Shh families are mutually inhibitory—Wnts repress Shh expression, whereas Shh represses Wnt signaling.

Such reciprocal regulatory interactions are critical in establishing the distinct boundaries between oral and dental ectoderm during tooth development and morphogenesis[4]. The pathways, however, are integrated at different levels in developing teeth, as members of different families regulate some of the same targets. For example, both BMP and FGF activate the expression of transcription factors Msx1 and Dlx2, and BMP competes with Wnt in Lef 1 expression[4]. It is

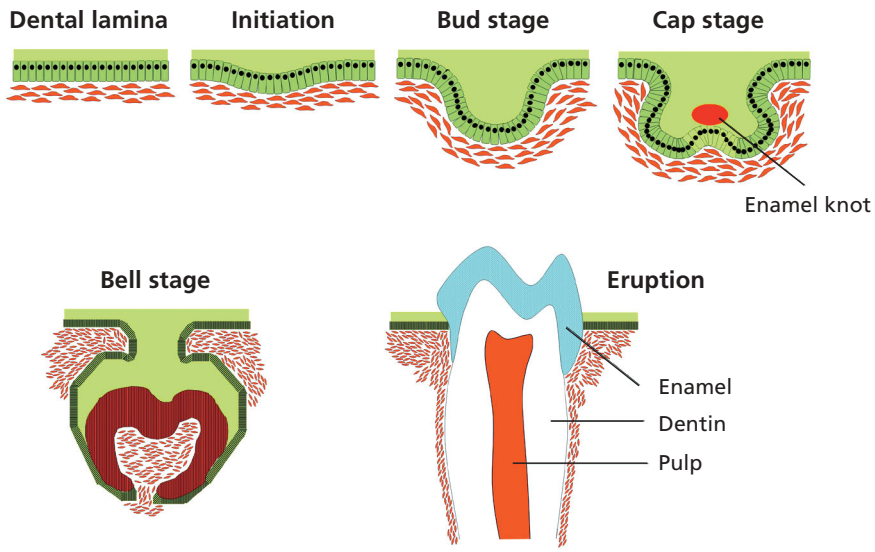

Figure 1 Reciprocal and reiterative signaling during tooth morphogenesis. Intricate, dynamic and reciprocal interactions occur between ectodermally derived enamel organ epithelium and neural crest–derived mesenchyme. Four key families of morphogens (BMPs, FGFs, Shh and Wnts) are involved in signaling between the epithelium and mesenchyme in an orchestrated spatial and temporal sequence. The tooth bud develops into the cap and bell stages before eruption. The enamel knot is a signaling center with a critical role in tooth morphogenesis.

noteworthy that the same families of morphogens are operative in discrete morphology of mammalian teeth[5]. Although all four families of morphogens are critical for embryonic tooth morphogenesis, in the adult, the BMPs alone can direct the regeneration and maintenance of dental tissues.

Bone morphogenetic proteins

Bone grafts have been used for over a century by orthopedic surgeons to aid recalcitrant bone unions. In 1965, Urist[6] made the key discovery that demineralized, lyophilized segments of rabbit bone induce new bone in intramuscular sites. Subsequently, BMPs were isolated from adult bone matrix in mammals by extracting demineralized bone matrix in 4M guanidine, heparin affinity chromatography and preparative electrophoresis, and the activity of reconstituted purified BMPs assayed by implantation with insoluble collagenous bone matrix *in vivo* (for a review, see ref. 7). The resultant stages of new bone development are reminiscent of embryonic bone morphogenesis, complete with the three key steps: chemotaxis, mitosis and differentiation of progenitor/stem cells.

The human genome encodes 20 BMPs[7]. BMPs are dimeric molecules critically dependent on the single intermolecular disulfide bond for biological activity. The monomeric subunit has about 120 amino acids, including seven conserved cysteine residues. The BMP family can be divided into four distinct subfamilies: first, BMP2 and 4; second, BMP3 and BMP3B, the latter also known as growth/differentiation factor 10 (GDF10); third, BMPs 5, 6, 7 and 8; and fourth, GDFs 5, 6 and 7, also known as cartilage-derived morphogenetic proteins 1, 2 and 3. BMP1 is not a member of the BMP family but rather is a procollagen C-proteinase involved in the proteolytic processing of soluble procollagen, leading to the self-assembly of insoluble collagen fibers in the extracellular matrix.

A single recombinant BMP can have pleiotropic effects on different steps in bone morphogenesis depending on its concentration: femtomolar concentrations promote chemotaxis; higher concentrations promote mitogenesis and differentiation[7]. The BMPs play pivotal roles in the development of the brain, eyes, heart, kidneys, skin, bones and teeth. Their actions include the establishment of body plan; initiation and maintenance during regeneration of bone, the formation of skeletal tissue during embryogenesis, growth and remodeling, and the induction and creation of new bone (**Fig. 2**). In the post-natal skeleton, BMPs are intimately associated with the collagenous extracellular matrix and are localized in periosteal cells and in mesenchymal cells of marrow stroma during fracture repair[8–10].

BMPs also play a critical role in tooth morphogenesis[1]. In craniofacial development, they have been implicated in the inductive interactions between dental epithelium and mesenchyme in a stage-dependent, reiterative manner. BMPs 2, 4 and 7 are expressed in dental epithelium, and recombinant BMPs 2 and 4 can be used as a substitute for dental epithelium in inducing mesenchyme differentiation. BMPs are also expressed in the enamel knot and are associated with differentiating odontoblasts and ameloblasts. BMP3 and BMP7 have been immunolocalized to developing PDL, cementum and alveolar bone[11,12]. On the other hand, BMP2 was localized only in alveolar bone during root morphogenesis[12]. A role for BMP3 in the cementoblast lineage has been suggested by its localization in the root-lining cells[13]. One of us (M. Nakashima) has also observed expression of members of the BMP family in rat incisor dental pulp during different stages of odontoblast development[14].

A comparison of other growth factors reveals that BMPs are (34–38%) related to the TGFβ family. BMPs and TGFβ ligands have cognate BMP type I and II receptors and TGFβ type I and II receptors, respectively, which function as protein kinases (**Fig. 3**). The BMP type I receptor protein kinase phosphorylates intracellular signaling substrates Smads (a fusion of the Sma gene in *C. elegans* and Mad gene in *Drosophila*) 1, 5 and 8. The TGFβ type I receptor kinase specifically phosphorylates Smads 2 and 3. The phosphorylated Smads 1, 5 and 8 (BMP-signaling Smads) and Smads 2 and 3 (TGFβ-signaling Smads) partner with Smad 4 to form signaling complexes in the cytosol that enter the nucleus to initiate the transcription of downstream targets (**Fig. 3**). Although BMPs and TGFβ ligands signal via distinct receptors, they act in collaboration during bone and tooth morphogenesis[4,53].

Progenitor/stem cells

Several studies indicate that progenitor/stem cells are present both in dental pulp and in the PDL. During wound healing, dental pulp cells have the potential to proliferate and to differentiate into odontoblasts to form dentin. Odontoblast formation has been studied in cultures of mature bovine, human and rodent pulp cells as well as in thick slices of human and rodent teeth[15]. It is accompanied by the expression of dentin matrix protein 1, dentin sialoprotein (Dsp) and dentin phosphoprotein (Dpp)[15]. In addition, both increased expression of osteocalcin (a marker for differentiated odontoblasts) and matrix mineralization occurs during the culture period[16–19]. When transplanted into ectopic sites in immunocompromised mice, cells isolated from human dental pulp give rise to dentin-like structures

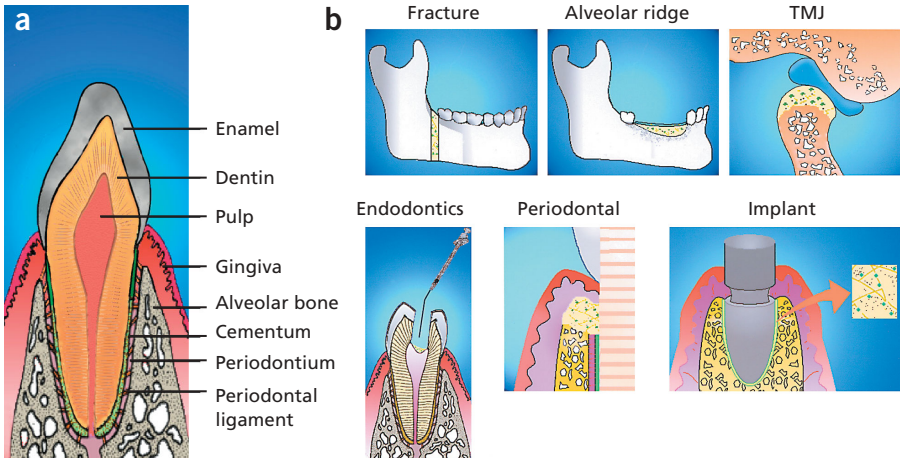

Figure 2 Craniofacial structures and treatments. (**a**) Structure of a human tooth. The tooth is surrounded by the periodontium and is anchored in the alveolar bone of the maxilla or mandible. The pulp has progenitor/stem cells for the repair and regeneration of dentin by dentin-forming odontoblasts in response to BMPs. (**b**) BMPs have been demonstrated in preclinical studies to have the potential for tissue engineering and regeneration of alveolar bone, alveolar ridge augmentation, cartilage repair in the temporomandibular joint (TMJ), oral implants, endodontic treatment and regeneration of periodontal tissues.

lined with odontoblast-like cells[20]. A recent paper also reports the presence of stem cells in exfoliated deciduous human teeth[21]. Together, these results provide evidence that the pulp contains pulp progenitor/stem cells.

Although specific markers for the dental pulp stem cells have yet to be characterized, cultured pulp cells express BMP2, BMP4, BMP6 and BMP7 mRNA[16,22,23] and their receptors[24] in a stage-dependent manner. In response to treatment with recombinant human BMP2, pulp-derived mesenchymal cells differentiate into dentin-forming odontoblasts[16]. When combined with total EDTA-soluble fraction of dentin, recombinant human BMP2 also stimulates odontoblast differentiation by dental papilla separate from that in the enamel organ[25].

Some progress has also been made in understanding the role of progenitor cells in repair of the PDL, which responds to changes in applied mechanical force and has a remarkable capacity for regeneration. In a normal PDL, several types of osteoblast and progenitor cell associated with bone formation have been identified, including fibroblasts, rests of Malassez (an epithelial remnant), and undifferentiated mesenchymal cells. In addition, cementoblasts have been found lining the root surface. Although the origin of cementoblasts that appear during periodontal regeneration is unknown, cells derived from the PDL and alveolar bone may migrate into the periodontal defect, proliferate and differentiate[26]. It has also been suggested that a small population of PDL cells in the mature periodontium can differentiate into an osteoblast or cementoblast phenotype, thereby laying down bone or cementum[13]. Other likely sources of cementoblasts or osteoblast progenitor cells include marrow stroma, perivascular, paravascular and endosteal fibroblasts[13].

An ongoing challenge for the field is to isolate progenitor stem cells with well-defined markers from both dental pulp tissue and PDL and to expand these cell types *in vitro*. Current research in several centers is focusing on the isolation of universal human stem cells with defined markers that potentially could be used to circumvent immune histocompatibility barriers. Such cells ultimately may allow the development of stem cells for use as shuttle vectors in cellular/gene therapy (see **Box 2**).

Extracellular matrix and biomimetic scaffolds

Tissue engineering of teeth and associated tissues requires that the scaffolding of the extracellular matrix be faithfully duplicated. Both developing and adult teeth are surrounded by the PDL and anchored to alveolar bone by cementum. These connective tissues have in common an extracellular matrix that includes collagens, proteoglycans and an assortment of noncollagenous glycoproteins. Studies in one of our

Box 2 Gene therapy and tooth repair

Although gene therapy in oral medicine remains some way off, the approach has clear advantages. First, because the half-life of BMPs is limiting and high concentrations are required to induce tissue regeneration, morphogen production from transduced tissues, rather than by direct application of the protein, may be advantageous. Several studies have already detailed the ability of rodent or human mesenchymal cells, transduced with a recombinant adenoviral vector carrying the gene for BMP2 (refs. 67–69) or BMP7 (refs. 70,71), to elicit new bone formation *in vitro* and *in vivo*. Ligands have also been incorporated into the surface of viral vectors to enable cell-specific delivery, which is both better targeted and less invasive than other methods, such as direct injection[72]. Second, it is clear that the ease of visual observation and accessibility of the mouth/oral cavity makes these tissues more amenable to gene delivery approaches than internal visceral organs and tissues. Thus, delivery methods such as electroporation or sonoporation have been used to transfer the *Gdf11* gene (which encodes BMP 11) to amputated dental pulp, stimulating the formation of reparative dentin[17,73]. An alternative approach is to use electroporation or sonoporation to transfect isolated progenitor/stem cells from dental pulp with BMP genes and subsequently implant the engineered cultured cells with an appropriate scaffold into the injured pulp. This *ex vivo* approach to gene transfer may stimulate reparative dentin formation more rapidly. In a recent paper, syngeneic dermal fibroblasts were transduced *ex vivo* with adenoviruses encoding BMP7, subsequently seeded onto gelatin carriers, and then transplanted into rats with large mandibular alveolar bone defects. The lesions treated with BMP7 demonstrated rapid chrondrogenesis, with subsequent osteogenesis, cementogenesis and predictable bridging of the periodontal bone defects[74]. Recent data indicate that ultrasound may have marked effects on the growth and function of bone and PDL cells *in vitro*[75].

laboratories (A.H. Reddi) have shown the critical role of the extracellular matrix substrata in bone induction by BMPs[27–29], particularly in craniofacial morphogenesis and regeneration, which is contact-mediated and occurs over a short range[30,31].

Recombinant BMP4 binds to heparan sulfate, heparin, and types I and IV collagen of the extracellular matrix. Thus, extracellular matrix components tether active morphogens to confer the optimal conformation and perhaps protect them from proteolysis[30]. Furthermore, binding soluble BMP4 to an extracellular matrix component converts the morphogen into a solid state, which then can initiate contact-mediated interactions. The interaction between the inductive signaling by morphogens and responding cells is modulated by the extracellular matrix. BMPs bind to collagens I and IV, type II procollagen, heparan sulfate, fibrillins, proteoglycans and the BMP-binding proteins noggin, chordin and DAN[31]. Recently, the BMP antagonist noggin has been demonstrated to interact with heparan sulfate and heparin in extracellular matrix[32].

The rate of release of growth factor from the scaffold can profoundly affect the results of tissue engineering strategies[33]. Polylactic-glycolic acid and polyethylene glycol copolymers, which are commonly used in implantable devices, are effective in releasing growth factors and thus are useful in BMP delivery. The pharmacokinetics of the recombinant BMP in conjunction with biomaterials, such as collagen and hydroxyapatite, may be different from the kinetics in alveolar bone periodontium and teeth because retention of BMPs at the site of implantation is dependent on the charge characteristics and isoelectric point of the morphogens[34].

The mineral phase in alveolar bone, cementum and teeth is carbonate-rich hydroxyapatite. The affinity of proteins for hydroxyapatite makes it a natural protein delivery system. A comparison of potential carriers of BMPs—including collagens, hydroxyapatite, tricalcium phosphate, glass beads and polymethylmethacrylate, a bone cement—has revealed that insoluble collagen and hydroxyapatite are optimal for bone induction[7]. However, in the case of hydroxyapatite, geometry of the scaffold is important. Discs of hydroxyapatite with BMPs are osteo-inductive, whereas granules are consistently feeble in bone induction, even though their chemical composition and pore sizes are identical[35,36]. The cellular and molecular mechanisms underlying this critical role of geometry of the scaffold with identical chemistry is unclear and requires further investigation.

Demineralized tooth matrix can also elicit new bone formation. Work in one of our laboratories (A.H. Reddi) has shown that the geometry of the demineralized tooth matrix derived from the lower incisors of rats influences the induced phenotype[37]. When whole demineralized incisors are implanted subcutaneously, cartilage forms in the pulp chamber and persists. However, when the apical portion of the tooth is amputated and the resulting tooth tubes are implanted, cartilage formation is transient in the middle of the tube and is replaced completely by bone. In this instance, angiogenesis and vascular invasion from both sides of the tooth tube result in bone formation throughout[37]. These experiments demonstrate the critical role of geometry of the matrix scaffold in determining developmental outcome.

The methods and the dose of irradiation used to sterilize the scaffolds of demineralized bone matrix for tissue engineering of bone are likely to have a profound effect on the outcome. One of us (A.H. Reddi) has also published work demonstrating that irradiation by a Cobalt 60 source at doses exceeding five mega RADs (radiation absorbed dose) may lead to premature mineralization of the implanted matrix[38]. It is noteworthy that endogenous BMPs in the demineralized bone matrix retain their biological activity after irradiation of up to 5 mega RADs.

The extracellular matrix in alveolar bone, periodontium and teeth releases morphogens locally to elicit bone repair. In this process, the tissue scaffolding, such as collagen, is crucial in healing segmental defects of long bones and alveolar bone in mandible and maxilla. Essentially, it functions as a delivery system and as an osteoconductive substratum. The surface geometry, the porosity, pore size and bulk properties are critical. The triad of morphogens, stem cells and scaffolds is thus a prerequisite for tissue engineering (**Fig. 4**).

Regeneration of the dentin-pulp complex

The dentin-pulp complex protects teeth from caries and trauma by maintaining the hydration of extracellular matrix. The goal of endodontics and conservative dentistry is to restore or regenerate the dentin-pulp complex to maintain the vitality and function (and aesthetics) of teeth[15,39,40]. Given the role of BMP4 in inductive epithelial-mesenchymal interactions during tooth development[41], and the known effects of demineralized tooth matrix on repair of amputated pulp (see M. Nakashima, ref. 42), BMPs might have a therapeutic role in endodontics.

The ideal therapy for repairing pulp would be anti-inflammatory and antibacterial, would stimulate proliferation of pulp stem cells and would induce their differentiation into odontoblasts to enhance healing potential and rapid dentin formation. Using an *in vivo* model, one

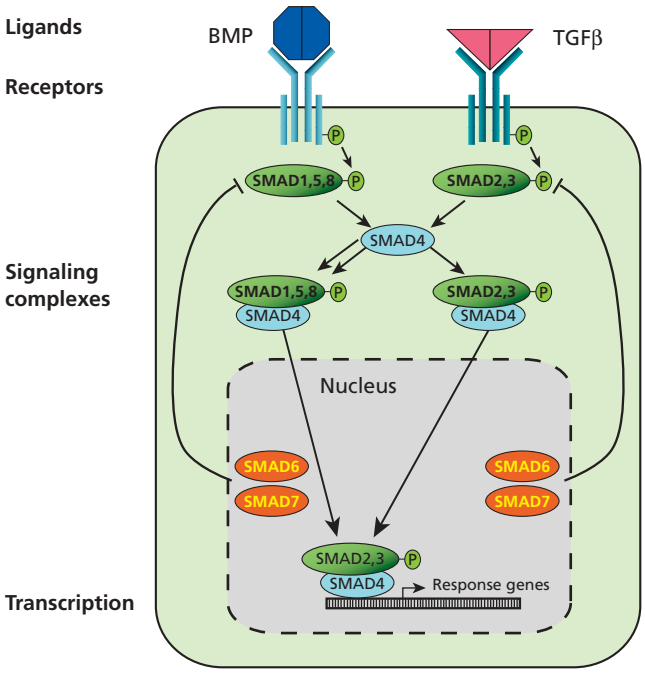

Figure 3 BMP and TGFβ signaling ligands, receptors and transcription. BMPs are dimeric ligands (with a cysteine knot in each monomer fold) that interact with both type I and II BMP receptors (BMPR-I and -II). In the signaling cascade, BMPR-II phosphorylates the Gs domain of BMPR-I. The collaboration between type I and II receptors forms the signal-transducing complex. BMP type I receptor kinase complex phosphorylates the trimeric signaling substrates Smad 1 or Smad 5 or Smad 8. TGFβ isoforms interact with TGFβ receptors that are distinct from BMP receptors. TGFβ receptor kinase phosphorylates Smad 2 or 3. This phosphorylation is inhibited and modulated by inhibitory Smads 6 and 7. Phosphorylated Smad 1, 5 or Smad 8 interacts with Smad 4 and enters the nucleus to activate the transcriptional machinery for early BMP-response genes. Phosphorylated Smad 2 or 3 partners with common co-Smad 4 and enters the nucleus to activate TGFβ responsive genes.

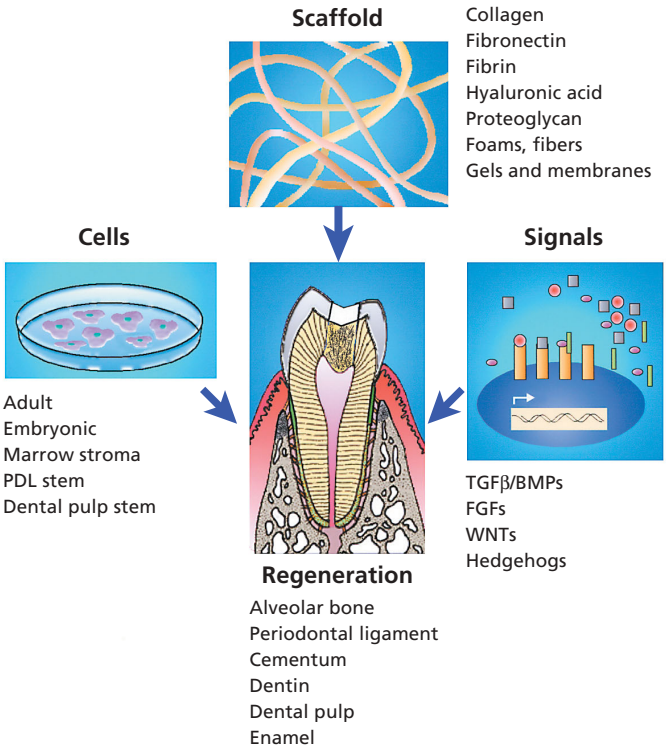

Figure 4 The three key elements for dental tissue engineering are signals for morphogenesis, progenitor/stem cells, and scaffolds of extracellular matrix components. The key morphogenetic signaling families are BMPs, FGFs, Shh and Wnts. The progenitor/stem cells include cells derived from marrow, dental pulp and PDL-derived cells. The extracellular matrix scaffold consists of collagens, fibronectin and proteoglycans, including hyaluronic acid. Synthetic foams, fibers, gels and membranes can be incorporated with biomimetic biomaterials. The triad of signals, stem cells and scaffolds can be used for regeneration of bone, PDL, cementum and dentin.

of us (M. Nakashima) has demonstrated that recombinant human BMP2 and BMP4 can induce new dentin[40]. Recombinant BMP delivered in a scaffold of demineralized dentin matrix induces classic tubular dentin (as in teeth) in amputated pulp, whereas BMP delivered using reconstituted type I collagen matrix induces instead osteodentin[42]. Reparative dentin is also induced on freshly cut healthy pulp tissue in nonhuman primate using recombinant human BMP7 with an insoluble type I collagen matrix[43,44]. The size and shape of the inductive material controls the size and shape of the reparative dentin[43]. The reparative dentin appears initially with cellular and soft tissue inclusions, a portion of which (comprising only about 20% of the reparative dentin) subsequently changes into a more tubular form of matrix with associated odontoblast-like cells attached to the mass of atubular matrix[44]. Therefore, the extracellular matrix scaffolding is a critical component and a prerequisite to odontoblast differentiation and tubular dentin formation[42,45]. Optimally engineered reparative dentin may consist of a combination of osteodentin and tubular dentin[46]. However, tubular dentin is critical for tooth function, including resistance to caries.

Periodontal regeneration

The periodontium, which consists of cementum, PDL and alveolar bone, functions to anchor the teeth to the jaws (mandible and maxilla). A critical challenge for clinical dentistry is the application of tissue engineering concepts to obtain total regeneration of the periodontium, deterioration of which during periodontal disease can ultimately lead to the loss of teeth. Although advances in the treatment of gingivitis have made the restoration of gingiva a reality, restoring alveolar bone and cementum is currently more problematic.

The ultimate goal of periodontal regeneration is the restoration of functional anchorage of teeth by the following sequential steps: restoration of PDL, including optimal orientation and insertion of Sharpey's fibers into exposed root surfaces and bone; formation of new cementum by cementoblasts on the surface of root; and finally restoration of alveolar bone to the cementum-enamel junction. Early approaches included curettage, open-flap debridement and bone grafts. However, the results have been variable and unpredictable because of the paucity of progenitor cells and the presence of microbes in the periodontal environment[47]. In addition, the periodontium is enriched in matrix metalloproteinases and may be detrimental to the inductive signals, including morphogens and growth factors, responding stem cells and scaffolds.

The migration of gingival epithelium onto the root surface is inhibitory to periodontal regeneration. The technique of guided tissue regeneration, in which a membrane barrier is inserted between the periodontal flap and root surface, has been key in preventing epithelial migration[48]. However, as in any surgical procedure, in some patients the outcome is not optimal. This may be improved by combining osteo-inductive bone grafts with a membrane barrier. Thus, further improvements in periodontal regeneration may be achieved through the use of BMPs alone or in combination with membrane barricades to prevent gingival epithelial migration.

In fact, the morphogenetic potential of BMPs makes them ideal candidates for use in periodontal regeneration. Optimizing the response of stem cells to BMP induction requires the use of a delivery system that is conducive to the migration and attachment of the responding stem cells onto the scaffolding. Using a baboon model, recombinant BMP7 and baboon type I collagen has been used as a biomimetic scaffold to regenerate surgically created furcation defects in molars. In this study, researchers reported the formation of alveolar bone and the creation of cementum and Sharpey's fibers, inserted at the optimal orientation into the root surface[49,50]. Similar results have been reported using recombinant human BMP2 and synthetic bioresorbable particles with autologous blood[51] to bind the particles (possibly owing to fibronectins) along with a gelatin and polylactic acid–polyglycolic acid copolymer scaffold[52]. More recently, periodontitis (induced by *Pseudomonas gingivalis*) in the molars of baboons has been shown to be treatable through the delivery of recombinant BMP7 via insoluble type I collagen, which restores the alveolar bone, cementum and the PDL[53]. Further work is in progress to optimize the dose of BMP7 to avoid solid fixation of teeth by fusion of cementum and alveolar bone[49].

The healing of a periodontal wound is complicated by several factors that limit predictable delivery of agents to the root surface. These include the perimucosal environment of the avascular mineralized tooth surface traversing the gingival soft tissue and a complex microbiota, which contaminates wounds at the soft-hard tissue interface and may affect release kinetics of delivered cells or molecules. Thus, several factors, including signaling molecules, cells, scaffolds, vasculature and microflora dictate the degree of periodontal regeneration achieved[47,54]. The use of morphogens and tissue engineering obviate donor site morbidity and variable osteoinduction typical of treatments using commercial demineralized bone matrix.

Craniofacial regeneration and implants

The remarkable capacity of BMPs to induce bone has set the stage for their application in craniofacial surgery for correcting acquired or

inherited craniofacial anomalies, the effects of head trauma, and to treat large bone defects after excision of neoplasms. As yet, there have been only a few studies in which BMPs have been applied to facilitate craniofacial regeneration.

Administration of a single recombinant BMP, such as BMP2, 4 or 7, can initiate the entire cascade of osteogenesis[7]. BMPs initiate the prompt regeneration of experimentally induced (critical size) defects of calvarium in rodents and primates at a dose of 100 μg/g matrix[55]. In systematic studies of baboons with critical craniofacial defects of 2.5 cm, dose-dependent regeneration was obtained using BMP-7 (ref. 49). However, when the concentration of BMP7 was increased to 1,000 μg/g of collagen carrier, a massive increase in turnover of bone was observed, with numerous osteoclasts. Thus, BMPs, when used in supraphysiological doses, can stimulate bone resorption and turnover, eliciting a counterproductive response rather than achieving the intended objective of bone formation. BMPs have also been used for repair of maxillary sinus and filling of regenerate tissue in extraction sockets[56–58].

Metallic implants might also benefit from combined use with BMPs. For an implant to be placed correctly, it must be stably anchored in alveolar bone. In certain instances, where an implant can be stabilized in native bone (but parts may not be completely covered by bone tissue), an oral surgeon can use a bone graft. BMPs may be used to stimulate bone growth in and around metallic implants to attain optimal integration of dental implants[59–65] in the jaw bone. BMPs could offset loosening of teeth caused by micromotion, thermal or pressure necrosis and/or osteolysis. Two studies have demonstrated the host-tissue response to be dependent on the dose of the BMPs and the collagen carrier[49,66].

Conclusions

Although our understanding of the molecular pathways underlying tooth morphogenesis and regeneration is increasing, the translation of this knowledge into dental tissue engineering strategies remains in its early stages. Despite an increasingly detailed picture of the complex and redundant cascades of signaling pathways underlying initiation, pattern formation, morphogenesis and cytodifferentiation, it remains unclear how these pathways specify the discrete tooth morphologies: incisors, canines, premolars and molars. Thus, the development of a whole, tissue-engineered tooth lies some way off. Clearly, further work is needed to characterize the progenitor/stem cell populations involved in the development of dental tissues, to map their differentiation pathways, and to determine how local conditions (*e.g.*, three-dimensional biomimetic scaffolds, presence of microflora, timing and concentrations of morphogens) affect developmental outcome. In the near term, it is likely that recombinant protein morphogens recently approved by the FDA, such as BMP2 and BMP7, could be applied in dentistry to facilitate the repair of craniofacial structures. Suitable scaffolds for endodontic treatment and craniofacial surgery are available now as well. Looking ahead, further characterization and analysis of dental pulp stem cells may permit new types of endodontic therapy (either alone or in combination with BMPs) and the refinement of biomimetic scaffolds may facilitate novel approaches to periodontal surgery.

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1. Thesleff, I. & Sharpe, P. Signalling networks regulating dental development. *Mech. Dev.* **67**, 111–123 (1997).
2. Chai, Y. & Slavkin, H.C. Prospects for tooth regeneration in the 21st century: a perspective. *Microsc. Res. Tech.* **60**, 469–479 (2003).
3. Cho, M.I. & Garant, P.R. Ultrastructural evidence of directed cell migration during initial cementoblast differentiation in root formation. *J. Periodontal Res.* **23**, 268–276 (1988).
4. Jernvall, J. & Thesleff, I. Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* **92**, 19–29 (2000).
5. Salazar-Ciudad, I. & Jernvall, J. A gene network model accounting for development and evolution of mammalian teeth. *Proc. Natl. Acad. Sci. USA* **99**, 8116–8120 (2002).
6. Urist, M.R. Bone: formation by autoinduction. *Science* **150**, 893–899 (1965).
7. Reddi, A.H. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat. Biotechnol.* **16**, 247–252 (1998).
8. Nakase, T. *et al.* Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. *J. Bone Miner. Res.* **9**, 651–659 (1994).
9. Bostrom, M.P. Expression of bone morphogenetic proteins in fracture healing. *Clin. Orthop.* S116–S123 (1998).
10. Bostrom, M.P. *et al.* Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. *J. Orthop. Res.* **13**, 357–367 (1995).
11. Helder, M.N. *et al.* Bone morphogenetic protein-7 (osteogenic protein-1, OP-1) and tooth development. *J. Dent. Res.* **77**, 545–554 (1998).
12. Thomadakis, G., Ramoshebi, L.N., Crooks, J., Rueger, D.C. & Ripamonti, U. Immunolocalization of bone morphogenetic protein-2 and -3 and osteogenic protein-1 during murine tooth root morphogenesis and in other craniofacial structures. *Eur. J. Oral Sci.* **107**, 368–377 (1999).
13. Saygin, N.E., Giannobile, W.V. & Somerman, M.J. Molecular and cell biology of cementum. *Periodontol.* **24**, 73–98 (2000).
14. Nakashima, M., Toyono, T., Murakami, T. & Akamine, A. Transforming growth factor-beta superfamily members expressed in rat incisor pulp. *Arch. Oral Biol.* **43**, 745–751 (1998).
15. Tziafas, D., Smith, A.J. & Lesot, H. Designing new treatment strategies in vital pulp therapy. *J Dent.* **28**, 77–92 (2000).
16. Nakashima, M., Nagasawa, H., Yamada, Y. & Reddi, A.H. Regulatory role of transforming growth factor-beta, bone morphogenetic protein-2, and protein-4 on gene expression of extracellular matrix proteins and differentiation of dental pulp cells. *Dev. Biol.* **162**, 18–28 (1994).
17. Nakashima, M., Mizunuma, K., Murakami, T. & Akamine, A. Induction of dental pulp stem cell differentiation into odontoblasts by electroporation-mediated gene delivery of growth/differentiation factor 11 (Gdf11). *Gene Ther.* **9**, 814–818 (2002).
18. Yokose, S. *et al.* Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcif. Tissue Int.* **66**, 139–144 (2000).
19. Couble, M.L. *et al.* Odontoblast differentiation of human dental pulp cells in explant cultures. *Calcif. Tissue Int.* **66**, 129–138 (2000).
20. Gronthos, S., Mankani, M., Brahim, J., Robey, P.G. & Shi, S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **97**, 13625–13630 (2000).
21. Miura, M. *et al.* SHED: stem cells from human exfoliated deciduous teeth. *Proc. Natl. Acad. Sci. USA* **100**, 5807–5812 (2003).
22. Takeda, K. *et al.* Expression of bone morphogenetic protein genes in the human dental pulp cells. *Bone* **15**, 467–470 (1994).
23. Gu, K., Smoke, R.H. & Rutherford, R.B. Expression of genes for bone morphogenetic proteins and receptors in human dental pulp. *Arch. Oral Biol.* **41**, 919–923 (1996).
24. Toyono, T., Nakashima, M., Kuhara, S. & Akamine, A. Temporal changes in expression of transforming growth factor-beta superfamily members and their receptors during bovine preodontoblast differentiation *in vitro*. *Arch. Oral Biol.* **42**, 481–488 (1997).
25. Begue-Kirn, C. *et al.* Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast *in vitro*. *Int. J. Dev. Biol.* **36**, 491–503 (1992).
26. Cho, M.I. & Garant, P.R. Development and general structure of the periodontium. *Periodontol.* **24**, 9–27 (2000).
27. Reddi, A.H. Bone matrix in the solid state: geometric influence on differentiation of fibroblasts. *Adv. Biol. Med. Phys.* **15**, 1–18 (1974).
28. Reddi, A.H. & Huggins, C.B. Cyclic electrochemical inactivation and restoration of competence of bone matrix to transform fibroblasts. *Proc. Natl. Acad. Sci. USA* **71**, 1648–1652 (1974).
29. Sampath, T.K. & Reddi, A.H. Importance of geometry of the extracellular matrix in endochondral bone differentiation. *J. Cell Biol.* **98**, 2192–2197 (1984).
30. Paralkar, V.M., Nandedkar, A.K., Pointer, R.H., Kleinman, H.K. & Reddi, A.H. Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. *J. Biol. Chem.* **265**, 17281–17284 (1990).
31. Reddi, A.H. Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. *Arthritis Res.* **3**, 1–5 (2001).
32. Paine-Saunders, S., Viviano, B.L., Economides, A.N. & Saunders, S. Heparan sulfate proteoglycans retain Noggin at the cell surface: a potential mechanism for shaping bone morphogenetic protein gradients. *J. Biol. Chem.* **277**, 2089–2096 (2002).
33. Lu, L., Yaszemski, M.J. & Mikos, A.G. TGF-beta1 release from biodegradable polymer microparticles: its effects on marrow stromal osteoblast function. *J. Bone Joint Surg. Am.* **83-A**, S82–S91 (2001).
34. Uludag, H., Gao, T., Porter, T.J., Friess, W. & Wozney, J.M. Delivery systems for BMPs: factors contributing to protein retention at an application site. *J. Bone Joint Surg.*

Am. **83-A**, S128–S135 (2001).
35. Ripamonti, U., Ma, S. & Reddi, A.H. The critical role of geometry of porous hydroxyapatite delivery system in induction of bone by osteogenin, a bone morphogenetic protein. *Matrix* **12**, 202–212 (1992).
36. Kuboki, Y., Jin, Q. & Takita, H. Geometry of carriers controlling phenotypic expression in BMP-induced osteogenesis and chondrogenesis. *J. Bone Joint Surg. Am.* **83-A**, S105–S115 (2001).
37. Reddi, A.H. & Huggins, C.B. Influence of geometry of transplanted tooth and bone on transformation of fibroblasts. *Proc. Soc. Exp. Biol. Med.* **143**, 634–637 (1973).
38. Wientroub, S. & Reddi, A.H. Influence of irradiation on the osteoinductive potential of demineralized bone matrix. *Calcif. Tissue Int.* **42**, 255–260 (1988).
39. Nakashima, M. The induction of reparative dentine in the amputated dental pulp of the dog by bone morphogenetic protein. *Arch. Oral Biol.* **35**, 493–497 (1990).
40. Nakashima, M. Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. *J. Dent. Res.* **73**, 1515–1522 (1994).
41. Vainio, S., Karavanova, I., Jowett, A. & Thesleff, I. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**, 45–58 (1993).
42. Nakashima, M. Induction of dentine in amputated pulp of dogs by recombinant human bone morphogenetic proteins-2 and -4 with collagen matrix. *Arch. Oral Biol.* **39**, 1085–1089 (1994).
43. Rutherford, R.B., Wahle, J., Tucker, M., Rueger, D. & Charette, M. Induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. *Arch. Oral Biol.* **38**, 571–576 (1993).
44. Rutherford, R.B., Spangberg, L., Tucker, M., Rueger, D. & Charette, M. The time-course of the induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. *Arch. Oral Biol.* **39**, 833–838 (1994).
45. Veis, A. The role of dental pulp—thoughts on the session on pulp repair processes. *J. Dent. Res.* **64 Spec No**, 552–554 (1985).
46. Rutherford, R.B. in *Principles of Tissue Engineering.* (eds. Lanza, R.P., Langer, R. & Vacanti, J.) 847–853 (Academic Press, San Diego, CA, 2000).
47. Polson, A.M. & Proye, M.P. Fibrin linkage: a precursor for new attachment. *J. Periodontol.* **54**, 141–147 (1983).
48. Gottlow, J., Nyman, S., Lindhe, J., Karring, T. & Wennstrom, J. New attachment formation in the human periodontium by guided tissue regeneration. Case reports. *J. Clin. Periodontol.* **13**, 604–616 (1986).
49. Ripamonti, U. *et al.* Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1 (hOP-1, bone morphogenetic protein-7). *Growth Factors* **13**, 273–289 (1996).
50. Ripamonti, U. & Duneas, N. Tissue morphogenesis and regeneration by bone morphogenetic proteins. *Plast. Reconstr. Surg.* **101**, 227–239 (1998).
51. Sigurdsson, T.J. *et al.* Periodontal repair in dogs: recombinant human bone morphogenetic protein-2 significantly enhances periodontal regeneration. *J. Periodontol.* **66**, 131–138 (1995).
52. Kinoshita, A., Oda, S., Takahashi, K., Yokota, S. & Ishikawa, I. Periodontal regeneration by application of recombinant human bone morphogenetic protein-2 to horizontal circumferential defects created by experimental periodontitis in beagle dogs. *J. Periodontol.* **68**, 103–109 (1997).
53. Ripamonti, U. *et al.* Bone induction by BMPs/OPs and related family members in primates. *J. Bone Joint Surg. Am.* **83-A**, S116–S127 (2001).
54. Ripamonti, U. & Reddi, A.H. Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins. *Crit. Rev. Oral Biol. Med.* **8**, 154–163 (1997).
55. Sun, Y., Ma, G. & Li, D. Repair of large cranial defect using allogeneic cranial bone and bone morphogenetic protein. *Zhonghua Zheng Xing Shao Shang Wai Ke Za Zhi* **11**, 8–9 (1995).
56. Nevins, M., Kirker-Head, C., Wozney, J.A., Palmer, R. & Graham, D. Bone formation in the goat maxillary sinus induced by absorbable collagen sponge implants impregnated with recombinant human bone morphogenetic protein-2. *Int. J. Periodontics Restorative Dent.* **16**, 8–19 (1996).
57. Margolin, M.D. *et al.* Maxillary sinus augmentation in the non-human primate: a comparative radiographic and histologic study between recombinant human osteogenic protein-1 and natural bone mineral. *J. Periodontol.* **69**, 911–919 (1998).
58. Boyne, P.J. Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects. *J. Bone Joint Surg. Am.* **83-A**, S146–S150 (2001).
59. Cook, S.D., Salkeld, S.L. & Rueger, D.C. Evaluation of recombinant human osteogenic protein-1 (rhOP-1) placed with dental implants in fresh extraction sites. *J. Oral Implantol.* **21**, 281–289 (1995).
60. Cochran, D.L. *et al.* Radiographic analysis of regenerated bone around endosseous implants in the canine using recombinant human bone morphogenetic protein-2. *Int. J. Oral Maxillofac. Implants* **12**, 739–748 (1997).
61. Cochran, D.L., Schenk, R., Buser, D., Wozney, J.M. & Jones, A.A. Recombinant human bone morphogenetic protein-2 stimulation of bone formation around endosseous dental implants. *J. Periodontol.* **70**, 139–150 (1999).
62. Sigurdsson, T.J., Fu, E., Tatakis, D.N., Rohrer, M.D. & Wikesjo, U.M. Bone morphogenetic protein-2 for peri-implant bone regeneration and osseointegration. *Clin. Oral Implants Res.* **8**, 367–374 (1997).
63. Hanisch, O., Tatakis, D.N., Boskovic, M.M., Rohrer, M.D. & Wikesjo, U.M. Bone formation and reosseointegration in peri-implantitis defects following surgical implantation of rhBMP-2. *Int. J. Oral Maxillofac. Implants* **12**, 604–610 (1997).
64. Bessho, K., Carnes, D.L., Cavin, R., Chen, H.Y. & Ong, J.L. BMP stimulation of bone response adjacent to titanium implants *in vivo*. *Clin. Oral Implants Res.* **10**, 212–218 (1999).
65. Sykaras, N., Triplett, R.G., Nunn, M.E., Iacopino, A.M. & Opperman, L.A. Effect of recombinant human bone morphogenetic protein-2 on bone regeneration and osseointegration of dental implants. *Clin. Oral Implants Res.* **12**, 339–349 (2001).
66. Sigurdsson, T.J. *et al.* Periodontal repair in dogs: evaluation of rhBMP-2 carriers. *Int. J. Periodontics Restorative Dent.* **16**, 524–537 (1996).
67. Cheng, S.L. *et al. In vitro* and *in vivo* induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. *Calcif. Tissue Int.* **68**, 87–94 (2001).
68. Lee, J.Y. *et al.* Effect of bone morphogenetic protein-2-expressing muscle-derived cells on healing of critical-sized bone defects in mice. *J. Bone Joint Surg. Am.* **83-A**, 1032–1039 (2001).
69. Olmsted, E.A. *et al.* Adenovirus-mediated BMP2 expression in human bone marrow stromal cells. *J. Cell Biochem.* **82**, 11–21 (2001).
70. Franceschi, R.T., Wang, D., Krebsbach, P.H. & Rutherford, R.B. Gene therapy for bone formation: *in vitro* and *in vivo* osteogenic activity of an adenovirus expressing BMP7. *J. Cell Biochem.* **78**, 476–486 (2000).
71. Krebsbach, P.H., Gu, K., Franceschi, R.T. & Rutherford, R.B. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone *in vivo*. *Hum. Gene Ther.* **11**, 1201–1210 (2000).
72. Unger, E.C., Hersh, E., Vannan, M., Matsunaga, T.O. & McCreery, T. Local drug and gene delivery through microbubbles. *Prog. Cardiovasc. Dis.* **44**, 45–54 (2001).
73. Nakashima, M., Tachibana, K., Iohara, K., Ito, M., Ishikawa, M., & Akamine, A. Induction of reparative dentin formation by ultrasound mediated gene delivery of growth/differentiation factor 11. *Human Gene Therapy* **14**, 591–597 (2003).
74. Jin, Q.M., Anusaksathien, O., Webb, S.A., Rutherford, R.B., & Giannobile, W.V. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *J. Periodontol.* **74**, 202–213 (2003).
75. Harle, J., Salih, V., Mayia, F., Knowles, J.C. & Olsen, I. Effects of ultrasound on the growth and function of bone and periodontal ligament cells *in vitro*. *Ultrasound Med. Biol.* **27**, 579–586 (2001).