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The Potential of Tissue Engineering and Regeneration for Craniofacial Bone

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Abstract

Bone regeneration is a complex, well-coordinated physiological process. Large quantities of bone regeneration are often required for craniofacial skeletal reconstruction of large bone defects created by trauma, tumor resection, infection, and skeletal abnormalities. Over the last two decades, a tissue engineering and regeneration approach has been developed as an alternative to conventional surgical treatments using bone grafts. Tissue engineering methods have several advantages including the potential to regenerate bone with natural form and function. This review presents several key elements of tissue engineering for craniofacial bone: the signaling molecules (proteins and genes); scaffolds or supporting matrices; and cells. Furthermore, the advantages, challenges, and risks related with each element will be discussed.

Keywords: Tissue engineering; Bone; Growth factor; Gene therapy; Scaffolds

Introduction

Conventionally, grafting of autogenous bone has been considered the gold standard for treating craniofacial bone defects. The use of autogenous bone grafts, however, may involve a series of disadvantages, such as limited availability and increased morbidity and surgical complications associated the donor site. Over the last two decades, a tissue engineering and regeneration approach has been developed as an alternative to conventional surgical treatments. Tissue engineering is an interdisciplinary field of study that applies the principles of engineering to biology and medicine toward the development of biological substitutes that restore, maintain, and improve normal function [1,2]. This strategy provides several potential benefits including the ability to closely mimic the microenvironment in an attempt to recapitulate normal tissue healing. Here, we review some key elements of tissue engineering for craniofacial bone: the signaling molecules (proteins and genes); scaffolds or supporting matrices; and cells. Furthermore, the advantages, challenges, and risks related with each element will be discussed.

Signaling Molecules

Growth factor (GF) protein delivery

Signaling molecules critical to the tissue engineering approach in that they coordinate interactions with cell populations and the extracellular matrix [3]. GFs, as primary signaling molecules, play important roles in regulating cell activities such as chemotaxis, migration, adhesion, proliferation, and differentiation. The strategy of tissue regeneration is to utilize GFs to induce and optimize the growth and differentiation of various cell types towards specific phenotypes [4]. For example, Many studies have identified the following GFs as therapeutic candidates for periodontal regeneration: Bone Morphogenetic Proteins (BMPs), Transforming Growth Factor- β (TGF-β), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Insulin-Like Growth Factor (IGF), Enamel-Matrix Derivatives (EMD), and Growth/Differentiation Factor-5 (GDF-5). Although there are many potential GFs for periodontal regeneration, those most commonly used will be discussed here.

BMPs: BMPs are known as a group of glycoproteins that are members of the TGF-β superfamily. The first discovery of a BMP was obtained from the induction of bone formation when animals were implanted extra orthotopically with demineralized bone powder and bone extracted proteins [5]. The primary function of BMPs is to induce embryonic skeletal development, and chondro-osteogenesis in physiologic and pathologic conditions [6]. Also, BMPs play an important role in cell migration, proliferation, differentiation and apoptosis for many cell types [7,8]. There are over thirty BMPs which have been identified [9]. In 2002, The US Food and Drug Administration (FDA) approved BMP-2 and BMP-7 for use in bone regeneration [10].

The osteoinductive ability of BMP-2 to stimulate periodontal regeneration has been extensively studied in preclinical trials [11]. The *in vivo* investigations have demonstrated significant improvement in regenerating alveolar bone, inducing bone growth in mandibular defects and stimulating bone generation in peri-implant defects using several types of carriers [12-14]. In human studies, BMP-2 has also demonstrated alveolar ridge augmentation, bone formation at the sinus floor, and accelerate bone formation at peri-implant bone defects [14]. Absorbable collagen sponges (ACS) containing recombinant human BMP-2 are currently approved for clinical use in certain oral surgery procedures, including sinus augmentation and localized alveolar ridge augmentation, under the name INFUSE Bone Graft (Medtronic, Minneapolis, MN) in the US and Induct OS™ (Wyeth, Maidenhead, UK) in Europe. GF delivery via an ACS releases the protein over a period of time and in a localized manner at the desired site while providing a scaffold on which new bone can grow. Subsequently, as the graft site heals, the ACS is absorbed and replaced by host bone [15].

Several delivery systems using BMP-7, also known as Osteogenic Protein (OP-1), have demonstrated predictability in cementogenesis

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and osteogenesis in periodontal defects and peri-implant bone regeneration in animal models [16-18]. Recent clinical studies have shown the promising results of BMP-7 in sinus floor elevations in patients [19]. OP-1 Implant (Stryker Biotech, Hopkinton, MA) is an osteoinductive bone graft material containing BMP-7 and bovine derived collagen (ratio of 3.5 mg BMP-7 to 1 g collagen). Although OP-1 Implant has not been approved for periodontal regeneration, it has already shown efficacy in the treatment of non-union fractures [20]. Overall, BMP-7 incorporated with a resorbable carrier has shown profound effects on enhancing periodontal tissue regeneration.

PDGF: PDGF was the first growth factor to be evaluated in preclinical periodontal and peri-implant regenerative studies [21]. The PDGF family is composed of four growth factors, PDGF-A,-B, -C and-D. Although all of them participate in wound healing process, only three isoforms PDGF-AA, -BB and -AB have been evaluated in periodontal therapy. Furthermore, it has been found that the PDGF-BB isoform is more effective than PDGF-AA and -AB in promoting Periodontal Ligament (PDL) cell mitogenesis [22]. PDGF-BB is US-FDA-approved for use in the treatment of localized periodontal defects. PDGFs influence a wide variety of cell types in terms of proliferation, migration, and matrix synthesis. For example, PDGFs have been shown to be potent mitogens that facilitate wound healing and stimulate bone repair by expanding osteoblastic precursor cells during the bone regeneration process [23].

In pre-clinical studies in dogs, alveolar bone defects of critical size were completely regenerated after treatment with guided tissue regeneration using PDGF-BB [24]. This finding was supported by studies showing enhanced fibroblast proliferation in early periodontal wound healing after treatment of alveolar bone defects in dogs with PDGF [25]. In a recent human study, a large multi-center Phase III clinical trial evaluated the benefits of PDGF-BB associated with synthetic β-tricalcium phosphate (β-TCP) in the treatment of periodontal bone defects in 180 patients [26]. Their study showed that the use of PDGF-BB is safe and effective in improving bone fill and attachment of gingival tissue to root surfaces of involved teeth. Subsequently, this study further led to the development of GF enhanced matrix, GEM 21S (Osteohealth, Shirley, NY). This material was developed for clinical use by utilizing innovative tissue engineering principles that combine a bioactive protein (highly purified recombinant human PDGF-BB) with an osteoconductive matrix, β-TCP. Currently, GEM 21S is the only commercially available product approved for periodontal regeneration that contains PDGF [23,26].

When PDGF has been used in clinical applications, it is usually mixed with grafting materials or GEM 21S. After the materials are packed into the bone defect the surgical site is covered by collagen membranes. Unfortunately, there is often very little controlled release of the protein. It was reported that with GEM 21S almost 100% of the PDGF was released from β-TCP within 90 min *in vitro*. Additionally, *in vivo* studies show that approximately 90% of PDGF was depleted from calvarial defect sites within 72 h of implantation [27]. In order to maximize the impact of growth factors in a tissue engineering approach, in general, tissues should be exposed for relatively long periods to the protein [28]. Recently, we reported a unique method for the delivery of PDGF which utilized a commercially available collagen membrane as a carrier [29]. The study demonstrated the achievement of a sustained release profile for PDGF and the subsequent effects of the released factor on cell functions *in vitro*. Our results indicated that a sustained release of PDGF from collagen membrane was observed for ~3 weeks with 100% of PDGF delivered. The influence of an *in situ* environment is missing from *in vitro* testing system therefore these results may not be completely reproducible *in vivo*. However, our delivery system may be applicable to clinical bone regeneration because it could allow tissues to be exposed to growth factors for a sustained period and thus enhance the potential for regeneration [30].

EMD: EMD contains hydrophobic enamel matrix proteins belonging to the amelogenin family. Early studies suggest that EMD is involved in the formation of acellular cementum during tooth development and that this matrix has the potential to induce regeneration of acellular cementum in periodontal disease. EMD stimulates cellular proliferation, protein synthesis, and mineral nodule formation in several cell types including PDL cells, osteoblasts, and cementoblasts. EMD is thought to act as a tissue-healing modulator that mimics the events that occur during root development and help stimulate periodontal regeneration. In an *in vivo* study, murine primary osteoblasts, pre-osteoblasts, and cementoblasts were treated with EMD and gene expression was assessed. The results showed that common bone markers such as collagen type I, osteopontin, and bone sialoprotein, were significantly upregulated [31]. Also, in another *in vivo* study where human pre-osteoblasts were treated with EMD, there was a significant upregulation of osteoblasts as indicated by an increase in alkaline phosphatase activity [32]. Based on human studies, EMD has demonstrated periodontal regeneration validated by histological analysis. The EMD therapy promoted significant bone defect fill when measured 3 years post-therapy, while paired control defects failed to show a change in radiographic bone level [33]. These results suggest that EMD stimulates cementogenesis during periodontal wound repair. A commercial EMD (Emdogain, Biora AB, Malmö, Sweden) received US-FDA approval and is now available for the treatment of periodontal defects.

GDF-5: Recently, GDF-5 has being considered as a possible therapeutic agent for periodontal regeneration. GDF-5 belongs to the BMP class of signaling molecules. Several studies suggest that GDF-5 is essential for the formation of bone, joints, tendons and ligaments in axial and appendicular skeleton. In animal studies, functional null mutations in GDFs led to specific skeletal tissue phenotypes which allows for studying the function GDFs [34].

In pre-clinical evaluation, GDF-5 significantly demonstrated increasing in the amount of newly formed bone in critical-sized calvarial defect compared with augmentation with standard grafting techniques [35]. In another animal study, histological assessment showed that GDF-5 induced bone formation in a mandibular through-and-through saddle type defect in canine models and also in lateral ridge augmentation [36,37]. A phase IIa randomized, controlled, clinical and histological study in 20 patients was conducted to evaluate the effect of GDF-5 in treating intra-bony periodontal defects. The result indicated that GDF-5 substantially enhanced periodontal regeneration [38]. Collectively, the studies evaluating the efficacy of GDF-5 for craniofacial and related indications show that: (1) GDF-5 enhances endosseous implant stability in trabecular bone, and (2) GDF-5 accelerates bone formation and osseointegration in the maxillary sinus and in mandibular alveolar defects. A summary of GF delivery studies is shown in Table 1.

Gene delivery

A major problem with the delivery of GF proteins is the limited bioactivity of those proteins due to degradation and difficulty in

GFs	Carriers	Species	Defect models	References
BMP-2	DMB	New Zealand Rabbit	Mandibular	[12]
	ACS	Beagle dog	Alveolar periodontal	[170]
	ACS + DMB	Mongrel dog	Alveolar ridge augmentation	[13]
	ACS	Human	Alveolar ridge augmentation	[14]
	ACS	Human	Sinus floor augmentation	[15]
BMP-7	Hydroxyapatite	Baboons	Calvarial	[16]
	Collagen	Beagle dog	Periodontal	[17]
	---	Mongrel dog	Extraction site	[18]
	Collagen	Human	Sinus augmentation	[19]
PDGF	DMB + e-PTFE	Beagle dog	Alveolar periodontal	[24]
	DMB + e-PTFE	Mongrel dog	Alveolar periodontal	[25]
	DMB	Human	Alveolar periodontal	[26]
	β-TCP	Human	Alveolar periodontal	[23]
EMD	---	Cementoblasts (*in vitro*)	---	[31]
	---	Pre-osteoblasts (*in vitro*)	---	[32]
	---	Human	Alveolar periodontal	[33]
GDF-5	Collagen	Mouse	Calvarial	[35]
	β-TCP	Beagle dog	Alveolar periodontal	[36]
	Particulated bone/block bone	Beagle dog	Mandibular	[37]
	β-TCP	Human	Alveolar periodontal	[38]

Absorbable Collagen Sponges (ACS); Bone Morphogenetic Protein 2 (BMP-2); β-tricalcium phosphate (β-TCP); demineralized/mineralized bone matrix (DMB); Enamel-Matrix Derivatives (EMD); expanded polytetrafluoroethylene (e-PTFE); Growth/Differentiation Factor-5 (GDF-5); and platelet derived growth factor (PDGF).

Table 1: Summary of GF studies for oral and craniofacial regeneration.

achieving a controlled release. Therefore, localized GF delivery remains a problem in clinical applications. One method to address these problems is the use of a gene therapy approach. Gene therapy is defined as the treatment of disease by transferring genetic materials to induce specific genes that direct an individual's own cells to produce a therapeutic agent [39]. Gene therapy has various advantages compared to traditional protein delivery: (1) Longer periods of bioactivity than that of a single protein, (2) Gene delivery decreases technical challenges related to *ex vivo* protein expression and purification, and (3) Transient and controlled delivery of genes encoding several GF proteins [40]. Thus, gene therapy approaches have the possibility to provide control over the timing, distribution, and level of multiple regenerative factors simultaneously expressed in a specific tissue. Many genes are associated with the multiple steps of bone regeneration and repair, and are potential candidates for gene therapy. For instance, the following genes have been considered as candidates: GFs including Bmps, Pdgf, Fgf, Tgf-β, parathyroid hormone, and vascular endothelial growth factor (Vegf), transcription factors including Runx2/Cbfal and Osterix, and extra-cellular matrix molecules including bone sialoprotein, dentin sialophosphoprotein, matrix Gla protein, osteopontin [41].

Many studies have reported the use of gene therapy with Bmps at specific sites with a dramatic increase of osteogenesis [42-46]. Chang et al. [47] showed that Bmp-2 delivery with autologous bone marrow stem cells enhanced periodontal regeneration. In a direct gene therapy application, adenoviral gene delivery for Bmp-2 with β-TCP scaffold significantly increases the mandibular bone repair and new bone formation in rats [48]. Zhao et al. [49] showed that the bioactivity of combinations of adenoviruses expressing Bmp-2, Bmp-4 and Bmp-7 significantly induced *in vitro* osteoblast differentiation and *in vivo* bone formation by synergistic stimulation. Other *in vivo* studies have shown that adenoviral-mediated Pdgf (Ad-Pdgf) delivery can enhance periodontal tissue regeneration of tooth-supporting wounds [50,51]. Chang et al. [52] also reported the ability of Ad-Pdgf to accelerate dental implant osseointegration and alveolar bone repair.

Because the existence of blood vessel formation is indispensable for normal bone formation, induction of angiogenesis for bone formation has also been investigated. Pen et al. [53] demonstrated that delivered Vegf acted synergistically with Bmp-4 to increase mesenchymal stem cell recruitment and survival, which led to stimulated bone formation. In addition, Huang et al. [54] demonstrated that the co-expression of angiogenic and osteoinductive factors can enhance bone formation and that vascularization is critical in the overall process of bone regeneration. They used human marrow stromal cells containing combinations of condensed plasmid DNA encoding Bmp-4 and Vegf with poly (lactic-co-glycolic acid) scaffolds. Utilizing another approach, Lee et al. [55] demonstrated that the simultaneous administration of naked DNA vectors encoding Vegf and bFgf could synergistically enhance collateral vessel growth and tissue perfusion in a murine model of hind limb ischemia.

Together, these studies highlight the potential for using gene therapy to express unique combinations of regenerative molecules for bone formation and tissue regeneration.

Vectors for gene delivery

In gene therapy, it is critical to establish effective carrier (i.e., vectors) systems that facilitate gene transfer to targeted cells. There are several systems and they are classified into viral and non-viral vectors. For bone regeneration, most studies of gene therapy have been conducted with viral vectors. Each vector has its own advantages and disadvantages but there are ideal conditions, which need to be met. An ideal vector should possess the following characteristics: no detrimental effects, protection of the transgene against degradation, avoidance of an immunological host response, preferential binding to specific target cells, transduction of dividing and non-dividing cells, integration of genes into cell DNA without disruption of normal cell function, expression of genes at an appropriate therapeutic level, ability to allow external control of protein expression, and ease of production at a reasonable cost [9,56-59].

Although none of the current vectors satisfy all these criteria, understanding the advantages and disadvantages of each vector can allow for selection of the system most appropriate for the particular study. The selection of an appropriate vector depends on the design of the experiment, whether it will be an *in vivo* or *ex vivo* study, the condition of nucleic acid and desired duration (transient expression or stable expression). A summary of vector types is shown in Table 2.

Viral vector: Many studies of gene therapy for bone regeneration have used viral vectors such as adenovirus, Adeno-Associated Virus (AAV), and retrovirus, with adenovirus being the most common. The major advantage of these viral vectors is their high transduction efficiency due to the natural tropism of viruses for living cells [60]. The main disadvantages of viral vectors are their immunogenic potential [61].

I. Adenovirus: The adenovirus contains double-stranded DNA and has no enveloping membrane. It is initially taken up by receptor-mediated endocytosis by binding to the coxsackie/adenovirus receptor on the cell membrane of regenerating cells [62]. The broad distribution of these receptors explains why adenoviruses can be used to infect such a wide range of cell types [63,64]. Subsequent to infection, instead of integrating into the host genome, adenoviruses remain in the nucleus as an episome that is gradually degraded as cells divide [65]. The major advantage of adenovirus is that it infects both dividing and non-dividing

Vectors	Genes	Species/Cells	Locations	References
Adenovirus	Bmp-2	Rat	Femur	[68]
		Osteoporotic sheep	Injury site	[69]
		Goat	Tibia	[73,81]
	Bmp-7	Rat	Alveolar bone defect	[74]
		Rat	Alveolar bone defect	[76]
	Pdgf-bb	Rat	Alveolar bone defect	[75]
AAV	Bmp-2	Mouse	Cranial defect	[83]
		Rat	Hind limb	[81]
	Bmp-4	Immunocompetent rat	Intramuscular	[80]
	Rankl/Vegf	Mouse	Femoral bone allograft	[82]
Retrovirus	Bmp-2	SCID mouse/BMSCs	Hind limb	[171]
	Bmp-4	Mouse/MDSCs	Subcutaneous back	[86]
Polyethylenimine	Bmp-4	Rat	Cranial defect	[91]
Polyethyleneglyco	Runx2/caAlk6	Mouse	Skull bone	[92]
Electroporation	Bmp-2	Mouse	Skeletal muscle	[94]
	Bmp-4	Rat	Gastrocnemius	[95]
Ultrasound	Bmp-11	Canine	Pulp tissue	[96]

Bone Marrow Stromal Cells (BMSCs); Bone Morphogenetic Protein 2 (BMP-2); constitutively active activin receptor-like kinase 6 (caALK6); Muscle-Derived Stem Cells (MDSCs); Platelet Derived Growth Factor (PDGF); receptor activator for nuclear factor-κB ligand (RANKL); runt-related transcription factor 2 (Runx2); and Vascular Endothelial Growth Factor (VEGF).

Table 2: Summary of gene therapy studies for bone/dental tissue engineering.

cell, infects a wide range of cell types and does not integrate into target cell genome. Therefore an adenoviral transduced gene is expressed for only a limited period of time [66]. A major limitation is the strong host immune response to viral capsid proteins. Viral backbone modification for reduction of immunogenicity has been investigated [67].

For gene delivery in bone many groups have used direct administration of adenovirus vector carrying Bmp-2 to promote bone formation [68-73]. Adenoviral vectors have been utilized for alveolar bone engineering at dental implant defects. A vector encoding for Bmp-7 induced alveolar bone formation in a defect site [74]. Application of adenoviral vector encoding Pdgf in periodontal defects resulted in stimulation of alveolar bone and cementum regeneration in bony defects [75]. Although both cartilage and bone formation were observed in this model after 10 days, complete bridging of the defect with new bone was observed after 35 days. Furthermore, the denuded tooth root surface in animals administered by adenoviral vector carrying Bmp-7 was covered with a thin layer of new cementum and showed evidence of fiber attachment. The periodontal alveolar bone defect model involved removal of bone overlying the mandibular first molar, and the periodontal ligament and cementum from the first and second molars, followed by implantation of virally transduced fibroblasts [76]. Also, they can induce immune responses to self-antigens [77]. The overexpression of self-transgenes may lead to significant autoimmune responses and unexpected side effects. Therefore, human gene therapy trials involving any replication-defective adenoviral vectors containing human genes need to be pursued with caution.

II. AAV: AAVs derive from the parvovirus family and are small viruses with a single-stranded DNA genome [78]. The recombinant AAV (rAAV)-based vector has been developed to overcome the problems arising in immune competent individuals, based on a nonpathogenic and replication-defective virus [79]. The major advantages are that AAV initiates little detectable immunological responses and infects both dividing and non-dividing cells. The AAV vector offers a very promising option for gene transfer within the musculoskeletal system because of its safety, longevity, efficiency, and the ability to carry out direct application in immune competent individuals [80]. The major limitations to their use in gene therapy are their poor capacity to accommodate foreign DNA and their difficulty to produce sufficient amounts of the virus for clinical application [9].

The feasibility of using rAAV vector encoding Bmp-2 to induce bone formation was demonstrated by heterotopic bone formation after injecting the virus in the hind limb of immunocompetent rats. Because of low transfection efficiency, a large bolus of rAAV vector was required to induce osteogenic activity [81]. Only a few studies have examined rAAV in gene therapy applications for bone regeneration. Luk et al. [80] showed that rAAV encoding Bmp-4 could stimulate bone formation after injection into an intramuscular site. Ito et al. [82] reported that implantation of bone allograft coated with the freeze-dried rAAV vectors encoding receptor activator of nuclear factor kappa-B ligand and Vegf generated remodeling and vascularization of the implant. Human MSCs were implanted in a segmental calvarial defect in mice and infected with the rAAV encoding Bmp-2 under Tetracycline-on regulation *in vivo*. In this system, the addition of doxycycline to the animals' drinking water led to the expression of BMP-2 and eventually to fracture healing [83].

III. Retrovirus: Although retroviruses are the most extensively used vectors for gene therapy applications [84], there are only a few reports of studies using them in bone regeneration. Retroviruses are an example of viruses contained in envelopes consisting of a lipid bilayer that encloses the viral capsid containing viral RNA and RNA transcriptase. These viral RNA use reverse-transcriptase to make a double-stranded copy of their genome that is randomly integrated into the host cell genome and then replicated as the cell divides [65]. After entering the host cell, the RNA is transcribed into DNA by the viral reverse transcriptase, and a complementary strand of DNA is subsequently synthesized, resulting in double-stranded DNA that is integrated into the host cell chromosome by the viral enzyme integrase. This allows the virus to use the replication and translation mechanisms of the cell to assemble and release new viral particles [9]. These vectors have significant advantages for sustained and efficient transgene expression that are ideal for the treatment of life-threatening hereditary disorders [40]. However, the most obvious limitation is that they are only able to transfect dividing cells [85]. Furthermore, the integrated retrovirus can disrupt normal cell function by insertion mutagenesis. This vector is most suitable for *ex vivo* gene therapy applications. Peng et al. [86] reported an optimal self-inactivating retroviral vector expressing Bmp-4 that maintains a high titer, efficiently transduces muscle-derived stem cells, and enables both high levels of inducible gene expression *in vitro* and robust regulated bone formation *in vivo*.

Non-viral vectors: Serious safety concerns have been raised about some commonly used viral vectors because of the acute immune response, immunogenicity, and insertion mutagenesis uncovered in gene therapy clinical trials. As a result, non-viral vectors have been given more consideration in the gene therapy field. Non-viral vectors are categorized into two general groups: (1) delivery mediated by a chemical carrier such as cationic lipid and polymer and (2) naked DNA delivery by a physical method, such as electroporation, ultrasound and gene gun. Some types of non-viral vectors have several advantages over viral vectors, including ease of manufacture, stability, low immunogenicity, and low likelihood of being inserted into the host cell genome [65]. However, the major disadvantage for non-viral delivery methods is that non-viral gene carriers exhibit relatively low transfection efficiency, and thus there have been only few reports of bone regeneration achieved in this manner.

I. Liposomes: Cationic liposome-mediated gene transfer or lipofection represents the most extensively investigated and commonly used non-viral gene delivery method [87]. However, these carriers can often be cytotoxic which constitutes a limiting factor for application of liposomes in gene delivery due to their capacity to interact with biological membranes [88]. Compared to other non-viral vectors, a cationic lipid-based reagent is more suitable for many cell lines, including the bone related cell lines MC3T3-E1 and C3H10T1/2 [89]. Recently, we have demonstrated that combining modified HIV-1 Tat peptide with cationic lipids dramatically enhanced transfection efficiency across a range of cell lines [90]. In addition, the efficiency of the Tat peptide combination was significantly higher than many commercial non-viral vectors *in vitro*. This vector may be a potentially attractive non-viral gene vector for bone tissue engineering.

II. Polymers: Cationic polymers have been used for bone regeneration. Polyethylenimine (PEI) was used to condense plasmid DNA encoding Bmp-4 [91]. The condensed plasmid was loaded onto poly(lactic-co-glycolic acid) scaffolds, which were placed in rat cranial defects. When compared with naked DNA–loaded scaffolds, the PEI with Bmp-4 significantly induced more bone. Itaka et al. [92] demonstrated substantial bone formation in mouse skull bone defect

with poly(ethylene glycol) (PEG)-block-catiomer delivering caALK6 and Runx2 genes.

III. Electroporation and ultrasound: Electroporation is one of the non-viral methods reported in orthopedic gene therapy. It delivers macromolecules into cells by using an electric pulse. Electroporation technique is efficient, generally safe, and can produce good reproducibility compared to other non-viral methods *in vivo*. When parameters are optimized, this method can generate transfection efficiency equal to that achieved by viral vectors [93]. There are reports that *in vivo* electroporation of Bmp-2 and Bmp-4 resulted in ectopic bone formation in a mouse and a rat model, respectively [94,95]. Ultrasound-mediated delivery of Bmp-11 to mechanically exposed canine pulp tissue was effective at promoting a large amount of reparative dentin formation *in vivo*, with minimal pulpal inflammation or necrosis [96].

Cells

Stem cells (SCs)

Cell-based therapy is critical to the success of tissue engineering and bone regeneration. Although the treatments involved in the reconstruction of craniofacial and periodontal defects have largely relied on autologous tissue grafts and/or artificial implants, the success of these approaches has been limited as a result of resorption of bone, limited graft quantity, donor-site morbidity, and insufficient biocompatibility. Recently, one of most interesting cell-based therapies, stem cell (SC) treatment has presented great potential for tissue engineering as well as gene-based therapies in craniofacial skeletal reconstruction of large bone defects [1,75,97]. In general, SCs are the foundation cells for every organ and tissue in the body, including the periodontium [98,99]. SCs are usually defined by two characteristics: (1) the potential for indefinite self-renewal to give rise to more SCs; and (2) the potential to differentiate into multiple cells to perform specific function(s) [100,101]. They are also used to promote bone formation through two main mechanisms; as vehicles or as bioreactors to deliver growth factors. During osteogenesis SCs have the ability to supply cells that can differentiate to a number of cell types and accelerate endogenous bone formation [2,40].

Mesenchymal stem cells (MSCs)

SCs can be derived from three main sources: embryonic SCs, adult SCs and, more recently, through genetic manipulation, induced pluripotent SCs. Embryonic SCs have great potential for use in regenerative techniques because these cells can be pluripotent – with the ability to differentiate into virtually all mature cell types, and can be maintained indefinitely in culture in an undifferentiated state. However, the use of embryonic SCs in regenerative therapies has been significantly limited by legal and ethical concerns surrounding the use of embryos for cell isolation. Adult, somatic or postnatal SCs reside amongst differentiated cells within a number of organs in the body where they play a role in tissue repair, renewal and maintenance. In general, adult SCs are more restricted in their differentiation capacity when compared with embryonic SCs. However, one advantage of adult SCs is the greater potential for their use in autologous transplantation. In this method adult SCs can be extracted from a patient and then used to treat that patient, thereby decreasing complications arising from immune rejection. However, many mesenchymal SC (MSC)-like cell populations, derived from various types of tissues, could be used as a source of allogeneic SCs because they display immunoprivileged properties with the capacity to inhibit immune responses [102].

MSCs were first termed as colony-forming-unit fibroblasts, and identified from bone marrow aspirates, spleen and thymus [103,104]. MSCs were also defined by three criteria: adherence to plastic; a specific surface-antigen expression pattern; and multipotent differentiation potential [105]. MSCs are one of the most highly studied types of adult SCs. These cells are capable of differentiating into cells of mesodermal (adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes and visceral stromal cells) [106-111], ectodermal (neurons and astrocytes) [112] and endodermal (hepatocytes) [113] origins.

The most common source of adult SCs is the bone marrow, containing hematopoietic SCs [114] and bone marrow SCs or MSCs [105,115]. MSCs have the therapeutic capacity to treat a range of musculoskeletal abnormalities, cardiac diseases and immune abnormalities [116]. Bone marrow MSCs have been the most widely studied MSCs, in large part because they are easily accessible in quantities appropriate for clinical applications [106,117,118]. These cells are clonogenic and have demonstrated the potential to form bone and cartilage *in vivo* [110,119]. Bone marrow MSCs have been used in a number of preclinical and clinical trials and in particular for orthopedic trials due to their strong differentiation potential [120-122]. MSCs have also been shown to form craniofacial and alveolar bone, cementum, and periodontal ligament *in vivo* after implantation into craniofacial and periodontal defects [97,123,124]. These results suggest that bone marrow may be a productive source of MSCs for bone and periodontal regeneration.

In light of this, researchers have begun to assess the potential for dental-derived MSC-like SC populations in periodontal regeneration. These SC populations have the advantage over bone marrow SCs in that they can be obtained from patients in the dental clinic rather than requiring an invasive bone marrow aspiration in a hospital setting. MSCs have been identified from multiple dental-derived tissues, such as periodontal ligament [125], dental pulp [126] human exfoliated deciduous teeth [127], apical papilla [128] and dental follicles [129]. Dental-tissue-derived MSC-like populations are just one of the many types of SCs residing in specialized tissues that have been isolated and characterized. The first type of dental stem cell was isolated from the human pulp tissue and termed dental pulp SCs (DPSCs) [126]. DPSCs are isolated by enzyme treatment of pulp tissues from MSCs with various characteristics [126,130]. Subsequently, three more SCs have been characterized and isolated: SCs from human exfoliated deciduous teeth (SHED) [127], periodontal ligament SCs (PDLSCs) [125], SCs from apical papilla (SCAP) [128,131]. Although SHED showed the capacity to undergo osteogenic [127] and adipogenic differentiation [127], unlike DPSCs, SHED is unable to regenerate a complete dentin-pulp-like complex *in vivo* [127]. PDLSCs, and recently progenitor cells from the dental follicle (DFPCs) [129], have been identified as additional dental-tissue-derived progenitor cell populations. They are reported to have the potential for bone regeneration, and the capacity to differentiate into osteogenic, chondrogenic and odontogenic cells [132]. Dental-tissue derived stem/progenitor cells have been used for tissue engineering studies in large animals to assess their potential in pre-clinical test [133]. To date, the developmental relationship between these different mesenchymal stem cell-like populations has yet to be clearly understood. Also, there has been no systematic comparison between bone marrow SCs and dental-tissue-derived SCs. However, in comparison with bone marrow SCs, the dental-tissue-derived SCs appear to be more committed to odontogenic rather than osteogenic development [132].

SC-based tooth tissue engineering has been a much discussed subject because cell-based therapy for the regeneration of tissue is considered a promising strategy for the future. To repair partially lost tissue such as PDL, dentin, and pulp, one or two [134] particular types of dental SCs may be sufficient to fulfill the need. Recently, publications have directly compared the regenerative capacity of different populations of MSC-like SCs [135-137]. Kim et al. [136] compared the alveolar bone regeneration achieved from implantation of periodontal ligament SCs with bone marrow SCs, and identified no significant difference in regenerative potential between these two cell populations. However, studies comparing the regenerative capacity of periodontal ligament SCs, dental pulp SCs and periapical follicular SCs in periodontal defects have identified that periodontal ligament SCs have the greatest regenerative capacity [135,137]. This new source of SCs could be useful in cell-based tissue engineering therapy and the eventual development of techniques for use in both regenerative periodontics and degenerative diseases. However, a more complete understanding of the cellular mechanisms of these dental SC populations is necessary.

While periodontal ligament SCs shows strong potential for use in periodontal regeneration, a limiting factor to their clinical use is that tooth extraction is required in order to isolate the cells. Research is ongoing into more easily accessible SC populations, one of which is induced pluripotent stem (iPS) cells. Recently, Wada et al. [138] demonstrated that iPS cells can be successfully generated from adult human gingival and periodontal ligament fibroblasts. The early signs regarding the use of iPS cells in periodontal regeneration look promising. Still, significantly more work is required in this area. Questions also exist surrounding the potential to regulate the differentiation of iPS cells once implanted because they have the ability to differentiate into virtually any cell type of the body.

Scaffolds

The development of bone and tissue engineering is directly correlated to changes in biomaterials technology. The nature and structure of scaffolds and matrices is critical in controlling osteoinductive capacity. The factors that determine an appropriate scaffold for bone formation include biodegradability, porosity, rigidity, and cell carrier capacity [122]. Proper oxygen supply, regulating cell differentiation, adhesion, and proliferation also have an influence on the amount of bone formation within the scaffolds, particularly over long periods [139]. Scaffolds and matrices have been extensively studied and many basic elements for their design have been proposed [140]. In their application to tissue engineering, the ideal properties of scaffolds and matrices are as follows [141], they should:

(1) be a barrier to restrict cellular migration and proliferation

(2) provide physical support for healing area

(3) potentially control release rates of gene therapy vectors

(4) Supply a suitable three-dimensional environment for signaling molecules.

Several three-dimensional (3D) biomaterials are available for tissue engineering over an extended period of time for cellular and tissue in-growth [78]. Both natural and synthetic scaffolds are used to regenerate tissue *in vivo*. Naturally derived scaffolds include autografts, allografts, and xenografts [142]. Autologous bone graft is one of the most commonly used materials and primary sources for bone healing. This graft surpasses other techniques because tissue derived from the same individual contains live cells and growth factors and these grafts do not cause immunoreactions [143]. However, this process needs highly invasive bone collection from healthy sites, and the autologous bone supply is limited [144]. Although autologous bone graft remains the standard therapy for large bone defects, this treatment is limited due to the high percentage of donor and recipient site complication.

In contrast to natural scaffolds, artificial scaffolds can be highly manipulated to customize the material for a particular application. Artificial scaffolds take advantage of property modifications, such as control of macrostructure and degradation time. These materials also carry little risk of contamination and do not require bone collection. They are often regarded as superior materials to natural scaffolds such as autologous bone grafts and allografts in terms of biosafety and invasiveness [145]. In fact, artificial scaffolds such as PGA [146], PLGA [76], CaP-based ceramics such as β-TCP [147], and hydroxyapatite (HA)-based scaffolds [148] have been used extensively for gene delivery studies [149].

The use of HA in the dental field has been demonstrated to restore periodontal defects and to carry and deliver growth factors, such as BMPs and FGF-2 [150]. Although no clinical or *in vivo* studies have used HA for gene and cell therapy strategies for periodontal engineering purposes, a recent in vitro study has shown an HA and collagen combination scaffold to be a suitable environment for the growth of human PDL cells, therefore indicating its potential for periodontal tissue engineering [151].

Moreover, inorganic CaP-based materials have been used as delivery systems. Such materials as β-TCP are synthetic scaffolds that can be used to repair osseous defects around teeth or dental implants by acting as a bone substitute or as a carrier for growth factor delivery and cells [147]. Tissue engineering methods with gene- and cell-therapy have used β-TCP as a carrier for bone reengineering approaches but its value for periodontal regeneration remains to be explored [48,152].

Hydrogels are originated from natural materials, such as collagen chitosan, fibrin, or alginate, and formed by the cross-linking or self-assembly of a variety of natural or synthetic hydrophilic polymers to produce structures containing more than 90% water. These materials are prepared from biodegradable polymers with negative charges. A positively charged growth factor, for example, interacts electrostatically with the polymer chain, permitting the factor to become physically immobilized in the hydrogel carrier. Scaffolds and matrixes should serve as supportive carriers that conduct a sustained release of bioactive molecules, thereby inducing stimuli for tissue formation.

Gene vector release from hydrogels is dependent on the physical structure and degradation of the hydrogel and its interactions with the vector [153]. Tabata et al. [154] created a delivery system for bioactive molecules that mimicked the natural release system *in vivo* by using a biodegradable gelatin hydrogel. This system succeeded in promoting bone repair in skull defects of animals by the controlled release of TGF-β1 and BMP-2. In addition, integrated MSCs prepared from the bone marrow of rabbit fibula with gelatin microspheres incorporating TGF-β permitted complete closure of a rabbit skull defect by newly formed bone tissue [154,155]. Together these studies show that the use of allografts or xenografts has the potential for use in applications where larger scaffolds are needed and it eliminates the need for a donor site and the subsequent associated morbidity.

Unlike autografts, allografts and xenografts do not contain living cells and do not provide osteoinductive signals because of the

purification and sterilization processes they undergo [156]. Moreover, they present a potential risk of contamination with viral and bacterial infections, and the biological risk of an immune response of the host tissue after implantation [157]. Also, ethical concerns have been raised. Eppley et al. [158] has discussed the ethical implications associated with body trading. Allografts and xenografts can fail, especially when used in large defects. Wheeler et al. [159] have reported that failure rates of large allograft reconstructions were as high as 60% at 10 years. These failures are associated with a multitude of biologic processes influencing the graft incorporation and functional capacity. In addition, artificial materials are usually sintered to increase mechanical strength [160,161], leading to decrease in biodegradability and contraction in size.

To overcome many of the problems described above, 3D fabrication technology was innovated [161]. The design and manufacture of 3D shapes using computer-aided design and computer-aided manufacturing (CAD/CAM) systems in the industrial world is very common [162,163]. Using this technology, Saijo et al. [124] recently reported on the clinical use of novel Inkjet-Printed Custom-Made Artificial Bones (IPCABs) for ten patients with maxillofacial deformities. The study demonstrated that IPCABs were safe and achieved dimensional compatibility along with good biodegradability and osteoconductivity. Hernigou et al. [164] reported that BMSCs need to be implanted within 3D organic or inorganic scaffolds to create a supporting bone matrix for differentiated MSC and more efficient bone formation. Ultimately, the appropriate combinations of cell-based gene therapy and tissue engineered scaffolds will lead to successful bone formation and tissue engineering.

Current Challenges

The therapeutic achievement of craniofacial regeneration will depend on determining the optimal conditions for a given localized area (The diagram in Figure 1 illustrates the mechanisms involved in tissue engineering for craniofacial bone). Modular delivery systems may have to be conceived that can be customized to match individual pathological situations. From the reviewed literature it is clear that most therapeutic agents studied are merely simple combinations of GFs with biomaterials. An optimal delivery system should not only release the most appropriate GFs at the ideal dose and kinetics, but also further offer a matrix for the ingrowth of osteoprogenitor cells and blood vessels. However, there are no perfect strategies, which combine optimized carrier compatibility, GFs immobilizing method, release kinetics, dosage levels, toxicity thresholds, and target specificities. Without a specific delivery method, most GFs released are only functioning in a suboptimal state. There are also host factors to consider, such as genetic background, lifestyle, physical activity, age, variable pathology, and additional medications. Therefore, simply adapting known release technologies to existing GFs will not yield high quality results and it can be very costly.

An innovative gene delivery method may provide an alternative to direct application of growth factors in tissue engineering. Our understanding of gene regulation of some proteins has been confirmed

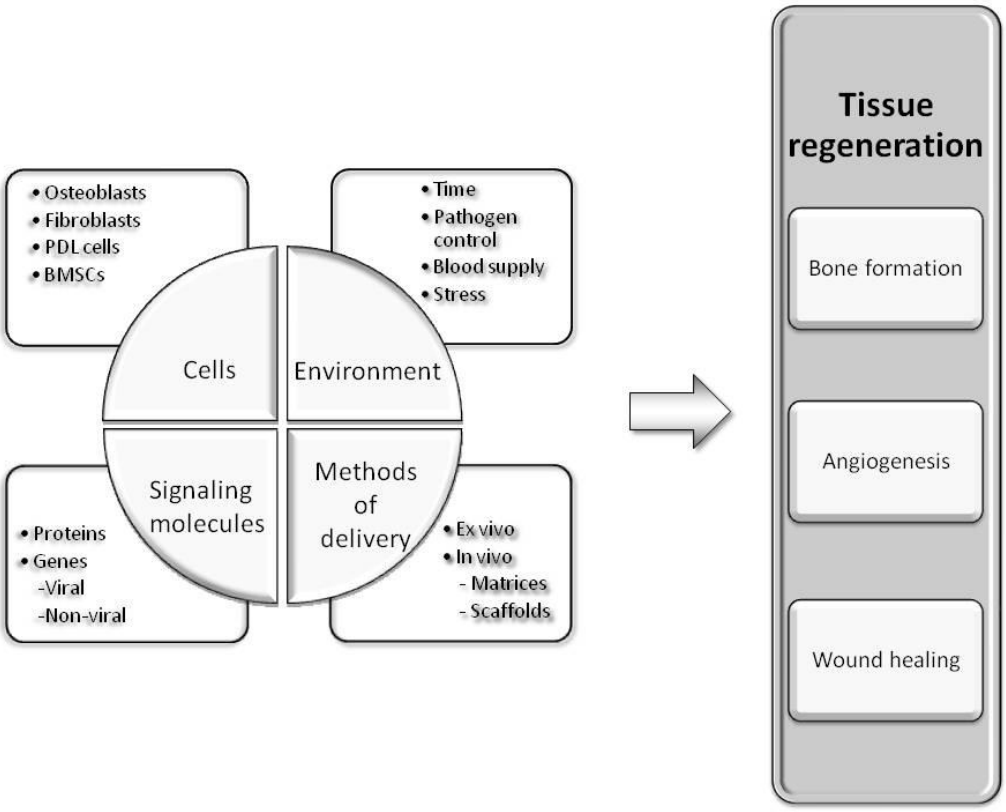

Figure 1: The strategy diagram of tissue engineering for craniofacial bone. Tissue engineering requires the following four essential elements: cells, signaling molecules, methods of delivery, and environment. Eventually, tissue regeneration including bone formation, angiogenesis, and wound healing would be lead. PDL cells, periodontal ligament cells; BMSCs, bone marrow stromal cells.

with experimental gene therapy studies; however, the safety and efficacy of using gene therapy technology for *in vivo* tissue engineering have yet to be determined. The need to avoid some of the risks of viral vectors in gene delivery have led to advances involving condensation of DNA with liposomes or other carriers which have the potential to enhance the uptake of non-viral DNA by cells [165]. However, as currently formulated, cellular uptake of non-viral vectors is an extremely inefficient process, estimated to be 10^{-9} that of viral vectors [166]. Although many gene delivery systems have developed and some are very valid, we are still far from the perfect gene carrier suitable for clinical use. Further improvements need to be made to improve efficiency, reduce toxicity, enhance target-specificity and prolong efficacy before clinical applications can be developed.

Various novel scaffold delivery systems have been examined and demonstrated possibilities to meet the challenges of current tissue engineering and bone regeneration therapy. Naturally derived scaffolds include autografts, allografts, and xenografts, as well as inorganic CaP-based materials such as β-TCP can be used as a bone substitute or as a carrier for GF and cell delivery. An appropriately shaped 3D printed scaffold is now also a widely available method utilized to fill a defect space. However, there remain some challenges related to cell and gene delivery. The carriers should ideally degrade within a few weeks to months, to minimize interference with the normal healing process. The delivery device should provide a dose- and time-controlled release of the bioactive agent, include high biocompatibility, low toxicity, cost effectiveness, and ease of manufacture [167]. In addition, efficiency of bone formation within the scaffolds is highly dependent on proper oxygen and blood supply, which controls cell adhesion, proliferation and differentiation in the long term. In the future, improvements of MSCs and scaffolds may lead to a more efficient cell therapy for bone tissue regeneration [168-171]. Also, further preclinical and clinical controlled studies are needed to establish the efficacy and safety of these methods.

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