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Review Article

Theme: Recent Advances in Musculoskeletal Tissue Engineering
Guest Editor: Aliasger K. Salem

Bone Regeneration Using Gene-Activated Matrices

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ABSTRACT. Gene delivery to bone is a potential therapeutic strategy for directed, sustained, and regulated protein expression. Tissue engineering strategies for bone regeneration include delivery of proteins, genes (viral and non-viral-mediated delivery), and/or cells to the bone defect site. In addition, biomimetic scaffolds and scaffolds incorporating bone anabolic agents greatly enhance the bone repair process. Regional gene therapy has the potential of enhancing bone defect healing and bone regeneration by delivering osteogenic genes locally to the osseous lesions, thereby reducing systemic toxicity and the need for using supraphysiological dosages of therapeutic proteins. By implanting gene-activated matrices (GAMs), sustained gene expression and continuous osteogenic protein production *in situ* can be achieved in a way that stimulates osteogenesis and bone repair within osseous defects. Critical parameters substantially affecting the therapeutic efficacy of gene therapy include the choice of osteogenic transgene(s), selection of non-viral or viral vectors, the wound environment, and the selection of *ex vivo* and *in vivo* gene delivery strategies, such as GAMs. It is critical for gene therapy applications that clinically beneficial amounts of proteins are synthesized endogenously within and around the lesion in a sustained manner. It is therefore necessary that reliable and reproducible methods of gene delivery be developed and tested for their efficacy and safety before translating into clinical practice. Practical considerations such as the age, gender, and systemic health of patients and the nature of the disease process also need to be taken into account in order to personalize the treatments and progress towards developing a clinically applicable gene therapy for healing bone defects. This review discusses tissue engineering strategies to regenerate bone with specific focus on non-viral gene delivery systems.

KEY WORDS: bone healing; collagen scaffold; gene-activated matrix; plasmid DNA and chemically modified RNA; transcript-activated matrix.

BONE HEALING MECHANISM: UNDERSTANDING THE BIOLOGY OF BONE AND BONE TISSUE FORMATION

Bone is a dynamic, highly vascularized tissue that provides essential mechanical and structural support to the body. Due to its high degree of vascularization, bone has a strong capacity to heal, supply osteoprogenitor cells, and mobilize minerals, especially calcium, when required. Bone healing involves a complex set of events encompassing a large number of genetic and molecular triggers, morphogens, signaling molecules, and transcriptional regulators that act in concert during specific stages of the healing process. The process of bone healing starts with the formation of a blood clot, followed by an inflammatory phase to remove potential antigens or foreign material from the wound bed (1). Any impairment in the clotting process due to local or systemic factors or due to the use of anticoagulation drugs may lead to impaired healing. The blood clot is then gradually replaced by granulation tissue with mediators such as vascular endothelial growth factor (VEGF) and angiopoietins playing a crucial role in this process (2). The highly vascularized granulation tissue is subsequently replaced by woven bone that then gradually matures (1). In the reparative phase, growth factors such as transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), and insulin-like growth factor-I (IGF-I)

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contribute to the induction of callus formation within the granulation tissue (3). There are two major mechanisms by which bone formation takes place. One is endochondral ossification in which endothelial cells proliferate and chondroblasts differentiate in the clot area due to lack of oxygen, ultimately resulting in the formation of hyaline cartilage (4). Chondroblasts after synthesizing the cartilaginous matrix become hypertrophic and produce VEGF and bFGF. New blood vessels are formed in this region which results in the transport of osteoprogenitor and hematopoietic cells, resulting in the replacement of cartilage with bone and bone marrow (4). The other process is intramembranous ossification in which preosteoblasts differentiate into osteoblasts which then secrete extracellular matrix proteins and deposit calcium to harden the matrix (5).

The final stage of bone healing is the resorption phase where macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) mediates the activity of macrophages and osteoclasts in the resorption of hard tissue debris (6). All these phases together contribute to the formation of a complete remodeled bone which is both structurally and functionally similar to the native bone.

TISSUE ENGINEERING STRATEGIES TO REGENERATE BONE

The healing capacity of bone in some situations might be limited or insufficient to heal large bone defects. Approaches such as bone grafting are widely used but have several limitations. Bone substitutes based on novel materials continue to be an area of active research; however, as yet, a successful product with predictable regenerative capacity has not been developed for widespread clinical use. Thus, most bone injuries are still not treated in a predictable manner. Each year, there are approximately one million cases of skeletal defects that need bone grafting to achieve union. Current treatments are based on autologous bone grafting which is considered the gold standard approach for bone healing and regeneration. Autografts are bone grafts transferred from a different part of the patient's own body, which provide excellent osteoconductive, osteoinductive, and osteogenic properties (7). However, due to second surgical site morbidity, associated pain, and limited supply, this strategy is reserved for only a restricted number of cases (7). Grafts originating from a different individual of the same (allografts) and different (xenografts) species run the risk of immune rejection and pathogen transmission from donor to host (8). The success rate in terms of graft incorporation is lower for allografts or xenografts when compared to autografts (9). Alternative sources for bone grafts include synthetic alloplasts made from metals, polymers, or ceramics (7). They too possess certain disadvantages such as poor integration with the native tissue at the site of the defect and potential failure due to fatigue or infection caused during implantation. In addition, ceramics are very brittle and cannot be used in locations of high stress or mechanical load (10).

Owing to the drawbacks of bone grafts, over the last few decades, identification of key molecules (such as growth factors, cytokines, and morphogens) involved in bone development and fracture healing is in progress and has led to the introduction and rapid expansion of biomimetic materials in the field of bone tissue engineering. Tissue engineering has been defined as "an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" (11). Successful healing of fractures requires integration of engineered tissue with surrounding host tissues and involves combined input from signaling generated by intercellular communications, cell–matrix interactions, and growth factors.

Tissue engineering strategies for bone regeneration have included recombinant protein therapy to deliver osteogenic cytokines and growth factors, delivery of nucleic acids (DNA, mRNA) encoding growth factors that promote bone growth, and transplantation of osteogenic cells at sites of bone defects. Osteogenic cells can be utilized *per se* or can be either transduced with viruses or transfected with non-viral vectors prior to implantation. These approaches may be further modified by combining with biomimetic biomaterial scaffolds in order to boost the therapeutic response and bone repair process. Scaffolds are supportive substrates that provide a three-dimensional platform for the cells to successfully transform into bone tissue. Biomaterial scaffold-based delivery systems need to be designed such that they can control and maintain the activity of the incorporated therapeutics and serve to prolong their residence time. Three-dimensional macroporous scaffolds fabricated from biodegradable polymers can act as osteoconductive substrates upon direct implantation *in vivo*, recruiting progenitor cells to the wound site.

Growth Factor and Cytokine Protein-Based Approaches

Growth factors act in a concentration, and time, dependent manner to control cell migration, differentiation, and/or proliferation by binding to their specific target cell surface receptors in order to repair damaged tissues (Table I). Osteoinductivity is achieved by the action of bone morphogenetic proteins (BMPs, BMP-2 to BMP-8), TGF-β, and other growth factors that stimulate osteogenesis (21). Pro-angiogenic factors, such as bFGF, VEGF, PDGF-BB, TGF-β, and angiopoietins 1 and 2, contribute to osteoconductivity (22). Multiple growth factors, including both osteoinductive and osteoconductive factors, have been shown to synergistically promote angiogenesis and enhance bone regeneration (23). A brief overview of the pathways through which these growth factors induce osteogenesis and angiogenesis is provided in Fig. 1. One significant drawback of current protein-based therapies is their lack of specificity for osteoblasts or bone-forming cells. Due to their short half-lives and rapid degradation, high doses of proteins (in milligrams) are necessary for direct clinical application despite very small quantities being required for localized osteoinduction. These supraphysiological doses, in addition to the functional heterogeneity of proteins, may result in unwanted complications. For example, high doses of BMP-2 can result in soft tissue swelling, radiculitis, and ectopic bone formation (31). Owing to these unpredictable adverse effects and high cost, the prospect of protein-based therapies is limited.

Table I. A List of Growth Factors and Their Role in Bone Regeneration

Growth factor/ protein	Known functions
EGF (12)	Growth and proliferation of mesenchymal and fibroblast cells, induction of granulation tissue formation and angiogenesis
PDGF-BB (13)	Chemo-attractant, induces mitogenesis, promotes angiogenesis, and extracellular matrix synthesis and deposition
TGF-β (14)	Regulation of cell proliferation, differentiation, and matrix synthesis
rhBMP (15)	Stimulation of angiogenesis and migration, induces proliferation and differentiation of mesenchymal stem cells into cartilage and bone-forming cells
VEGF (16)	Promotion of chemotaxis of MSCs, indirectly induces proliferation and differentiation of osteoblast precursor cells
bFGF (17,18)	Stimulation of migration and proliferation of endothelial cells, hypertrophic chondrocyte differentiation, and osteoblast/ osteoclast recruitment to the growth plate
Activin (19)	Induction of osteoblastic cell proliferation and collagen synthesis
IGF-I (20)	Induction of cellular proliferation and matrix synthesis

EGF epidermal growth factor, *PDGF-BB* platelet-derived growth factor-BB, *TGF-β* transforming growth factor beta, *rhBMP* recombinant human bone morphogenetic proteins, *VEGF* vascular endothelial growth factor, *bFGF* basic fibroblast growth factor, *IGF-1* insulin-like growth factor 1

Cell-Based Approaches

Cell therapy is an approach based on transplantation of key cells which can synthesize the desired therapeutic growth factors at the implanted site. Isolated autologous cells that are expanded *ex vivo* can be genetically modified to produce growth factors and can be transplanted into the defect (32). Cell-based bone tissue engineering includes both somatic and undifferentiated stem cells (33). In contrast to stem cells, somatic cells have limited potency, lack self-renewal ability, and are committed to the production of only a single cell type. Hence, they have very limited use in complex tissue engineering approaches. As a result, a significant portion of current research is concentrated on the use of stem cells. Human mesenchymal stem cells (MSCs) have been primarily isolated from adipose tissue, bone marrow, umbilical cord, and teeth (dental pulp and periodontal ligaments). These cells are inherently capable of differentiating into bone tissue cells under appropriate stimuli including appropriate gene transfection. Genetically modified MSCs can be cultured to

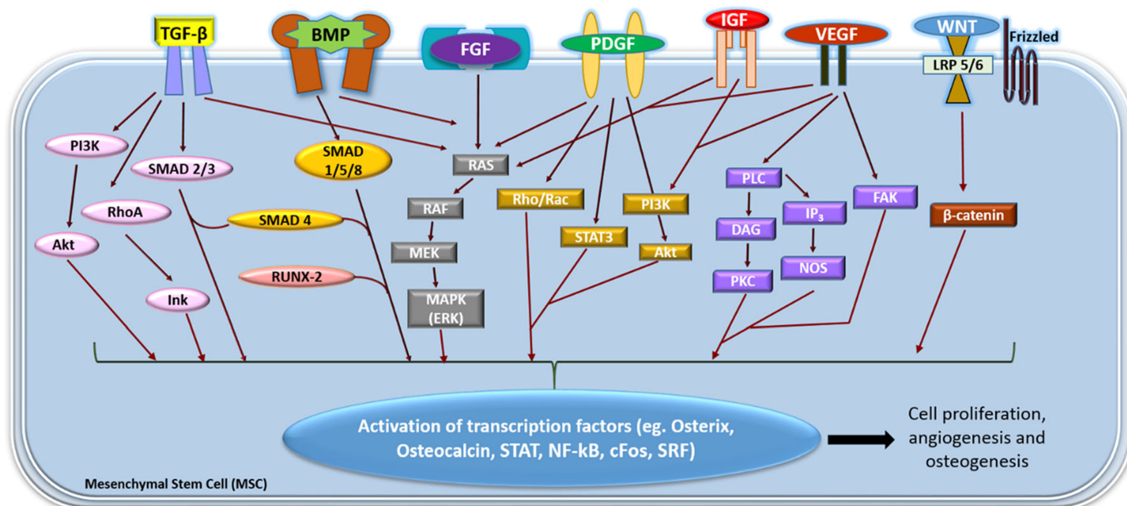

Fig. 1. Schematic demonstrating the signaling networks and their crosstalk involved in the differentiation of mesenchymal stem cells (MSCs) into osteoblasts. TGF-β induces osteogenic differentiation by activating PI3K, SMAD 2/3, and RhoA pathways (24). FGF-2 and BMP-2 can synergistically differentiate MSCs into osteoblasts *via* RAS/RAF/MEK/MAPK and SMAD pathways (25). PDGF triggers angiogenesis through crosstalk between MAPK, Rho/Rac, STAT3, and PI3K pathways (26). IGF can induce osteogenesis by activating MAPK and PI3K pathways (27). VEGF can induce angiogenesis by activating PLC, IP_3, and FAK pathways (28,29). WNT signaling through β-catenin can induce osteogenic differentiation of MSCs (30). Legend: *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase, *Akt/PKB* protein kinase B, *SMAD* suppressor of mothers against decapentaplegic, *RhoA* Ras homolog gene family, member A, *RUNX2* Runt-related transcription factor 2, *MAPK* mitogen-activated protein kinase, *STAT* signal transducer and activator of transcription, *PLC* phosphoinositide phospholipase C, *DAG* diacylglycerol, *IP_3* inositol 1,4,5-trisphosphate, *NOS* nitric oxide synthase, and *FAK* focal adhesion kinase

produce varying combinations of growth factors such as bFGF and VEGF, BMP-2 and BMP-7, and VEGF and BMP-4 (34). Before implantation at the defect site, gene transfer to these cells can be performed *ex vivo* using viral vectors and then combined with polymers, such as collagen type I, to create cell/polymer constructs (35). Various studies have also reported the use of *ex vivo* expanded autologous bone marrow-derived osteoprogenitor cells grown on macroporous hydroxyapatite scaffolds for implantation at lesion sites. This treatment, devoid of any cellular genetic modifications, was intended for the repair of large bone defects in long bones and demonstrated repair and functional recovery of segmental bone defects (36).

GENE THERAPY FOR BONE REGENERATION

Gene therapy is considered an effective means of delivering growth factors in a sustained fashion while overcoming the limitations of using high protein doses. Gene delivery is more cost-effective than bolus protein delivery and it can be finely controlled (37). Furthermore, simultaneous delivery of multiple genes is possible and customization is relatively straightforward. We have previously evaluated the feasibility and efficacy of co-delivering genes encoding FGF-2 and BMP-2 to promote osteogenesis in human adipose-derived mesenchymal stem cells (hADMSCs) (25). We showed that when compared to delivery of pDNA (*FGF-2*) or pDNA (*BMP-2*) alone, co-delivery of both genes resulted in significant enhancement in the secretion of BMP-2 protein from the transfected hADMSCs. Post-transfection, a significantly higher expression of osteogenic markers, such as runx-2 and osteocalcin, was observed in hADMSCs that were simultaneously transfected with *FGF-2* and *BMP-2* genes, compared to delivery of single genes (25). *In vitro* mineralization data further confirmed the synergistic effects of co-delivering *FGF-2* and *BMP-2* genes (25). Altogether, gene therapy is considered to be an effective alternative to protein therapy. The three essential material components of gene therapy include pDNA encoding the desired therapeutic protein, a vector to facilitate the cellular uptake of the pDNA, and the *in situ* or *ex vivo* target tissue or cells that produce the desired protein upon transfection.

Non-Viral Gene delivery

Although viral gene therapy has proven to be efficaceous in several animal studies, perceived safety concerns continue to present challenges with respect to conducting human clinical trials and therefore clinical application of viral gene therapy for non-lethal conditions is generally undesirable. In spite of lower transfection efficiencies compared to that of viral vectors, non-viral vectors are safer and can be clinically translated for potential bone regeneration applications. Hence, the main focus of this review is on the utilization of non-viral vectors, with a primary focus on polymers as gene delivery vectors.

Ideally, a vector mediating successful gene therapy should possess a number of crucial attributes that allow for therapeutic levels of transgene expression for an adequate duration of time. The transgenes must be delivered to the target cell nuclei in a manner that ensures efficient transfection associated with minimal cytotoxicity and safety concerns. In addition, it should ideally be target cell-specific and have a controllable timeframe of protein expression (38). Unfortunately, despite their strong safety profiles, non-viral gene delivery systems suffer from having lower transfection efficiencies, which has previously hampered their potential (39). Tremendous growth in the field of nanotechnology in the last decade has led to the production of safer non-viral vectors with improved transfection efficiencies. Several studies have explored the *in vivo* and *ex vivo* delivery of genes (encoding growth factors or transcriptional factors) using non-viral vectors for bone regeneration. Non-viral gene delivery agents have advantages over viral vectors in that the responses to the treatment are less immunogenic, less toxic, and there are lower pathogenic, carcinogenic, or other mutagenic concerns, often associated with viral vectors, thus making them safer for clinical applications (39). Moreover, the gene expression induced is transient and the expression levels can be tightly regulated as needed depending on the acute or chronic nature of the disease. It is also relatively easy to alter and optimize the properties of these gene carriers so as to create a balance between transfection efficiency and cytotoxicity. pDNA can be complexed *via* electrostatic interactions with liposomes, polymers, or other polycations to form either lipoplexes or polyplexes. Among the numerous non-viral gene vectors studied *in vitro* and *in vivo*, polyethylenimine (PEI), especially the branched 25-kDa PEI polymer, is one of the most successful gene transfer agents to date (40). PEI is believed to exhibit higher transfection efficiencies than many other non-viral vectors due to a phenomenon known as the "proton sponge effect" (41) and the level of transfection efficiency attained with PEI is considered comparable with that of viral vectors.

In Vivo Versus Ex Vivo Gene Therapy

There are two main methods of gene transfer for tissue regeneration: (1) transfection of MSCs *ex vivo* and subsequent transplantation into the defect site (42) and (2) direct application of osteogenic genes to the defect site *in vivo*. Plasmids containing cDNA equivalents of therapeutic genes can be delivered *per se*, or the plasmid can be complexed with polycations to form nanoplexes. These methods could be used in conjunction with scaffolds to enhance bone regeneration.

A high transfection efficiency of host cells is required for the *in vivo* gene delivery approach to be effective. With this approach, it is difficult to obtain targeted gene delivery to specific cells, as the cells surrounding the target tissue of interest may also be transfected. In *ex vivo* gene transfer, the isolated and culture-expanded cells can be transfected *in vitro* and are then implanted into the defect. *Ex vivo* gene therapy can target specific cell populations of interest and permits selection, control, and scrutiny of the genetically altered cells prior to re-implantation. However, compared with *in vivo* gene therapy, *ex vivo* approaches are generally more surgically invasive, technically complex, and expensive, thus reducing their scope for clinical translation (43). Therefore, there is growing interest in the development of non-viral vectors with higher transfection efficiencies that can deliver pDNA encoding osteogenic factors *in vivo*.

Gene-activated matrices (GAMs) are inert scaffold systems containing viral or non-viral gene delivery vectors that have been widely investigated and used in wound healing and tissue engineering approaches (44). Following gene delivery, the recombinant protein could be expressed *in situ* by endogenous wound repair cells. Although secreted in small amounts, protein expression for prolonged periods of time can promote bone regeneration (45). GAMs temporarily serve as three-dimensional templates for tissue formation and guide the growth of new functional tissue. They can encapsulate and retain the gene within the sponge matrices for longer durations thereby enhancing matrix deposition and blood vessel formation in the developing bone tissue (46). In contrast, direct injection of the gene mediates shorter cellular expression and does not significantly affect tissue formation (47). Research performed by our group demonstrated the ability of a GAM comprising a collagen scaffold injected with PEI/pDNA (encoding PDGF-B) complexes to regenerate bone (48). The *in vivo* regenerative capacity of the GAM was evaluated in 5-mm-diameter critical-sized calvarial defects in rats and demonstrated a 44-fold higher new bone volume percentage in calvarial defects when compared to implanted empty scaffolds (48).

ROLE OF GAMS IN TISSUE ENGINEERING

Scaffold Design Criteria for Gene Delivery Systems

GAMs are ideally composed of biodegradable and biocompatible polymeric materials that are bioresorbable upon *in vivo* implantation into osseous defects. The scaffold matrix provides structural support for osteogenesis or bone remodeling by the infiltrating reparative cells expressing the transgene (Fig. 2). Three-dimensional GAMs are typically highly porous in nature and can be fabricated with different desired geometries that can regenerate functional bone tissue while maintaining its original size and shape. A biologically active scaffold specifically engineered for bone tissue regeneration must possess a number of key qualities necessary for enabling new tissue formation (49). Ideally, biomaterials composing GAMs need to be osteoconductive, mechanically compatible, and capable of integrating with the surrounding native bone during the repair process. The matrix must be suitable for creating and maintaining appropriate space and a favorable environment *in vivo* to support tissue development and control the size and shape of the space-filling regenerating tissue. It must also be structurally and mechanically stable and homogenously porous with optimal pore size suitable for promoting cell adhesion and growth. For bone healing involving guided bone regeneration, it is critical that the scaffold matrices possess suitable physical and mechanical properties for supporting cell survival and proliferation. The ability of the scaffolds to engineer the bone tissue and control its structure is defined by the mechanical properties and degradation rate of the scaffolds. For this reason, the scaffold must maintain architectural integrity after placement *in vivo* that is essential for the gradual filling-in of critical-sized defects during the process of bone formation (50). The biocompatible scaffold must degrade (generating non-toxic degradation products) at a rate such that the new bone tissue is formed, gradually replacing the scaffold. The scaffold should allow for migration and attachment of progenitor cells from the surrounding tissue into the scaffold. It is desirable that the chemical composition of the scaffold contains binding sites (ligands) appropriate for specific cell populations. In addition, the pore size must be large enough to allow maximal cell entry and migration yet small enough to retain a high specific surface area for cell attachment and matrix deposition (51). It is also necessary that the scaffolds should possess optimal pore volume fraction (high porosity, usually >90%) together with an interconnected pore network for efficient transport of metabolites and nutrients (52). The pore size, pore shape, and the porosity of the scaffold are critical parameters governing the availability of total specific surface area as well as ligand distribution to the cells. Initiation of the bone regenerative process involves an interplay between the scaffolds and infiltrating cells involving structural, mechanical, and biological cues. For bone regeneration applications, a

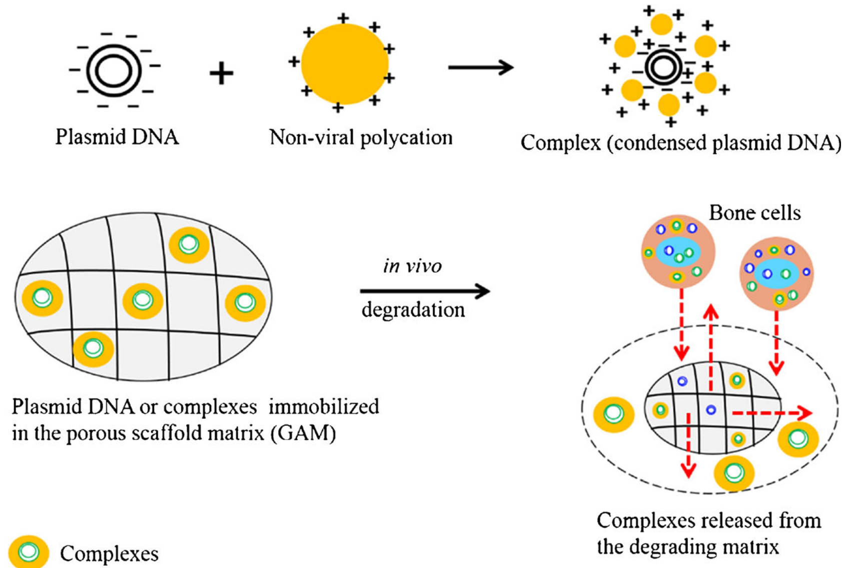

Fig. 2. Schematic of the gene-activated matrices (GAMs) demonstrating the proposed mechanism of action for bone regeneration

series of scaffolds with tailored structural, mechanical, and biological properties can be fabricated that facilitate cellular adhesion, migration, and tissue development (Table II). This may significantly enable engineering of the bone tissue with pre-defined structures.

Soft Tissue Healing

Some bone defects, such as those resulting from trauma, also require reconstructive therapy of soft tissue injuries associated with the trauma. This includes repair of blood vessels, cartilage, muscles, ligaments and tendons, neural tissue, and skin. The process of bone formation alone requires the integration of a number of physiological processes such as angiogenesis. New blood vessels are essential for supplying oxygen and providing nutrients necessary to sustain cell vitality required of the highly metabolically active cells involved in repair. They also serve to carry inflammatory and mesenchymal tissue progenitor cells to, and waste and breakdown products from, the wound site (74). By modifying

Table II. A List of Different Polymeric Scaffolds Implicated in Tissue Engineering Applications with Their Respective Modifications

Scaffold material	Modification description	Outcome
Collagen-GAG (53,54)	Addition of GAG to collagen scaffolds, constant cooling rate during the freezing process prior to lyophilization	GAG effectively improves attachment, migration, and infiltration of cells throughout the porous scaffold; uniform porous structure and less variation in mean pore size
Collagen-GAG (55)	Collagen concentration (0.25%, 0.5%, and 1%) and crosslink density (dehydrothermal crosslinking processes at 105°C for 24 h and 150°C for 48 h)	Significant improvement in the biological and mechanical properties of the scaffold with increased collagen amount (1%) and crosslinking (at 150°C for 48 h); enhanced pore size, permeability, compressive strength, cell number, and cell metabolic activity
Hyaluronic acid-based polymer (56–58)	Chemical modification through total esterification of carboxyl groups	Insoluble polymer with good stability against acidic hydrolysis; covalent binding of hydroxyl functional moieties; promotes cell adhesion, proliferation, ECM production, osteogenic differentiation, and mineralization
Hydroxyapatite/β-tricalcium phosphate ceramic implants (59)	MSCs loaded onto the porous carrier	Stronger bone formation superior to the carrier alone
Collagen-PGA (60,61)	Collagen sponge mechanically reinforced by incorporation of PGA fiber (dehydrothermal crosslinking)	Enhancement in compression strength; sustained release of pDNA complex; significant attachment of fibroblasts, greater cell proliferation and infiltration; reduction in sponge shrinkage
Gelatin-PLGA (62)	PLGA microspheres loaded into gelatin scaffolds	Increased mechanical strength and flexibility; delivery of multiple genes with distinct release kinetics
PLA, PGA, PLGA (63)	Type of polymer, molecular weight, intrinsic viscosity	High porosity with low molecular weight, PLGA with low intrinsic viscosity; superior mechanical properties with higher lactic acid content
PLGA (64)	Partial fusion of NaCl porogen in the solvent casting-particulate leaching process	Scaffolds with enhanced pore interconnectivity and compressive modulus
PLLA (65)	Scaffold surface modification using gelatin spheres as porogen	Higher compressive modulus; significant improvement in initial cell adhesion and proliferation, cell spreading and matrix secretion
Hyaluronan (66)	Modification with gelatin using disulfide crosslinking	Hyaluronan-gelatin sponge promoted cell attachment, growth, and spreading
PLGA (67)	Coating PLGA microspheres with polydopamine	Increased incorporation and slowed release of pDNA from the scaffold
Collagen (68)	Calcium phosphate coating for collagen scaffolds	Improved mechanical properties (higher compressive modulus/stiffness)
Collagen (69,70)	Nano-hydroxyapatite inclusion in the scaffold	Enhanced cell function and osteointegration; significantly increased scaffold stiffness and pore interconnectivity
PCL (71)	Coupling resveratrol through a hydrolysable covalent bond with the carboxylic acid groups on PCL surface grafted with acrylic acid	Significant increase in osteogenesis
Alginate (72)	Mixing octacalcium phosphate (OCP) with alginate solution	Increased elastic modulus and pore size with increasing OCP concentration
Collagen (73)	Specific binding of biotinylated PEI-pDNA complexes to avidin-modified collagen	Enhanced transfection efficiency by immobilizing complexes in the matrix through biotin/avidin bond; inhibits aggregation of complexes; higher loading efficiency and bioavailability of complexes

vascularization through exogenous delivery of various growth factors, including VEGF, PDGF, BMPs, and FGF, bone regeneration and healing can be enhanced (75). This could also be a viable therapeutic approach for healing of soft tissues. The highly vascularized soft tissue envelopes, periosteum, and endosteum, restore normal blood supply to the fracture site. By combining osteogenic and angiogenic inductive growth factors, complete wound healing can be potentially achieved. Work by Shea *et al*. showed that polymeric PLGA scaffolds incorporating DNA encoding VEGF resulted in local and sustained delivery of the growth factor at the site of implantation (76). The treatment led to increased blood vessel density at the local tissue site. In a separate animal study, Mooney et al. showed that when pDNA encoding human recombinant PDGF-B was encapsulated into PLGA matrices, an increase in granulation tissue and vascularization was observed (47). There was a statistically significant increase in the granulation tissue thickness and the number and area of blood vessels from 2 to 4 weeks, thus demonstrating continuous expression of the delivered gene and its promotion of tissue formation over time. Enhancement of skeletal muscle repair was reported by Pierce's group using collagen-gelatin matrix-immobilized gene vectors encoding either FGF-2 or FGF-6 (77). When delivered to excisional muscle defects, these biomatrices were responsible for promoting angiogenesis that later remodeled to form arteries. Along with enhancing the density of endothelial cells and muscular arterioles at the treatment sites, myotube regeneration and muscle repair were also facilitated. For cartilage engineering applications, Zhang and colleagues carried out a study employing porous chitosan-gelatin scaffolds containing DNA encoding TGF-β1 (78). This GAM proved effective for the proliferation of chondrocytes and also increased the synthesis of major ECM components, thereby promoting cartilage regeneration. The GAM approach can be similarly utilized for gene delivery in the regeneration of other soft tissues (79).

Hard Tissue Regeneration with a Focus on the Use of GAMs

The engineering of bone tissue requires transient gene expression of growth factors for a time period spanning weeks. A variety of natural, synthetic, and semi-synthetic polymers can be used as substrates for gene delivery. An advantage of employing synthetic polymers as depot systems is that they can be tailored specifically with properties favorable for bone tissue regeneration. These modifications provide control over the amount of pDNA available to cells over time; thus, the time frame of gene expression can be modulated. GAMs may also contain therapeutic gene(s) encapsulated or entrapped into polymeric nanospheres or microspheres for further optimization of release and uptake kinetics. When formulating pDNA into these spherical particles, either the uncondensed (naked) or condensed form (with polycations) can be incorporated (80). Alternatively, lyophilized pDNA can either be mixed with the polymer particles or pre-encapsulated into polymer microspheres before processing the polymer particles or microspheres into porous scaffolds (81). The latter approach may result in a more even distribution of pDNA throughout the matrix, with release being regulated by the degrading microspheres. These two approaches may be combined to provide delivery of multiple genes (encoding different growth factors), each with a distinct release rate and delivery kinetics from the same structural scaffold unit. The different phases of bone healing rely on the action of multiple growth factors at distinct stages, for recruitment, proliferation, and differentiation of MSCs (82). Using the aforementioned combinatorial approach for tissue-specific controlled dose and rate of delivery, these signals can be then temporally and spatially manipulated so as to enhance the cellular events necessary for bone regeneration. The formulation properties such as polymer concentration, polymer molecular weight, and the method of preparation can be used to control the loading efficiency, release kinetics, and the bioavailability of the released pDNA. The kinetics of gene construct release can also be varied by altering the polymer degradation rate using various polymer formulations. The polymer constructs can be fabricated from synthetic polyesters such as PLGA, PGA, and polycaprolactone and natural polymers such as chitosan, alginate, hydroxyapatite, calcium phosphate, collagen, and hyaluronan (Table III). These polymers can also be used in combination (94).

GENE-ACTIVATED TITANIUM SURFACES FOR ENHANCED OSSEOINTEGRATION

In dentistry, the introduction of dental implants has revolutionized the way oral rehabilitation and reconstruction is achieved. Through osseointegration, the process by which the titanium surface of the dental implants forms direct bonding with the surrounding bone, dental implants are able to support and retain dental prostheses improving patient's esthetics and function in a significant manner (95,96). Though osseointegration is a highly predictable process, failure does occur and ways to enhance osseointegration are constantly being explored (97). Our group developed a novel approach of coating the titanium surface with polymer-pDNA nanoplexes to enhance osseointegration (98). We showed that discs coated with PEI-pDNA (BMP-2) nanoplexes prepared at an *N*/*P* ratio of 10 (N/P-10) resulted in 75% cell viability and 14% transfection efficiency in bone marrow stromal cells (BMSCs) *in vitro*. Compared to controls, the transfection of BMSCs by PEI-pDNA (BMP-2) nanoplexes from the titanium surface resulted in enhanced expression of osteogenic markers including runx-2, alkaline phosphatase, and osteocalcin (98). Compared to controls, enhanced calcium deposition as determined by qualitative (alizarin red staining) and quantitative (atomic absorption spectroscopy) assays was noted in transfected cells on day 30 posttreatment (98). The results highlight the potential of this novel approach of using gene-activated titanium surfaces to enhance osseointegration.

RNA-ACTIVATED MATRICES FOR BONE REGENERATION

As discussed earlier in this review, the inherent barrier to non-viral gene delivery is the need for the polymer-pDNA complex or the pDNA alone to cross the nuclear membrane to enter the nucleus, where they utilize the machinery for transcription of the encoded DNA into mRNA. This rate

Table III. A List of Different Types of GAMs Investigated for Induction of Bone Formation

Scaffold material	Vector	Transgene	Model
Collagen (83)	pDNA	PTH 1-34 or/and BMP-4	Rat femoral defect
Collagen (44)	pDNA	PTH 1-34	Dog tibial defect
PLGA (84)	PEI-pDNA complexes	BMP-4	Rat cranial defect
Poly(propylene fumarate) (85)	Triacrylate/amine polycationic polymer-pDNA polyplexes complexed with gelatin microparticles	BMP-2	Rat cranial defect
Collagen (48)	PEI-pDNA complexes	PDGF-B	Rat cranial defect
Collagen or autologous bone graft (86)	BMP-2 condensed with liposomal vector	BMP-2	Pig cranial defect
Collagen (87)	CaP-pDNA precipitates	BMP-2	Rat tibial defect
Collagen (88)	pDNA	PTH 1–34	Lumbar interbody fusions in sheep
Collagen (89)	pDNA	$VEGF_{165}$	Rabbit radial defect
Calcium phosphate cement (90)	pDNA complexed with poly(ethyleneglycol) (PEG)-block-polycation	caALK6 and Runx2	Mouse cranial defect
Collagen (91)	pDNA	Osteogenic protein-1 (OP-1 or BMP-7)	Rat lumbar interbody arthrodesis
Collagen/calcium phosphate (92)	pDNA complexed with PAMAM dendrimer	$VEGF_{165}$	Mouse intra-femoral defect
Hydroxyapatite (93)	pDNA condensed with cationic liposomes	BMP-2	Rabbit cranial defect

(PEG-b-P[Asp-(DET)]) PEG-b-polyasparagine carrying the *N*-(2-aminoethyl) aminoethyl group $(CH_2)_2NH(CH_2)_2NH_2$ as the side chain), *caALK6* constitutively active form of activin receptor-like kinase 6, *Runx2* runt-related transcription factor 2

limiting step which requires the target cells to be in a constantly dividing state to allow for nuclear entry significantly affects the overall transfection efficiency of the non-viral gene delivery system and also minimizes its applications. We have shown that synthesizing and utilizing chemically modified RNA (cmRNA) encoding BMP-2 instead of pDNA as the active molecule in combination with PEI as a vector resulted in significantly higher bone regeneration in a rat calvarial defect model (99). Issues related to stability and immunogenicity inherent to RNA were addressed by strategic modifications of RNA. The PEI-cmRNA (BMP-2) outperformed PEI-pDNA (BMP-2) across a wide range of *in vitro*- and *in vivo*-based metrics (99). This study and a subsequent investigation that validated these findings may pave the way for a new field of cm RNA-based therapy for bone regeneration (100).

ADVANTAGES AND DISADVANTAGES OF GAMS FOR BONE REGENERATION: BIOCOMPATIBILITY/SAFETY OF GAMS

Utilizing non-viral GAMs for tissue engineering has been shown to be effective in generating sustained expression of recombinant growth factors by transfected infiltrating wound repair cells, as well as being more cost-effective, and may be safer for use clinically when compared to recombinant protein or viral therapy. The implanted GAMs facilitate gene expression and protein production for an extended period of time thereby stimulating an enhanced therapeutic response for osteogenesis and bone repair. Localized gene therapy has also been reported to eliminate or reduce systemic toxicity resulting from dose dumping which can occur with current protein therapies (101,102). The *in vitro* production of pDNA is relatively simple, produced in large quantities, and economical as compared to commercial protein production (103). In addition, it has been shown that localized gene delivery using the GAM approach directs the production of endogenous proteins which are less altered and thereby less immunogenic, in a targeted, controlled manner at the site of implantation. The *in situ* production of proteins by transfected bone repair cells ensures efficient cell surface receptor targeting. Consequently, significantly lower doses of proteins are required to attain similar or even higher levels of therapeutic effect for enhanced bone regeneration, when compared to protein delivery. With GAMs, a long-term healing effect due to relatively sustained transgene expression can be achieved *in vivo* (73). Gene transfer from GAMs causes specific cells to differentiate into desired cell types over time and eliminates the need for repeated administrations (67). Furthermore, incorporation of gene vectors into the porous scaffold affords protection from physiological degradation and delays clearance from the wound site thereby maintaining long-term availability of transgenes to cells infiltrating the GAMs. However, there is a lack of knowledge with respect to the amount of proteins actually produced by transfected cells and the amount needed for therapeutic effects in a given clinical situation. Further research is required to determine optimal levels and timing of protein expression needed during the bone healing process. Regarding clinical applications, the lack of preclinical data in large animal models due to the highly expensive and time-consuming nature of the studies continues to be a major barrier. Even if encouraging data is obtained readily by simple, available technologies, the funding and the regulatory environment raise issues and pose additional impediments to progress. As mentioned earlier, compared to viral gene delivery systems, GAM-based non-viral gene delivery systems have the best potential to overcome key barriers to

clinical translation, so long as safety and efficacy can be demonstrated using these materials in appropriate animal and human studies in the future.

FUTURE PROSPECTS AND CHALLENGES IN CLINICAL APPLICATIONS

Critical-sized bone defects may not be capable of self-healing and regeneration without intervention. This necessitates treatments that facilitate rapid new bone development and reduce healing times. Gene therapy is being investigated in preclinical models as a way to treat tissue loss and enhance the regenerative process. Gene transfer can be performed using a variety of viral and non-viral vectors as gene carriers. Depending on the type of defect, either *in vivo* or *ex vivo* methods can be implemented. In terms of safety, and therefore potential for clinical translation, non-viral vectors may be preferred over viral vectors. In bone tissue engineering, GAM technology is a highly versatile approach for affecting sustained localized gene delivery and extended expression of tissue inductive growth factors. The polymeric scaffold design of GAMs can control tissue development by controlling the release and maintaining sufficient availability of growth factors at the site of injury. The GAM design provides a powerful and useful tool to study, regulate, and manipulate cell functions in the bone developmental process thereby providing important details for our understanding of the biology of bone tissue formation and regeneration.

Although the combination of tissue engineering scaffolds with gene therapy has immense potential to target many cellular processes involved in promoting bone regeneration, there are several challenges which need to be addressed. One challenge is to fabricate matrices that possess all the desired properties needed to achieve optimal bone regeneration such as porosity, biocompatibility, mechanical integrity, mechanical stability, osteoconductivity, and osteoinductivity. Novel matrices need to be developed that are capable of maintaining structural integrity while simultaneously providing ease of access to infiltrating bone progenitors. When compared to the viral vectors, polymeric vectors generally display inferior transfection efficiencies. Hence, there is a need to improve upon current formulations, possibly through mimicking viral modes of transduction, such that GAMs possess the ability to transfect cells at levels comparable to viral vectors.

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REFERENCES

1. Stroncek JD, Reichert WM. Overview of wound healing in different tissue types. In: Reichert WM, editor. Indwelling neural implants: strategies for contending with the in vivo environment. Boca Raton (FL); 2008.
2. Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The role of vascular endothelial growth factor in wound healing. J Surg Res. 2009;153(2):347–58. doi:10.1016/j.jss.2008.04.023.
3. Marzona L, Pavolini B. Play and players in bone fracture healing match. Clin Cases Miner Bone Metab. 2009;6(2):159–62.
4. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell B. 2008;40(1):46–62. doi:10.1016/j.biocel.2007.06.009.
5. Zimmermann B. Degeneration of osteoblasts involved in intramembranous ossification of fetal rat calvaria. Cell Tissue Res. 1992;267(1):75–84.
6. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res. 2008;87(2):107–18.
7. Kumar P, Vinitha B, Fathima G. Bone grafts in dentistry. J Pharm Bioallied Sci. 2013;5 Suppl 1:S125–7. doi:10.4103/0975-7406.113312.
8. Greenwald MA, Kuehnert MJ, Fishman JA. Infectious disease transmission during organ and tissue transplantation. Emerg Infect Dis. 2012;18(8):e1. doi:10.3201/eid1808.120277.
9. Delloye C, Cornu O, Druez V, Barbier O. Bone allografts—what they can offer and what they cannot. J Bone Joint Surg (Br). 2007;89b(5):574–9. doi:10.1302/0301-620x.89b5.19039.
10. Nuss KM, von Rechenberg B. Biocompatibility issues with modern implants in bone—a review for clinical orthopedics. Open Orthop J. 2008;2:66–78. doi:10.2174/1874325000802010066.
11. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260(5110):920–6.
12. Marquez L, de Abreu FA, Ferreira CL, Alves GD, Miziara MN, Alves JB. Enhanced bone healing of rat tooth sockets after administration of epidermal growth factor (EGF) carried by liposome. Injury. 2013;44(4):558–64. doi:10.1016/j.injury.2012.10.014.
13. Caplan AI, Correa D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. J Orthopaed Res : Off Publ Orthopaed Res Soc. 2011;29(12):1795–803. doi:10.1002/jor.21462.
14. Poniatowski LA, Wojdasiewicz P, Gasik R, Szukiewicz D. Transforming growth factor Beta family: insight into the role of growth factors in regulation of fracture healing biology and potential clinical applications. Mediat Inflamm. 2015;2015:137823. doi:10.1155/2015/137823.
15. Rao SM, Ugale GM, Warad SB. Bone morphogenetic proteins: periodontal regeneration. N Am J Med Sci. 2013;5(3):161–8. doi:10.4103/1947-2714.109175.
16. Street J, Bao M, deGuzman L, *et al*. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A. 2002;99(15):9656–61. doi:10.1073/pnas.152324099.
17. Nakamura T, Hara Y, Tagawa M, *et al*. Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. J Bone Miner Res. 1998;13(6):942–9. doi:10.1359/jbmr.1998.13.6.942.
18. Schmid GJ, Kobayashi C, Sandell LJ, Ornitz DM. Fibroblast growth factor expression during skeletal fracture healing in mice. Dev Dyn. 2009;238(3):766–74. doi:10.1002/dvdy.21882.
19. Sakai R, Eto Y. Involvement of activin in the regulation of bone metabolism. Mol Cell Endocrinol. 2001;180(1–2):183–8.
20. Trippel SB. Potential role of insulinlike growth factors in fracture healing. Clin Orthop Relat Res. 1998;(355 Suppl):S301–13.
21. Zhao GQ. Consequences of knocking out BMP signaling in the mouse. Genesis. 2003;35(1):43–56. doi:10.1002/gene.10167.
22. Hirschi KK, Skalak TC, Peirce SM, Little CD. Vascular assembly in natural and engineered tissues. Ann N Y Acad Sci. 2002;961:223–42.
23. Reyes R, De la Riva B, Delgado A, Hernandez A, Sanchez E, Evora C. Effect of triple growth factor controlled delivery by a brushite-PLGA system on a bone defect. Injury. 2012;43(3):334–42. doi:10.1016/j.injury.2011.10.008.
24. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96. doi:10.1038/nrm3758.

25. Atluri K, Seabold D, Hong L, Elangovan S, Salem AK. Nanoplex-mediated codelivery of fibroblast growth factor and bone morphogenetic protein genes promotes osteogenesis in human adipocyte-derived mesenchymal stem cells. Mol Pharm. 2015;12(8):3032–42. doi:10.1021/acs.molpharmaceut.5b00297.
26. Hayrapetyan A, Jansen JA, van den Beucken JJJP. Signaling pathways involved in osteogenesis and their application for bone regenerative medicine. Tissue Eng B Rev. 2015;21(1):75–87. doi:10.1089/ten.teb.2014.0119.
27. Bikle DD, Wang Y. Insulin-like growth factor-I and bone. IBMS BoneKEy. 2011;8(7):328–41.
28. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med. 2012;2(7):a006502. doi:10.1101/cshperspect.a006502.
29. Jung S KJ. Angiogenesis—the key to regeneration, regenerative medicine and tissue engineering. Prof. Jose A. Andrades (Ed.), InTech. 2013. doi: 10.5772/55542.
30. Rodriguez-Carballo E, Gamez B, Ventura F. p38 MAPK signaling in osteoblast differentiation. Front Cell Dev Biol. 2016;4:40. doi:10.3389/fcell.2016.00040.
31. Shimer AL, Oner FC, Vaccaro AR. Spinal reconstruction and bone morphogenetic proteins: open questions. Injury. 2009;40 Suppl 3:S32–8. doi:10.1016/S0020-1383(09)70009-9.
32. Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Curr Stem Cell Res Ther. 2008;3(4):254–64.
33. Brown PT, Handorf AM, Jeon WB, Li WJ. Stem cell-based tissue engineering approaches for musculoskeletal regeneration. Curr Pharm Des. 2013;19(19):3429–45.
34. Rao RR, Stegemann JP. Cell-based approaches to the engineering of vascularized bone tissue. Cytotherapy. 2013;15(11):1309–22. doi:10.1016/j.jcyt.2013.06.005.
35. Chang SC-N, Wei FC, Chuang H, *et al.* Ex vivo gene therapy in autologous critical-size craniofacial bone regeneration. Plast Reconstr Surg. 2003;112(7):1841–50. doi:10.1097/01.PRS.0000091168.73462.1A.
36. Kon E, Muraglia A, Corsi A, *et al.* Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res. 2000;49(3):328–37.
37. Kessler PD, Podsakoff GM, Chen X, *et al.* Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. Proc Natl Acad Sci U S A. 1996;93(24):14082–7.
38. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4(7):581–93. doi:10.1038/nrd1775.
39. Abbas AO, Donovan MD, Salem AK. Formulating poly(lactide-co-glycolide) particles for plasmid DNA delivery. J Pharm Sci. 2008;97(7):2448–61. doi:10.1002/jps.21215.
40. Boussif O, Lezoualc'h F, Zanta MA, *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A. 1995;92(16):7297–301.
41. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 2005;7(5):657–63. doi:10.1002/jgm.696.
42. Lieberman JR, Daluiski A, Stevenson S, *et al.* The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. J Bone Joint Surg - Ser A. 1999;81(7):905–17.
43. Steinert AF, Noth U, Tuan RS. Concepts in gene therapy for cartilage repair. Injury. 2008;39 Suppl 1:S97–113. doi:10.1016/j.injury.2008.01.034.
44. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med. 1999;5(7):753–9. doi:10.1038/10473.
45. Bonadio J. Review: local gene delivery for tissue regeneration. e-biomed: J Regen Med. 2000;1(2):25–9.
46. Huang YC, Connell M, Park Y, Mooney DJ, Rice KG. Fabrication and in vitro testing of polymeric delivery system for condensed DNA. J Biomed Mater Res A. 2003;67(4):1384–92. doi:10.1002/jbm.a.20036.
47. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nat Biotechnol. 1999;17(6):551–4. doi:10.1038/9853.
48. Elangovan S, D'Mello SR, Hong L, *et al.* The enhancement of bone regeneration by gene activated matrix encoding for platelet derived growth factor. Biomaterials. 2014;35(2):737–47. doi:10.1016/j.biomaterials.2013.10.021.
49. Putnam AJ, Mooney DJ. Tissue engineering using synthetic extracellular matrices. Nat Med. 1996;2(7):824–6.
50. Bose S, Roy M, Bandyopadhyay A. Recent advances in bone tissue engineering scaffolds. Trends Biotechnol. 2012;30(10):546–54. doi:10.1016/j.tibtech.2012.07.005.
51. Zeltinger J, Sherwood JK, Graham DA, Mueller R, Griffith LG. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. Tissue Eng. 2001;7(5):557–72. doi:10.1089/107632701753213183.
52. Yannas IV. Tissue and organ regeneration in adults: Springer; 2001.
53. Murphy CM, Haugh MG, O'Brien FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials. 2010;31(3):461–6. doi:10.1016/j.biomaterials.2009.09.063.
54. O'Brien FJ, Harley BA, Yannas IV, Gibson L. Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. Biomaterials. 2004;25(6):1077–86. doi:10.1016/S0142-9612(03)00630-6.
55. Tierney CM, Haugh MG, Liedl J, Mulcahy F, Hayes B, O'Brien FJ. The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. J Mech Behav Biomed Mater. 2009;2(2):202–9. doi:10.1016/j.jmbbm.2008.08.007.
56. Campoccia D, Doherty P, Radice M, Brun P, Abatangelo G, Williams DF. Semisynthetic resorbable materials from hyaluronan esterification. Biomaterials. 1998;19(23):2101–27.
57. Lisignoli G, Zini N, Remiddi G, *et al.* Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic acid based polymer scaffold. Biomaterials. 2001;22(15):2095–105. doi:10.1016/S0142-9612(00)00398-7.
58. Radice M, Brun P, Cortivo R, Scapinelli R, Battaliard C, Abatangelo G. Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors. J Biomed Mater Res. 2000;50(2):101–9.
59. Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res. 1998;16(2):155–62. doi:10.1002/jor.1100160202.
60. Hiraoka Y, Kimura Y, Ueda H, Tabata Y. Fabrication and biocompatibility of collagen sponge reinforced with poly(glycolic acid) fiber. Tissue Eng. 2003;9(6):1101–12. doi:10.1089/10763270360728017.
61. Hosseinkhani H, Yamamoto M, Inatsugu Y, *et al.* Enhanced ectopic bone formation using a combination of plasmid DNA impregnation into 3-D scaffold and bioreactor perfusion culture. Biomaterials. 2006;27(8):1387–98. doi:10.1016/j.biomaterials.2005.08.017.
62. Banerjee I, Mishra D, Maiti TK. PLGA microspheres incorporated gelatin scaffold: microspheres modulate scaffold properties. Int J Biomater. 2009;2009:143659. doi:10.1155/2009/143659.
63. Sheridan MH, Shea LD, Peters MC, Mooney DJ. Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. J Control Release. 2000;64(1–3):91–102. doi:10.1016/S0168-3659(99)00138-8.
64. Murphy WL, Dennis RG, Kileny JL, Mooney DJ. Salt fusion: an approach to improve pore interconnectivity within tissue engineering scaffolds. Tissue Eng. 2002;8(1):43–52. doi:10.1089/107632702753503045.
65. Liu X, Won Y, Ma PX. Porogen-induced surface modification of nano-fibrous poly(L-lactic acid) scaffolds for tissue engineering. Biomaterials. 2006;27(21):3980–7. doi:10.1016/j.biomaterials.2006.03.008.
66. Liu Y, Shu XZ, Gray SD, Prestwich GD. Disulfide-crosslinked hyaluronan–gelatin sponge: growth of fibrous tissue in vivo. J Biomed Mater Res A. 2004;68A(1):142–9. doi:10.1002/jbm.a.10142.
67. Avilés MO, Lin CH, Zelivyanskaya M, *et al.* The contribution of plasmid design and release to in vivo gene expression

following delivery from cationic polymer modified scaffolds. Biomaterials. 2010;31(6):1140–7. doi:10.1016/j.injury.2012.10.014.
68. Al-Munajjed AA, O'Brien FJ. Influence of a novel calcium-phosphate coating on the mechanical properties of highly porous collagen scaffolds for bone repair. J Mech Behav Biomed Mater. 2009;2(2):138–46. doi:10.1016/j.jmbbm.2008.05.001.
69. Al-Munajjed AA, Plunkett NA, Gleeson JP, *et al*. Development of a biomimetic collagen-hydroxyapatite scaffold for bone tissue engineering using a SBF immersion technique. J Biomed Mater Res B Appl Biomater. 2009;90(2):584–91. doi:10.1002/jbm.b.31320.
70. Gleeson JP, Plunkett NA, O'Brien FJ. Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration. Eur Cell Mater. 2010;20:218–30.
71. Li Y, Danmark S, Edlund U, *et al*. Resveratrol-conjugated poly-epsilon-caprolactone facilitates in vitro mineralization and in vivo bone regeneration. Acta Biomater. 2011;7(2):751–8. doi:10.1016/j.actbio.2010.09.008.
72. Shiraishi N, Anada T, Honda Y, Masuda T, Sasaki K, Suzuki O. Preparation and characterization of porous alginate scaffolds containing various amounts of octacalcium phosphate (OCP) crystals. J Mater Sci Mater Med. 2010;21(3):907–14. doi:10.1007/s10856-009-3911-1.
73. Orsi S, De Capua A, Guarnieri D, Marasco D, Netti PA. Cell recruitment and transfection in gene activated collagen matrix. Biomaterials. 2010;31(3):570–6.
74. Velazquez OC. Angiogenesis and vasculogenesis: inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J Vasc Surg. 2007;45(Suppl A):A39–47. doi:10.1016/j.jvs.2007.02.068.
75. Vo TN, Kasper FK, Mikos AG. Strategies for controlled delivery of growth factors and cells for bone regeneration. Adv Drug Deliv Rev. 2012;64(12):1292–309. doi:10.1016/j.addr.2012.01.016.
76. Jang JH, Rives CB, Shea LD. Plasmid delivery in vivo from porous tissue-engineering scaffolds: transgene expression and cellular transfection. Mol Ther. 2005;12(3):475–83. doi:10.1016/j.ymthe.2005.03.036.
77. Doukas J, Blease K, Craig D, *et al*. Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. Mol Ther. 2002;5(5 Pt 1):517–27. doi:10.1006/mthe.2002.0579.
78. Guo T, Zhao J, Chang J, *et al*. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation. Biomaterials. 2006;27(7):1095–103. doi:10.1016/j.biomaterials.2005.08.015.
79. Tyrone JW, Mogford JE, Chandler LA, *et al*. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. J Surg Res. 2000;93(2):230–6. doi:10.1006/jsre.2000.5912.
80. Zhang XQ, Intra J, Salem AK. Conjugation of polyamidoamine dendrimers on biodegradable microparticles for nonviral gene delivery. Bioconjug Chem. 2007;18(6):2068–76. doi:10.1021/bc070116l.
81. De Laporte L, Shea LD. Matrices and scaffolds for DNA delivery in tissue engineering. Adv Drug Deliv Rev. 2007;59(4–5):292–307. doi:10.1016/j.addr.2007.03.017.
82. Jones E, Yang X. Mesenchymal stem cells and bone regeneration: current status. Injury. 2011;42(6):562–8. doi:10.1016/j.injury.2011.03.030.
83. Fang J, Zhu YY, Smiley E, *et al*. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. Proc Natl Acad Sci U S A. 1996;93(12):5753–8.
84. Huang YC, Simmons C, Kaigler D, Rice KG, Mooney DJ. Bone regeneration in a rat cranial defect with delivery of PEI-condensed plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4). Gene Ther. 2005;12(5):418–26. doi:10.1038/sj.gt.3302439.
85. Chew SA, Kretlow JD, Spicer PP, *et al*. Delivery of plasmid DNA encoding bone morphogenetic protein-2 with a biodegradable branched polycationic polymer in a critical-size rat cranial defect model. Tissue Eng A. 2011;17(5–6):751–63. doi:10.1089/ten.TEA.2010.0496.
86. Park J, Lutz R, Felszeghy E, *et al*. The effect on bone regeneration of a liposomal vector to deliver BMP-2 gene to bone grafts in peri-implant bone defects. Biomaterials. 2007;28(17):2772–82. doi:10.1016/j.biomaterials.2007.02.009.
87. Endo M, Kuroda S, Kondo H, Maruoka Y, Ohya K, Kasugai S. Bone regeneration by modified gene-activated matrix: effectiveness in segmental tibial defects in rats. Tissue Eng. 2006;12(3):489–97. doi:10.1089/ten.2006.12.489.
88. Patil PVGG, Bonadio J, *et al*. Interbody fusion augmentation using localized gene delivery. Trans Orthop Res Soc. 2000;25:360.
89. Geiger F, Bertram H, Berger I, *et al*. Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. J Bone Miner Res. 2005;20(11):2028–35. doi:10.1359/jbmr.050701.
90. Itaka K, Ohba S, Miyata K, *et al*. Bone regeneration by regulated in vivo gene transfer using biocompatible polyplex nanomicelles. Mol Ther. 2007;15(9):1655–62. doi:10.1038/sj.mt.6300218.
91. Bright C, Park YS, Sieber AN, Kostuik JP, Leong KW. In vivo evaluation of plasmid DNA encoding OP-1 protein for spine fusion. Spine (Phila Pa 1976). 2006;31(19):2163–72. doi:10.1097/01.brs.0000232721.59901.45.
92. Keeney M, van den Beucken JJJP, van der Kraan PM, Jansen JA, Pandit A. The ability of a collagen/calcium phosphate scaffold to act as its own vector for gene delivery and to promote bone formation via transfection with VEGF165. Biomaterials. 2010;31(10):2893–902. doi:10.1016/j.biomaterials.2009.12.041.
93. Ono I, Yamashita T, Jin H-Y, *et al*. Combination of porous hydroxyapatite and cationic liposomes as a vector for BMP-2 gene therapy. Biomaterials. 2004;25(19):4709–18. doi:10.1016/j.biomaterials.2003.11.038.
94. Do AV, Akkouch A, Green B, *et al*. Controlled and sequential delivery of fluorophores from 3D printed alginate-PLGA tubes. Ann Biomed Eng. 2016. doi:10.1007/s10439-016-1648-9.
95. Kim MH, Park K, Choi KH, *et al*. Cell adhesion and in vivo osseointegration of sandblasted/acid etched/anodized dental implants. Int J Mol Sci. 2015;16(5):10324–36. doi:10.3390/ijms160510324.
96. MacDonald DE, Rapuano BE, Schniepp HC. Surface oxide net charge of a titanium alloy comparison between effects of treatment with heat or radiofrequency plasma glow discharge. Colloid Surf B. 2011;82(1):173–81. doi:10.1016/j.colsurfb.2010.08.031.
97. Chen H, Liu NZ, Xu XC, Qu XH, Lu EY. Smoking, radiotherapy, diabetes and osteoporosis as risk factors for dental implant failure: a meta-analysis. PloS one. 2013;8(8). doi:10.1371/journal.pone.0071955.
98. Atluri K LJ, Seabold D, Elangovan S, Salem A. Gene activated titanium surfaces promote in vitro osteogenesis. Int J Oral Maxillofac Implants. 2016: In Press.
99. Elangovan S, Khorsand B, Do AV, *et al*. Chemically modified RNA activated matrices enhance bone regeneration. J Control Rel : Off J Control Rel Soc. 2015;218:22–8. doi:10.1016/j.jconrel.2015.09.050.
100. Balmayor ER, Geiger JP, Aneja MK, *et al*. Chemically modified RNA induces osteogenesis of stem cells and human tissue explants as well as accelerates bone healing in rats. Biomaterials. 2016;87:131–46. doi:10.1016/j.biomaterials.2016.02.018.
101. Langer R. Drug delivery and targeting. Nature. 1998;392(6679 Suppl):5–10.
102. Terrell TG, Working PK, Chow CP, Green JD. Pathology of recombinant human transforming growth factor-beta 1 in rats and rabbits. Int Rev Exp Pathol. 1993;34 Pt B:43–67.
103. Horn NA, Meek JA, Budahazi G, Marquet M. Cancer gene therapy using plasmid DNA: purification of DNA for human clinical trials. Hum Gene Ther. 1995;6(5):565–73. doi:10.1089/hum.1995.6.5-565.