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Emerging local delivery strategies to enhance bone regeneration

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Abstract

In orthopedics and dentistry there is an increasing need for novel biomaterials and clinical strategies to achieve predictable bone regeneration. These novel molecular strategies have the potential to eliminate the limitations of currently available approaches. Specifically, they have the potential to reduce or eliminate the need to harvest autogenous bone, and the overall complexity of the clinical procedures. In this review, emerging tissue engineering strategies that have been, or are currently being, developed based on the current understanding of bone biology, development and wound healing will be discussed. In particular, protein/peptide based approaches, DNA/RNA therapeutics, cell therapy, and the use of exosomes will be briefly covered. The review ends with a summary of the current status of these approaches, their clinical translational potentials and their challenges.

Keywords

tissue engineering; bone regeneration; growth factors; gene therapy; exosomes

1. Introduction

Bone is a sophisticated connective tissue that primarily comprises a mineralized organic matrix with remaining contributions from organic components and water. Bone protects the internal organs from external forces and acts as an effective reservoir of key minerals such as calcium and phosphorus that are involved in a multitude of functions in the human body. In addition, native bone cells secrete hormones such as fibroblast growth factor 23 (FGF23) that regulate phosphorous excretion, also making bone an endocrine organ [1]. Further, bone houses the red bone marrow, where hematopoiesis occurs. In humans, bone develops by one of the following two mechanisms: intramembranous or endochondral ossification.

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In the intramembranous bone formation, the mesenchymal stem cells (MSCs) consolidate and directly differentiate into osteoblasts which will lay down osteoid (composed primarily of type I collagen and other ground substances), that mineralizes first to woven bone and eventually matures into lamellar bone. Flat bones such as bones of the skull, scapula and clavicle develop by intramembranous bone formation. In contrast, in the endochondral bone formation process, the MSCs differentiate first to chondroblasts which generates a cartilaginous analogue of the future bone, which will then be replaced by bone. Most of the long bones in the human body develop by this latter mechanism [2].

Bone modeling is the process by which the bone gets its form and shape. In this process, bone formation is uncoupled, meaning the bone formation and resorption occur at different sites as independent processes. This occurs primarily in childhood but continues throughout the lifetime of an individual. The net effect of bone modeling is an increase in bone mass and attainment of specific shapes [3]. In contrast, bone remodeling is the process that occurs throughout the life time of an individual and in this case, the resorption and formation are coupled, interconnected and occur at the same site. This process actively remodels 2%–5% of the cortical bone every year [4], [5]. Though bone resorption and formation are balanced initially, with age, the resorption dominates, with a net decrease in bone mass. In conditions such as osteoporosis, the resorption is even more pronounced making the individual prone to fractures. Both in modeling and remodeling, key bone cells such as osteoblasts, osteoclasts and osteocytes all play a significant role, orchestrating the events [3], [6].

In addition to conditions such as osteoporosis where the bone formation–resorption balance is affected, there are a myriad of medical and dental conditions such as trauma, malignancy or periodontal disease, where bone loss is imminent. Loss of bone, depending on the condition, can affect humans in several ways, ranging from increasing the fracture tendency to loss of a tooth or multiple teeth with associated social and economic implications. Though bone has significant regenerative potential, surgical intervention and use of a bone replacement grant is warranted in defects that cannot heal on their own. Throughout the world, a significant number of such procedures are done in medicine and dentistry that involve the use of bone replacement grafts. In fact, next to blood, bone is the second most transplanted tissue in humans and according to one estimate, over 2.3 billion dollars were attributed to bone replacement grafts in the United States in the year 2015 and is expected to grow beyond 3.5 billion dollars by 2024 [7]. In dentistry, with the growing number of dental implants being placed globally, the need to predictably regenerate bone has also increased substantially [8].

The inherent limitations of using autogenous bone (from the same patient) are the need to harvest bone from a second surgical site (and its associated morbidity) and the lack of availability. The alternatives to autogenous grafts include allografts, xenografts and alloplasts and they come with their own set of limitations and barriers, including the lack of inherent osteogenic potential leading to unpredictability in attaining clinical outcomes. In addition, in spite of stringent quality and infection control followed by the tissue banks, a small possibility of infection from the use of allografts cannot be completely ruled out [9].

2. Bone tissue engineering

Tissue engineering is an interdisciplinary field where the principles of engineering and life sciences are applied with the main objective of developing biological substitutes that can restore, maintain, or improve tissue function [10]. In order to engineer a tissue, it is critical to understand the biology of the tissue from two different perspectives—development and wound healing. A thorough understanding of the components and key players in the above-mentioned processes has enabled scientists to develop materials and strategies with the potential to achieve specific objectives. This process of going back to nature and being inspired by biology and bringing the time-tested processes to the lab is termed 'biomimicry' and the resulting biomaterials termed 'biomimetic materials'. As of now, the majority of studies that took the biomimetic approach have utilized the deconstructive approach, in which the individual players in a particular process are first identified, followed by elucidation of the functions of each of these players. The major challenge with this approach that focuses on only one entity at a time is to finally stitch all the gained information from these separate investigations together to come up with an all-encompassing strategy that can enhance the outcome. In the following sections, select tissue engineering strategies are discussed broadly (figure 1), in the context of bone regeneration. The advantages and limitations of each of the approaches described in this review are summarized in table 1.

2.1. Protein- and lipid-based approaches

Bone regeneration is a complex and dynamic process in which several players are involved. Apart from cells and matrix/scaffolds, numerous proteins in the form of multifunctional cytokines and growth factors play their definite roles at specific time points in an organized spatiotemporal fashion. BMPs are one such group of growth factors that belong to the TGF-β superfamily and play an important role in bone formation and bone homeostasis in adults. Though there are approximately 20 different BMPs that have been identified to date, BMP-2 is the most studied and tested for its bone regeneration potential. After the demonstration of bone regenerative capacity of BMP-2 in preclinical studies, several human clinical trials that followed evaluated its clinical efficacy in humans [11], [12]. Currently, recombinant human BMP-2(rhBMP-2) is cleared by the food and drug administration (FDA) of U.S.A for sinus augmentation and ridge preservation indications after its safety and efficacy were demonstrated in clinical trials. The major limitation with the use of rhBMP-2 is the high cost associated with its production. Additionally, in order to compensate for the reduced bioavailability of rhBMP-2 at the target site (due to proteolysis mediated rapid clearance), it is employed in supraphysiological doses, raising safety concerns. Though not reported in dentistry, several adverse effects including death from respiratory difficulty related to postoperative inflammation and edema were reported in the orthopedic field [13], [14]. It is also important to note that *in vitro*, rhBMP-2 increased the invasiveness and metastatic nature of oral squamous cell carcinoma cell lines [15].

Apart from rhBMP-2, several other proteins and peptides have been explored in preclinical and human clinical research for their role in expediting fracture healing and bone regeneration. Table 2 summarizes some of the explored protein and peptide agents. Another attractive strategy that is currently being explored is antibody mediated osseous regeneration

(AMOR). The idea behind this is to immobilize anti-BMP-2 antibodies in the scaffold that could capture endogenous BMP-2 protein molecules at the implant site *in vivo* and enhance bone regeneration. Few recent reports have shown promising bone regeneration outcomes using this approach in calvarial bone defects in animals [16], [17].

Apart from proteins and peptides that are currently being studied for their role as active agents of bone regeneration, endogenous lipid mediators that are involved in inflammation resolution are also currently being explored. Lipoxin A4 (LxA4), resolvin E1 (RvE1) and, more recently, benzo-lipoxin A4, a stable analog of lipoxin A, were all shown to be effective in regenerating bone in a preclinical periodontitis model [18–21]. These novel strategies underscore the importance of inflammation resolution in tissue regeneration and will play an important role in bone tissue engineering in the near future.

2.2. Gene (DNA)-based approaches

Gene therapy is the process of introducing exogenous deoxyribonucleic acid (DNA) encoding specific target proteins into cells of various tissues and converting them into protein synthesizing units. Once the DNA is taken up by cells, the DNA has to travel through the cytoplasm and enter into the nucleus, where they go through the process of DNA transcription into RNA, followed by translation of RNA to the protein of interest (in the cytoplasm) [22]. Gene therapy can be accomplished by *in vivo* or *ex vivo* approaches (figure 2). In the *in vivo* approach, the DNA uptake is made to occur inside the body at the target site, while, in the *ex vivo* approach, the target DNA is introduced first into the target cells in a controlled environment outside the body and then the cells containing the exogenous DNA are introduced into the site of interest, usually along with a scaffold/carrier. The advantage of this latter approach is that the number of cells that take up the delivered DNA molecules can be better controlled and it is targeted to specific cell types. But the downsides of this approach are higher costs and the impracticality of the approach, hampering its clinical translation.

The cells can take up the DNA by itself, but to promote its uptake by cells, it is customary to complex DNA with a vehicle or vector that is often cationic to facilitate electrostatic binding with the DNA. Refer to figure 3(a) for an illustration of the underlying mechanism of nonviral gene therapy. The vectors can be of two types: viral and nonviral and, accordingly, the techniques that utilize them are termed viral and nonviral gene therapy, respectively. Viruses have the inherent ability to infect cells and viral gene therapy harnesses this innate efficient capacity of viruses to introduce the target DNA (by a process called ‘transduction’) into cells. The goal in this approach is to eliminate the pathogenicity of the virus but retain its transduction efficacy. To date, in the field of viral gene therapy, different vectors have been explored, including adenovirus, adeno-associated virus, and herpes simplex virus. To achieve bone regeneration, several studies in the past had utilized viral vectors to efficiently deliver DNA encoding growth factors or morphogens such as bone morphogenetic proteins (BMPs) [23], [24], LIM-domain proteins (LMPs) [25], Runx2 [26], and cyclooxygenase-2 [27]. A large majority of these preclinical studies utilized the *ex vivo* approach of gene delivery and demonstrated a good degree of successful bone regeneration. As mentioned before, though the *ex vivo* approach is more controlled and targeted, the cost associated

with this approach will be much higher, than *in vivo* approaches. Preclinical studies using viral vectors encoding platelet delivered growth factor (PDGF) and employing an *in vivo* approach, also demonstrated enhanced bone regeneration in alveolar and peri-implant bony defects [28], [29].For detailed information on viral gene therapy for musculo-skeletal tissue engineering, the reader can refer to detailed reviews on this topic [30], [31].

In nonviral gene therapy, instead of viral particles, several synthetic vectors are being explored, including polymers, liposomes and other poly-cations. The goal here is to select a highly positively charged vector that when allowed to interact with negatively charged DNA, forms stable nano-sized complexes with an overall net positive charge that will enhance its uptake (along with the DNA) by cells (by a process called 'transfection'), that have negatively charged cell membranes. 'Transfection' is a nonviral counterpart of 'transduction' and transfection efficiency denotes the efficiency of DNA uptake by cells. An earlier study that explored nonviral gene therapy for bone regeneration utilized plasmid DNAs encoding BMP-4 and/or first 34 amino acids containing peptide of parathyroid hormone and demonstrated successful bone regeneration, when delivered separately and synergistic bone formation, when delivered together [32]. They did not use any vector to deliver the DNA of interest but rather used a collagen scaffold to tether the DNA molecules ('naked DNA') that were directly implanted into the bony defects.

Polyethylenimine (PEI), a cationic polymer is one of the most efficient and most commonly employed nonviral gene delivery vectors [33]. A gene activated matrix (GAM) was developed targeting bone regeneration but this time, instead of naked DNA, nano-sized complexes of PEI-DNA was synthesized by allowing the interactions of positively charged PEI molecules with negatively charged DNA molecules. Then, these nano complexes were distributed within a collagen matrix and were thoroughly characterized [34]. When this GAM strategy was applied *in vivo* in a rat calvarial bone defect model, defects implanted with collagen scaffold containing PEI-DNA (PDGF-B) complexes showed significantly more new bone formation than other control groups tested [35]. Such collagen matrices that carry DNA into the defect are commonly termed gene activated matrices or GAMs. Refer to figure 1 for a mechanistic illustration of GAM. Gene therapy, though promising, has its own challenges. Viral gene therapy, though shown to be safe in the preclinical studies, is plagued by safety concerns, whereas, in nonviral gene therapy, the lower transfection efficiency, compared to its viral counterpart, is the major limiting factor. For detailed information on nonviral gene therapy for periodontal regeneration indication, the reader is referred to a comprehensive review on this topic [36]. In order to enhance transfection efficiency, physical methods such as electroporation [37] and sonoporation [38] are currently being explored in preclinical fracture models with encouraging results, in the context of bone regeneration. Electroporation or sonoporation is known to increase the cellular permeability, which in turn increases the uptake of DNA by cells. Table 3 summarizes some of the preclinical studies that employed viral and nonviral gene therapy for bone regeneration application.

2.3. Transcript-based approaches

As discussed in section 2.2, in gene therapy, the DNA that enters the cells has to cross the nuclear membrane to reach the nucleus which is the primary site of action (figure 2). Of the different steps involved, the final step of nuclear entry by the DNA molecule is considered to be a rate limiting step in gene therapy, especially in nonviral approaches. The entry occurs effectively during cell division and therefore, in nondividing cells, DNA entry is hindered. To overcome the barriers associated with DNA therapy, several research groups explored the possibility of using RNA encoding the target protein, instead of DNA. As the name suggests, in transcript (RNA) therapy, the site of action is cytoplasm and not the nucleus and therefore nuclear entry is not required. As soon as the RNA enters the cells, they can be directly translated by ribosomes into proteins [58]. Refer to figure 3(b) for a mechanistic illustration of cmRNA therapy.

RNA by itself is unstable, immunogenic and also needs modifications to simulate its post-translational modifications for it to be translated into proteins. Over the last decade, with the help of several investigations, it is clear now that with specific modifications in RNA, we can improve its stability, translation ability and at the same time, reduce its immunogenicity [59]. The resulting RNA is termed by some as chemically modified RNA or simply cmRNA.

It was successfully demonstrated that by implanting collagen scaffolds containing complexes of chemically modified RNA (cmRNA) encoding BMP-2 and PEI (as a vector) in rat calvarial defects, significantly higher new bone formation was observed [60]. In this first-time demonstration of the use of cmRNA for a tissue engineering application, it was shown that the PEI-cmRNA (BMP-2) group outperformed its DNA counterpart [PEI-pDNA (BMP-2)] in several parameters assessed, both *in vitro* and *in vivo*. Subsequent publications from different research groups focusing on cmRNA as the regenerative agent further validated this strategy [61–66]. In a follow-up study, we compared cmRNA (BMP-2) with cmRNA (BMP-9) for their effectiveness to regenerate bone. Though cmRNA (BMP-9) demonstrated superior *in vitro* osteogenic potential, *in vivo*, the bone volume regenerated was not statistically higher than in the cmRNA (BMP-2) group highlighting that cmRNA delivery is a platform technology that can be used to deliver different growth factors effectively [67]. Apart from cmRNA, RNA interference, a process by which the gene expression is regulated by small RNAs such as small interfering RNA (siRNA) can be successfully harnessed to regenerate bone [68]. The use of siRNA is based on the idea of delivering these small RNA molecules to inhibit the expression of select genes such as noggin to enhance bone regeneration [69]. Additionally, microRNAs, the endogenous small, noncoding RNAs that play a role in gene regulation at the post-transcriptional level can be successfully employed for bone regeneration [70], [71]. Several *in vivo* studies using a range of bone defect models including calvarial defects and employing a variety of scaffolds to deliver different microRNAs or its antagonists, assessed their efficacy in the context of bone regeneration [72–75].

2.4. Exosomes

Exosomes are extracellular vesicles that are 30–150 nm in size [76]. Their size, nature of formation and composition distinguish these vesicles from other vesicles secreted by

the cells. Exosomes are formed in multivesicle compartments by the inward budding of the endosomal membrane and subsequently secreted by fusion of the vesicle to the plasma membrane. Multiple cellular mediators have been shown to trigger this process. Based on their origin and components, several subsets of exosomes could be identified. However, generally exosomes contain RNA, microRNA(miRNA) and DNA, along with plasma membrane and cytosolic proteins [77]. These nano packets of information can be endocytosed by effector cells to trigger a cellular response designated by the parental cell to the effector cell [78]. Although originally believed to be mediators of cellular homeostasis by secreting cellular waste [79], recent studies on exosome function have highlighted their important roles in modulating cellular aspects of immunology, cancer biology and regenerative medicine [79], [80].

Within this exosomal membrane, RNA (both messenger (Mrna) and miRNA), cytosolic proteins as well as trans membrane proteins are present [81]. Amongst the various exosome constituents, miRNA and noncoding RNA have been reported to be in abundance [82]. These miRNA act as regulators of signaling pathways, including those involved in repair and regeneration. Further, the nuclear and cytosolic protein content of the exosomes is selective and representative of the parent cell. Thus, there exists the potential to engineer the parent cell for the release of function specific exosomes, which can in turn be utilized to direct exosome-mediated changes in target cells. Studies have shown the potential of MSC derived exosomes to control various aspects of regeneration including, proliferation, differentiation [83], migration [84], angiogenesis [85] and apoptosis [86].

Depending on the source and target cell type, exosomes are endocytosed by either clathrin or cave-olin mediated endocytosis [87]. This process triggers endocytosis mediated signaling cascades in target cells mediated by the extracellular receptor kinase family (ERK) and mitogen activated protein kinase family (MAPK) [88], [89]. The endocytosis of exosomes also results in the transference of their miRNA and protein cargo intracellularly [78]. After this discovery, there has been an increased focus on their applications in regenerative medicine as inducers of cell proliferation [90], angiogenesis [91], [92] and as immunomodulators for cancer therapy [93–95]. The miRNA and protein composition of the exosome is unique to the parent cell type it is sourced from and can vary in content and activity depending on the state of the source cell [96], [97].

Tissue engineering strategies have aimed at developing novel biomaterials for bone regeneration applications for the past several years. Although many innovative strategies have been developed, none have been able to successfully replace existing bone graft materials. Despite the various drawbacks that bone grafts materials possess including donor and batch dependent variability in consistency [98] that results in unpredictable clinical performance, they continue to remain the primary clinical choice of material for regenerative applications. Emerging evidence indicates that transplanted MSCs act in a paracrine manner [99–101] exerting their influence through extracellular vesicles [94], [102], [103]. Therefore, there exists a possibility for MSCs to serve as a viable alternative to stem cell therapy. As one approach, various cell types, including MSCs can be genetically modified to generate exosomes with function-specific constituents, like specific miRNAs in exosomes.

Such technical innovations differ from conventional exosomal delivery approaches and embrace the innate characteristics of the source exosomes by not considering them as delivery vehicles alone. Recent studies provide positive evidence for the long-term stability of exosomes [95], [104] and their ability to protect against immune rejection and graft versus host disease [94], [105]. Hence, the use of exosomes in medicine can have far-reaching implications not only in the field of regenerative medicine but also in other therapies as well.

2.5. Stem cell-based approaches

Stem cells are defined by their self-renewal and multi-potency (ability to differentiate into specialized cell types) characteristics. Adult stem cells that exist within the bone are what contribute to its inherent regenerative capacity. The idea behind using adult MSCs such as bone marrow derived stromal/stem cells (BMSCs) for bone regeneration is that by increasing the number of these cells (the key players in bone regeneration) at the local target site, we can promote regeneration. Though it sounds simple, there are several challenges that have to be overcome to make this an effective and practical approach. The challenges start with identification of the right kind of cells. It is known that only a small percentage of cells that exist in bone marrow aspirates are true bone progenitor cells and that their number varies significantly [106]. This led to explorations on ways to increase the concentration of progenitor cells that can be used in bone tissue engineering. One approach is to expand the cells *ex vivo* to increase its numbers and then regraft them into the defect site. Many additional strategies were proposed to enhance the proliferative potential of these cells such as addition of growth factors, extracellular matrix and dynamic culture environment [82], [83], [107], [108]. Over-expressing human telomerase reverse transcriptase gene in adult stem cells was also attempted to eliminate replicative senescence [109]. Ways to increase the differentiation potential of these adult mesenchymal cells and their targeting for bone regeneration are also currently being investigated [108]. Other adult stem cells such as adipose derived stem cells (ADSCs) are also considered good candidate stem cells for bone tissue engineering applications [110]. ADSCs are an attractive alternative to BMSCs as the yield of these cells from the adipose aspirates is higher than BMSCs yield from bone marrow aspirates [111]. The other potential candidate is iPS cells, which are pluripotent and therefore have to be osteo-differentiated before use to avoid tumorigenesis [112], [113]. For more information on stem cells use in tissue engineering, refer to a detailed review on this topic [106].

In dentistry, cell-based therapeutics for bone regeneration is being explored in humans for alveolar bone augmentation/preservation and sinus augmentation indications [114]. In an earlier study, using a combination of PRP, mononuclear cells from bone marrow and bone scaffold, researchers successfully demonstrated the safety and applicability of cell therapy to achieve maxillary bone augmentation in humans, prior to dental implant placement [115]. In a separate first-in-human randomized controlled clinical trial (RCT), implantation of tissue repair cells (mixture of expanded MSCs (CD90 +), monocytes/macrophages (CD14 +) and mononuclear cells from the original bone marrow aspirate) delivered in a gelatin sponge into extraction sockets resulted in significantly more bone regeneration, than the saline soaked sponge (control) group [116]. In a separate human RCT with a one year follow-up,

researchers delivered MSCs (CD90 +) with β-tricalcium phosphate (TCP) scaffold, and successfully demonstrated the safety and efficacy of this approach in regenerating new bone in the maxillary sinus lift procedure [117].

2.6. Scaffolds

Scaffolds form an integral component of tissue engineering. They provide the necessary support system and conduit for the cells and the neovasculature to makes its way through, favoring regeneration. Materials to restore bony defects and deformities have evolved over time with first generation materials primarily made out of metals mainly to restore physical functions to a more recent generation of biomaterials that have the capacity to restore both form and function. To be effective, scaffolds for bone tissue engineering should possess certain characteristics and satisfy certain requirements. Being biodegradable and biocompatible are biological requirements of a scaffold, while adequate mechanical properties, space maintenance, pore size and interconnectivity of pores within a scaffold are required physical and structural properties of a scaffold for bone tissue engineering [118].

Apart from the physical characteristics of the scaffold that influence regeneration, it is now clear that the rigidity of the scaffold can directly influence the path of differentiation of MSCs. In a classic study, Engler and coworkers have shown that human MSCs cultured on soft collagen coated gels (similar to brain) committed to a neurogenic lineage, while MSCs cultured on rigid matrices (mimicking bone) committed to an osteogenic lineage [119].

Broadly, scaffolds can be classified based on the source and composition into two kinds: natural and synthetic. Some examples of naturally derived polymers used as scaffolds include collagen, chitosan, silk and alginate. Additionally, a wide range of synthetic materials from polymers such as poly-L-co-D, L-lactide to ceramics such as hydroxyapatite are being explored for their use in bone regeneration. For detailed information on the design characteristics and type of scaffolds, readers are referred to a recent comprehensive review on this topic [120].

Apart from utilizing scaffolds as a passive temporary physical structure, several studies explored the possibility of utilizing them as drug and biomolecule delivery systems, thereby making them biologically active. Three common incorporation strategies include covalent binding, physical entrapment or adsorption and incorporation into micro/nanospheres that are then incorporated into the scaffold [118]. Each of these strategies will result in a specific type of release profile of the active ingredient with the latter approach providing better control over the release rate of the growth factor and also allows for the release of multiple factors, or the sequential release of factors [118]. A study demonstrated enhancement in osteogenesis in the rabbit bone defect model, when they used a hyaluronic hydrogel that physically entrapped growth and differentiation factor-5 (GDF-5) and released the factor in a sustained manner for up to 28 d [121]. In a different study, poly(lactic-co-glycolic acid) (PLGA) nanoparticles and alginate microcapsules delivered from collagen was utilized to deliver BMP-2 and vascular endothelial growth factor (VEGF), that resulted in enhanced bone regeneration in the rat calvarial defect model [122]. The type of delivery system is selected based on the type of factor or biomolecule that is delivered and its biological requirement.

Past studies in this field incorporated the following factors with scaffolds intended for bone regeneration: BMP-2, VEGF, VEGF + FGF-2, VEGF + BMP-2, PDGF, BMP-2+TGFβ, BMP-6+TGFβ_3 or BMP-9 [123]. As mentioned in section 2.1, the important issue with proteins such as growth factors is the rapid decline in the therapeutic concentration at the implanted site due to proteolysis and degradation. Scaffolds can be designed to release growth factors gradually rather than releasing them in bulk. This will also reduce the need for higher dosage of growth factors, thereby one can potentially reduce its adverse effects [96], [124]. Several different strategies of incorporating growth factors into scaffolds for bone regeneration have been explored, including bulk incorporation, biomimetic binding, surface adsorption, multilayer coating, nanoparticles, and biomineralization [125].

In addition to GDF, natural and predominantly synthetic scaffolds incorporating several different types of antimicrobials have also been explored both *in vitro* and *in vivo* [126]. Some of the antibiotics include gentamycin, tetracycline, vancomycin, and ciprofloxacin. In addition, other pharmacological agents such as dexamethasone, ibuprofen, and bisphosphonates such as alendronate and zolen-dronate were also successfully incorporated into scaffolds [126]. It is also clear from studies that local delivery of systemic bone anabolic medications such as teriparatide and abaloparatide can result in local bone regeneration [127], [128]. A recent study conjugated isoniazid onto synthetic polymer scaffold as a means to achieve bone regeneration and to treat bone tuberculosis [129]. It is therefore clear that there are significant ongoing efforts to make scaffolds bioactive by way of incorporating different biological and pharmaceutical agents . There are also efforts to develop ‘smart biomaterials’, which will respond to external stimuli like light, changes in pH or temperature. The responsiveness of these smart materials can be exploited *in vivo* to release a specific factor at the appropriate time point [130], but the translatability of these novel drug and biomolecule eluting scaffolds to clinics will depend heavily on their performance in human clinical trials, in the context of their safety and efficacy.

The other exciting and rapidly evolving area in the development of novel scaffolds for bone tissue engineering is 3D bioprinting. It typically involves the following steps [131]: 1. Computer aided design of the construct to be printed for the required dimensions and other specifications (type of cells, type of matrix etc.), 2. Printing the construct. 3. Culturing the construct (if it contains cells) in a bioreactor before implantation. Broadly, there are three ways by which bone bioprinting can be performed [131]. Inkjet printing uses special printer heads to print the scaffold initially as liquid that hardens over time. Bioplotting is similar to inkjet printing but, in this case, a continuous filament of the starting material will be injected out of a syringe [132]. The third utilizes stereo lithography, where the constructs are created one layer at a time and they are made to solidify by curing with an ultra-violet light source. A recent study tested the incorporation of human BMSCs in a 3D bioprinted polysaccharide-based hydrogel and demonstrated that not only did the BMSCs survive but they also successfully differentiated into the osteogenic lineage [133]. Preclinical testing of commercially available 3D printed synthetic bone graft (without cells) and in-house printed synthetic 3D scaffold with bone marrow progenitor cells led to encouraging results [134], [135].

3. Conclusions

Tissue engineering is a rapidly expanding area and products of tissue engineering are making their way into clinical practice after regulatory approvals, including products for bone tissue engineering. One good example is the introduction and current usage of rhBMP-2 for oral and craniomaxillofacial applications. Though the field of bone tissue engineering is growing, several challenges still exist. As mentioned earlier, the deconstructive approach of testing one component at a time has its advantages and limitations. Though such approaches are much more straightforward to evaluate and to get the regulatory clearance, an all-encompassing strategy that includes all the key players such as biomimetic scaffolds, cells and mediators is often required to take the predictability and regenerative potential to the next level [114]. The other challenges with existing strategies are the high manufacturing cost and low efficiency of some of the laboratory techniques such as cell isolation, culture/expansion, seeding, and 3D bioprinting. Therefore, improving the cost-effectiveness of these laboratory techniques is critical for the success of future bone tissue engineering products.

The oral cavity is easily accessible and therefore a good system to test the products of bone tissue engineering but the constant presence of oral microbes during the healing process makes it a challenging model. Therefore, considerations regarding the effect of microbes and their products and the role of inflammation resolution on tissue regeneration should be given, when developing future materials and strategies, especially of dental applications. With regard to novel materials for critical sized defects, striking the right balance between sound mechanical and biological properties is a constant challenge. Though we are successful in regenerating smaller defects, the difficulty is in scaling up the strategy to treat larger critical sized defects with high predictability, *in vivo*. In addition, when it comes to preclinical testing of novel materials, using larger animal models will enhance the validity and translatability of the findings to human situations and can therefore hasten the rate of clinical translation.

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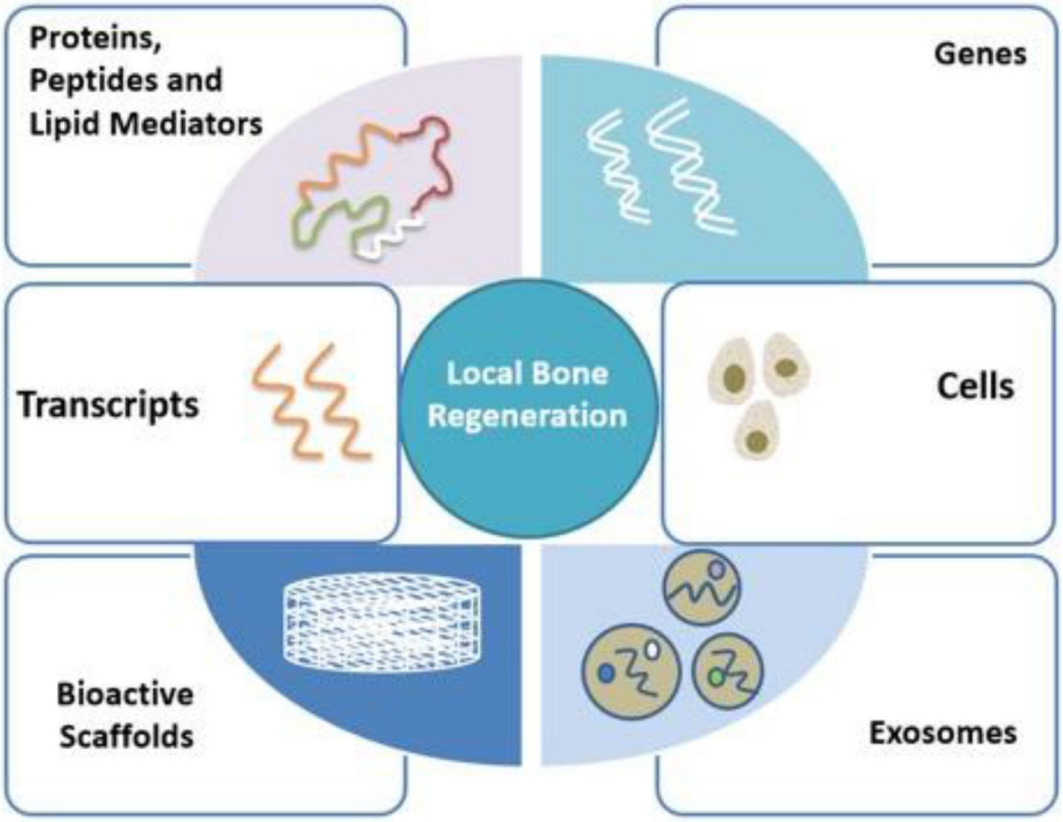

Figure 1.
Schematic depicting the locally deliverable tissue engineering approaches for bone regeneration.

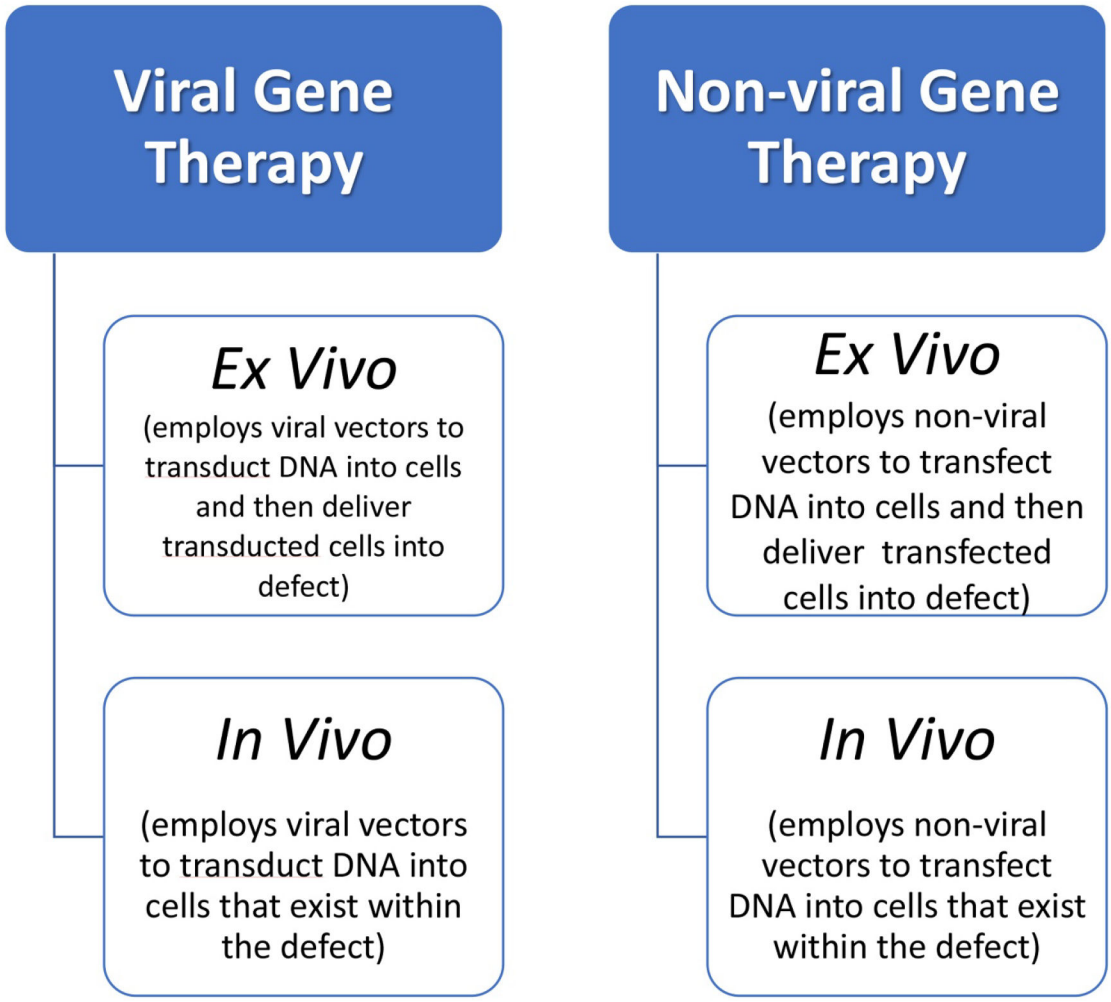

Figure 2.
Schematic depicting the types of gene therapy approaches.

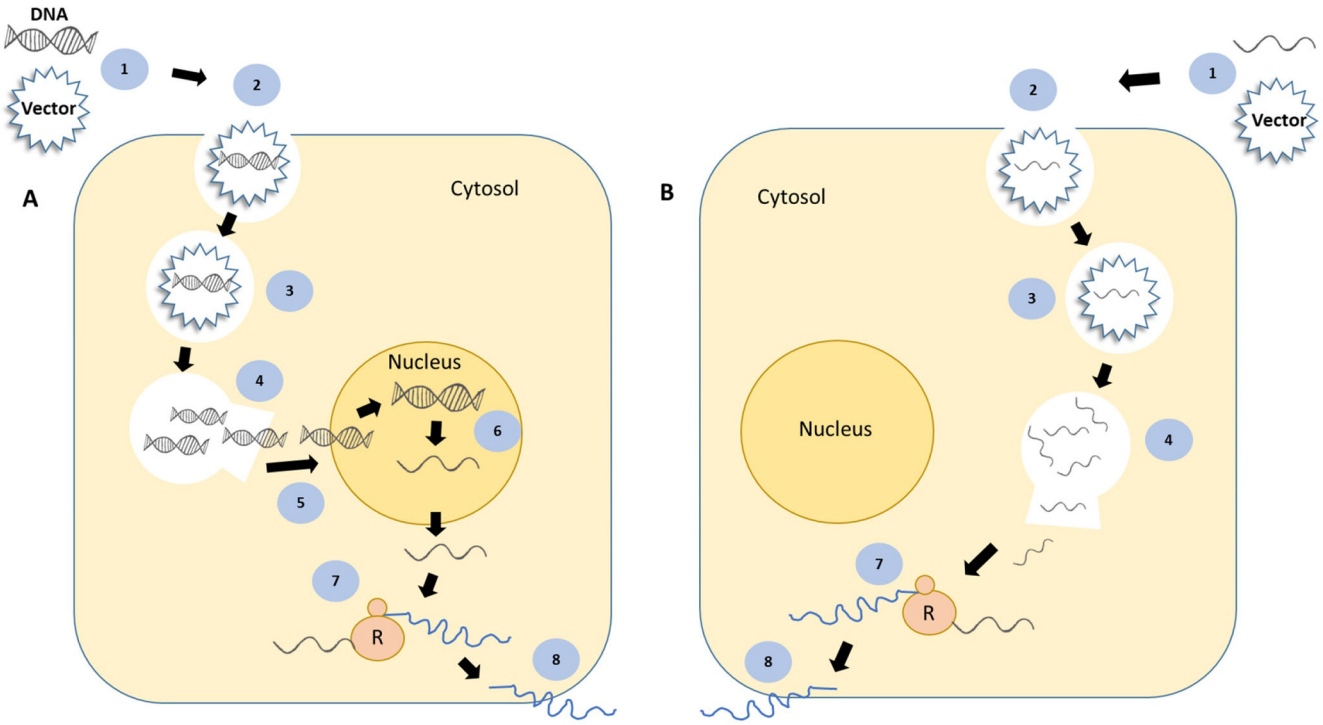

Figure 3.

Schematic illustrating the mechanism of action of gene (A) and transcript (B) therapeutics. The steps common to both the strategies are: complexation of DNA or RNA with a vector (1), endocytosis and cellular entry (2), presence in endo-lysosome (3), DNA/RNA escape from endo/lysosome (4), translation of RNA into protein by ribosomes (R) (7) and protein secretion and release (8). The steps unique to gene therapy are nuclear entry of DNA (5) and transcription of DNA into RNA (6).

Table 1.

Summary of key advantages and limitations of the approaches described in this review.

Approach	Advantages	Limitations
Protein-based therapeutics	• Potent stimulant of bone regeneration • Ease of use • Long history of use in humans	• High cost • Low bioavailability • Used in supraphysiological dose • Adverse effects
Gene-based therapeutics viral approaches	• Effective transduction of cells • Allows for sustained local delivery of proteins • Gene expression is regulated	• Safety concerns • Insertional mutagenesis risk (integration of DNA with the host genome)
Non-viral approaches	• Safe and inexpensive • Ease of manufacture	• Lower transfection of cells • Some nonviral vectors can exhibit cytotoxicity
Transcript-based approaches	• Lack of need for nuclear entry • Works well on dividing and nondividing cells	• High cost • Needs chemical modification to improve stability and translation
Cell-based approaches	• Can self-renew, proliferate and differentiate (stem cells) • Lower immune rejection (adult stem cells)	• High cost and limited availability • Procurement and isolation issues • Possible immune rejections (embryonic stem cells) • Ethical dilemmas
Bioactive scaffolds	• Can successfully deliver single or multiple regenerative factors or other molecules like antibiotics at the implanted site • Provides mechanical support	• Inert by themselves • Some scaffolds resorb very slowly inhibiting regeneration • High cost (for 3D printed scaffolds)
Exosomes	• Natural drug and molecular delivery system • High specificity and efficiency	• Difficulty in separation and characterization • Lack of distinct biomarkers

Table 2.

Proteins and peptides explored for their role in bone regeneration.

Type	Explored proteins and peptides (examples)
Growth factors/morphogens	
Single factor [16], [17]	Erthropoietin, PDGF-BB, b-FGF, SDF-1, VEGF, wnt-3, β-catenin, BMP-2, and BMP-7.
Multiple factors [17]	SDF-1 + BMP-2, BMP-2 + VEGF, and BMP-2 + TGF-β.
Mixture of factors [18], [19]	Platelet rich plasma, platelet gels and platelet concentrates.
Peptides [16], [20]	PTH-Rp, calcitonin gene-related peptide, osteogenic growth peptide, thrombin peptide 508, PepGen P-15 and RGD containing peptides.
Antibodies and inhibitors [17]	Anti-TNF-α antibody, anti-sclerostin antibody, RANKL inhibitor.

Abbreviations: PDGF-BB: platelet derived growth factor—BB; b-FGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; SDF-1: stromal cell derived factor-1; TGF-β: transforming growth factor—beta; PTH-Rp: parathyroid hormone related peptide; RGD: arginine, glycine, and aspartate; TNF-α: tumor necrosis factor—alpha; RANKL: receptor activator of nuclear factor-kappa B ligand.

Table 3.

Select preclinical studies that assessed viral and nonviral gene therapy approaches for bone regeneration.

Viral gene therapy studies				
Approach *(in vivo or ex vivo)*	**Vector**	**Animal/model**	**Gene product**	**Reference**
In vivo	Adenovirus	New Zealand white rabbit femoral defect	BMP-2	[39]
Ex vivo (bone marrow cells)	Adenovirus	Rat femoral defect	BMP-2	[40]
Ex vivo (bone marrow stromal cells)	Adenovirus	Rat mandibular defect	BMP-2	[41]
Ex vivo (bone marrow derived MSCs)	Modified adenovirus vector	Rat subcutaneous ectopic bone formation	BMP-2	[42]
In vivo	Adenovirus	Rat femoral defect	VEGF-A	[43]
Ex vivo (adipose derived MSCs)	Adenovirus	Rat femoral defect	BMP-2	[44]
Ex vivo (bone marrow derived MSCs)	Adenovirus	Goat tibial defect	BMP-2	[45]
Ex vivo (MSCs)	Adenovirus	Osteoporotic female sheep tibial defect	BMP-2	[46]
In vivo	Adenovirus	Rat femoral defect	BMP-2	[23]
In vivo	Adenovirus	Rat femoral defect	BMP-2	[47]
In vivo	Adenovirus	Rat femoral defect	Runx-2	[48]
Ex vivo (dermal fibroblasts)	Adenovirus	Equine metacarpal/-metatarsal osteotomies	BMP-2	[49]
Ex vivo (gingival fibroblasts)	Adenovirus	Rat cranial defect	BMP-2	[50]
Nonviral gene therapy studies				
In vivo	Naked plasmids	Rat femoral defect	BMP-4 or PTH (1–34)	[32]
In vivo	PEI modified with linoleic acid	Rat subcutaneous implant model	BMP-2 and b-FGF-2	[51]
In vivo	Cationized gelatin microspheres	Rat cranial defect	BMP-2	[52]
In vivo	PEI and nano HA	Rat cranial defect	BMP-2 and VEGF	[53]
Ex vivo (bone marrow stromal cells)	(K)16GRGDSPC	Rat segmental femoral defect	TGF- β	[54]
In vivo	PEI or FuGENE6	Rat cranial defect	caALK6 and Runx2	[55]
In vivo	Branched triacrylate/amine polycationic polymer	Rat cranial defect	BMP-2	[56]
In vivo	PEI	Rat cranial defect	PDGF-B	[35]
In vivo	PEI	Diabetic rabbit radial defect	FGF-2 and BMP-2	[57]

Abbreviations: hydroxyapatite (HA), activin receptor-like kinase 6 (caALK6) and runt-related transcription factor 2 (Runx2).