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Tissue Engineering Strategies for Promoting Vascularized Bone Regeneration

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Abstract

This review focuses on current tissue engineering strategies for promoting vascularized bone regeneration. We review the role of angiogenic growth factors in promoting vascularized bone regeneration and discuss the different therapeutic strategies for controlled/sustained growth factor delivery. Next, we address the therapeutic uses of stem cells in vascularized bone regeneration. Specifically, this review addresses the concept of co-culture using osteogenic and vasculogenic stem cells, and how adipose derived stem cells compare to bone marrow derived mesenchymal stem cells in the promotion of angiogenesis. We conclude this review with a discussion of a novel approach to bone regeneration through a cartilage intermediate, and discuss why it has the potential to be more effective than traditional bone grafting methods.

Keywords

Bone regeneration; angiogenesis; osteogenesis; controlled release; stem cells; endochondral ossification

1. Introduction

Bone grafts are crucial for the treatment of a number of conditions that represent a great global burden, including segmental bone defects caused by trauma, tumor excision or chronic osteomyelitis, nonunions, avascular necrosis and spinal fusions[1,2]. The current gold standard treatment is autografts. However, these are limited in availability, prolong the operation times and are often associated with donor site morbidity[3]. As an alternative,

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allografts are widely available. However, they place the patients at risk for infections and rejection by the immune system[3]. More importantly, allografts demonstrate decreased integration with the host tissue when compared to autografts[4], with high failure rates of approximately 25% and reaching up to 60% in patients requiring large grafts[5,6]. Allografts, as well as synthetic grafts, are associated with complications such as osteonecrosis[7–9] as these options lack an endogenous vascular network to facilitate host integration. Recently, vascularized bone grafts have been developed to address these limitations[10,11].

The first vascularized bone grafts developed were pedicled grafts, or bone that is transported with its blood supply (or pedicle). Theoretically, such grafts are considered “live”, and include all of the components needed for graft survival and improved bone incorporation, as well as avoid some of the complications associated with allograft, including graft failure and infection. Pedicled grafts, which are commonly used in the treatment of carpal bone pathology[10] and femoral head avascular necrosis[11], remain connected to the blood supply site of origin, and are therefore limited by their need for proximity to the treatment site. Common harvest sites include the iliac crest, greater trochanter or the fibula for the hip, and the distal radius for the carpal bones. Surgeons must also be able to achieve tension free anastomoses, estimate an adequate pedicle length, and continue monitoring the graft site for blood leakage after the procedure in order to avoid serious complications[11].

Free vascularized bone grafts, which keep the vascular connections of vessels to and from a freed segment of bone, were subsequently developed as an alternative to pedicled bone grafts[10]. However, they are technically difficult and have a relatively high rate of failure, involve intricate handling of the vessels, require specialized equipment, and are associated with morbidity at the harvest site. Therefore, given the difficulties with these vascular grafts, neither is widely used clinically, and there is a need for better solutions to promote vascularized bone regeneration.

The emerging field of tissue engineering holds promise for the development of a much simpler solution for vascularized bone regeneration compared to the complicated pedicled and free vascularized bone grafts while overcoming the problems of structural integration and limited revascularization associated with allografts. In this review, we address the currently available tissue engineering strategies that aim to enhance vascularized bone regeneration. We will start by describing the critical role that angiogenic growth factors play in directing neovascularization during bone regeneration and the importance of scaffolds in generating controlled/sustained release. We will then discuss the therapeutic uses of stem cells as an alternative growth factor delivery method for vascularized bone regeneration, and the potential of cartilage scaffolds in promoting better bone regeneration compared to the currently available bone grafts.

2. Angiogenic growth factors in pre-clinical/clinical trials

Tissue regeneration is largely dependent on cell signaling that is mediated by cellular interactions with growth factors. The therapeutic application of growth factors spans many indications including bone regeneration. Growth factors are capable of promoting osteogenesis by recruiting mesenchymal stem cells (MSCs) and inducing osteoblastic

differentiation in addition to stimulating the migration, differentiation and proliferation of stem cells to aid in vascularized bone formation. We will focus on angiogenic growth factors as a means to direct revascularization during bone repair.

2.1. Vascular endothelial growth factor (VEGF)

VEGF has been the target of many investigations for over 3 decades. Scientists identified VEGF target receptors on endothelial cells and found it to be a key player in physiological and pathological angiogenesis[12–15]. Furthermore, the role of VEGF in vascularization as it relates to endochondral bone formation has long been recognized. Hypertrophic chondrocytes in the epiphyseal growth plate express VEGF[16,17], and upon its release, blood vessels invade the cartilage and facilitate bone formation. Experiments with VEGF inactivation showed suppression of blood vessel invasion and significant changes to the growth plate architecture including expansion of the hypertrophic chondrocyte zone[17]. Furthermore, these effects could be reversed by terminating anti-VEGF treatment. Specifically, vascular invasion and resorption of hypertrophic cartilage resumed and the growth plate architecture returned to normal[17].

Subsequently, therapeutic application of VEGF has been proven to enhance both endochondral ossification and intramembranous bone formation. Studies have shown accelerated bone regeneration and improved vascularization with VEGF therapy[18,19] and its critical role in healing has been confirmed by evidence of the development of non-unions in experiments with VEGF inhibition[20]. VEGF is also believed to increase stem cell recruitment to damaged or diseased bone tissue[21,22]. However, the use of VEGF alone does not yield optimal bone regeneration, which is evident from a study comparing VEGF to platelet-rich plasma in the treatment of critical sized bone defects in New Zealand white rabbits[23]. Platelet rich plasma is known to contain VEGF in addition to many other growth factors including platelet derived growth factor (PDGF), transforming growth factor beta 1 and 2 (TGF-β1 & TGF-β2), insulin-like growth factor (IGF), epidermal growth factor, and endothelial cell growth factor[23]. This study concluded that while VEGF was sufficient to improve revascularization, a combination of growth factors led to better bone repair compared to VEGF alone.

The limitation of monotherapy with VEGF therapy has been demonstrated in other studies. A study by Peng *et al* found that VEGF failed to stimulate bone healing in skull defects in rats despite an increased number of endothelial cells in the non-unions[24]. Similarly, in a study of fracture healing, it was shown that the extent of vascularization in the non-union group was comparable to the union group in the early stage of repair and even increased after 14 days. Instead, they found that osteogenic proteins, BMP-2 & BMP-4, were decreased. Taken together these studies suggest that there is an essential balance between angiogenic and osteogenic growth factors during bone healing and excessive concentrations of VEGF may favor endothelial cell differentiation over osteogenesis[25,26].

Also hindering the translation of VEGF-based therapies to the clinic are practical considerations involved with growth factor delivery. VEGF has a short half-life of 6–8 hours[23], which means that controlled delivery is required to ensure sustained activity. Effective VEGF signaling also requires receptor clustering, so designing a delivery system

that enables this biological effect could improve outcomes[27,28]. Conversely, excessive VEGF may put the patients at risk for malignancy, since VEGF is associated with tumor development[29].

2.2 Fibroblast growth factor-2 (FGF-2)

FGF-2 is another growth factor that has been extensively studied for its key role in angiogenesis. It has been proven to play a role in both, the induction of angiogenesis and the mitogenesis of mesenchymal progenitors and osteoblasts[30,31]. A recent review by Hankenson *et al* highlights the considerable number of preclinical studies conducted in both small and large animal models to demonstrate the potential of FGF-2 to promote angiogenesis and improve fracture healing[32]. Importantly, the effectiveness of FGF-2 in the induction of angiogenesis and fracture healing is related to its dosage and release kinetics. Studies have shown the minimum required dose to be 100 μg when delivered as a single dose, but could be decreased to 1.4 μg when methods of controlling its release were used[33,34]. These data emphasize the importance of growth factor delivery methods and controlled release.

2.3 Platelet derived growth factor (PDGF)

A growth factor with demonstrated clinical success is PDGF. It is currently available in the market in the form of a gel for the treatment of chronic foot ulcers in diabetics. It also holds potential in promoting vascularized bone regeneration. In addition to being a chemotactic factor for osteoblasts and stimulating them to undergo proliferation[35], PDGF has also been reported to increase the expression of VEGF in endothelial cells[36]. Systemic administration of recombinant human platelet derived growth factor-BB (rhPDGF-BB) has been shown to improve bone density and biomechanical strength in osteoporotic rat models and improve fracture healing by increasing torsional strength[37,38]. Similarly, locally administered rhPDGF-bb accelerated bone regeneration in a rabbit osteotomy model through an increase in the size and density of the callus[39] and improved bone healing in dogs with periodontal defects[40]. In human clinical studies, rhPDGF-BB has been used in combination with allograft bone matrix to treat advanced periodontal lesions and promote alveolar bone formation[41,42]. Furthermore, a triple blinded randomized controlled clinical trial involving 11 clinical centers and 180 patients demonstrated that rhPDGF-BB applied in combination with a beta-tri-calcium phosphate carrier enhanced bone formation[43].

2.4 Placental growth factor (PlGF)

PlGF is part of the VEGF family and is a VEGF homolog[44]. It acts via activation of VEGFR-1, and is thought to potentiate the angiogenic response to VEGF, although there remains controversy surrounding its mechanism of action and net effect on angiogenesis[45]. Interestingly, when Maes *et al* examined the role of PlGF in fracture healing in mice, they found it to be responsible for recruiting inflammatory cells to the fracture site, a process important to promoting vascularization early during the healing cascade. Moreover, they discovered that it stimulates MSCs to proliferate and differentiate into osteoblasts to aid in bone formation and stimulates osteoclast progenitor cells to differentiate into osteoclasts to aid in bone remodeling[46].

2.5 Insulin-like growth factor (IGF)

IGF has a documented role in regulating endothelial cell migration and tubular formation[47,48], but its role in promoting vascularized bone regeneration remains unclear. *In vivo* studies on segmental defect models in Sprague-Dawley rats[49] and Swiss alpine sheep[50] showed that IGF has the ability to promote fracture healing. Furthermore, IGF is a critical mediator of the skeletal response to parathyroid hormone which has been shown to promote fracture healing[51–53]. However, preliminary data indicate that conditional deletion of IGF from osteocytes may result in accelerated boney bridging of a fracture gap indicating that osteocyte-derived IGF may have an inhibitory role during fracture repair[54]. Further investigation into this model is required to understand the molecular mechanism of IGF during fracture repair, in particular how the timing and source of IGF may influence progression of healing[55].

IGF also plays a documented role in bone growth and development[55], which may have analogous implications on fracture healing. Inhibition of the IGF-1 gene by conditional knockout in mice resulted in decreased trabecular and cortical bone mineral density, shortening of hypertrophic chondrocyte zone in the growth plate, and decreased periosteal expansion, suggesting that IGF affects both endochondral ossification and intramembranous bone formation[54]. In a detailed study of the hypertrophic chondrocyte zone of the growth plate using diffraction phase microscopy, Cooper *et al* found that IGF may play a critical role in coordinating chondrocyte enlargement[56].

2.6 Sonic hedgehog (SHH)

Although classically associated with developmental pathways[57], SHH has also been shown to increase neovascularization. In a study using an ischemic hind limb aged mouse model, Pola *et al* demonstrated that SHH induced significant neovascularization[58]. SHH may induce angiogenesis indirectly by activating VEGF, the angiopoietins (Ang-1 and Ang-2), PDGF-BB, and TGF-β which are important growth factors for vessel stabilization and maturation[58,59]. Furthermore, a co-culture study conducted using human primary osteoblasts and outgrowth endothelial cells found that SHH may offer an advantage over VEGF in that its effects have been observed as early as 24 hours following treatment, indicating a potential therapeutic benefit in accelerating vascularization[59].

SHH may also facilitate osteogenesis in addition to angiogenesis. A series of co-culture studies conducted by Dohle *et al*. showed that SHH increased the expression of many osteogenic differentiation markers, improved mineralization, and increased alkaline phosphatase activity[60]. Similarly, Ho *et al* found multivalent SHH was sufficient to robustly stimulate mitogenesis and differentiation of bone marrow-derived MSCs into an osteoblastic lineage[61].

2.7 Angiopoietins (Ang)

Several recent reviews have discussed angiopoietins and their relationship with the tyrosine kinase receptors (Tie). Tie-1 and Tie-2 receptors are only expressed on vascular endothelial cells and are considered to be crucial for vascular maturation[62–64]. In a study performed on transgenic mice, Thurston *et al* found that the combination of VEGF and Ang-1 resulted in

the formation of blood vessels that were highly differentiated and covered by periendothelial cells. As a result, they were more resistant to leakage when compared to the vessels that were formed in the VEGF only group[65–67]. A review by Fagiani *et al* draws attention to the fact that Ang-1 and VEGF control different aspects of the angiogenesis pathway; Ang-1 stimulates blood vessel remodeling and maturation, whereas VEGF is in charge of vessel development and growth[62]. The effect of Ang-2, on the other hand, is dependent on the presence or absence of VEGF. It is known to enhance neovascularization in the presence of VEGF and to separate the endothelial cells from the perivascular cells, and result in vascular regression in the absence of VEGF[68–70].

The benefits of angiogenic growth factors are numerous. However, despite increasing evidence supporting the role growth factors play in enhancing angiogenesis, the growth factors we have discussed thus far have met with varying degrees of success. Factors limiting their clinical translation include the lack of clinical data describing the optimal choice or combination of growth factors necessary to ensure positive interactions, the right time to deliver each factor and the method of delivery.

3. Delivery of Growth Factors

A possible explanation of what may be hindering growth factors from effectively playing out their role is the method by which we deliver them. As an important example, therapeutic delivery of VEGF has raised concern because continuous infusion of VEGF can result in complications, including, severe vascular leakage leading to hypotension[71] and increased risk of developing hemangioma like-vasculature[29,72]. Utilizing a well-designed delivery system that better recapitulates the native biology of VEGF, could improve its therapeutic effect by stabilizing it against rapid degradation and by modulating its release in an appropriate spatial and temporal manner.

Scaffolds in tissue engineering perform two functions: 1) to provide structural support by acting as an artificial extracellular matrix enabling cell infiltration, adhesion, and proliferation, 2) to serve as a platform for the delivery of growth factors. The later function generates ‘bioactive’ scaffolds that direct cellular behavior by regulating stem cell differentiation and tissue development. ‘Biomimetic’ polymers are synthetic systems that aim to model the native function of the extracellular matrix. One essential role of the native extracellular matrix is to create sustained, and often cell-mediated release, of growth factors. Together bioactive and biomimetic polymers can produce scaffold-based systems that provide engineered growth factor delivery. Important considerations in this design are: scaffold fabrication technique, form factor of the growth factor, and the ability to engineer desired release kinetics. (Figure 1)

3.1 Physical Entrapment

Physical encapsulation is perhaps the most simplistic method of growth factor delivery and can be accomplished by entrapping growth factors into the scaffold during synthesis, provided the polymerization process does not denature the protein[73]. A downside of this design is that this method often results in a simple burst release profile of the growth factors, which is often not ideal for stimulating a sustained biological response. Prolonged physical

entrapment can also result in the loss of growth factor bioactivity. Release profiles can be moderately altered by tuning the pore size of the scaffold based on the size of the protein or generating a biodegradable scaffold[74,75].

3.2 Covalent Binding

Alternatively, growth factors can be covalently bound into polymeric network of scaffolds. This system is advantageous because it ensures localization of the desired tissue response since the growth factor will not diffuse away from the source, and reduces the amount of growth factor that needs to be added since it is not systemically depleted. In order for the cells to access the growth factor, proteins are often presented in a pendant like fashion from the scaffold backbone. This can be accomplished via rapidly clikable Michael Type addition reaction in which cysteine residues within the peptide sequence react with the vinyl sulfone groups engineered onto the synthetic polymer backbone. This strategy was used to covalently link VEGF variants to poly(ethylene glycol) based scaffold resulting in formation of a dense vascular network[76,77].

Challenges associated with this delivery system are often centered on ensuring that fusion of the growth factor to the scaffold allows for downstream pathway activation. Specifically, in order to allow for signaling, the site for attaching the growth factor to the scaffold must be away from the receptor-binding domain. This can be complicated when the full-length recombinant form of the protein is used. One technique to engineer appropriate protein presentation is to utilize only the short, bioactive oligopeptide sequence of a protein to induce its functionality. This strategy has been widely used to create biomimetic scaffolds that allow cells to bind directly to the scaffold by incorporating variants of the tripeptide sequence from fibronectin (Arg-Gly-Asp, ‘RGD’) that has been identified as the functional domain mediating cell interaction through integrins. Recently, this concept has been adapted to enhance osteogenic differentiation of MSCs and induce angiogenic response by fusing only the active sequence of the full length SHH protein, located in the N-terminus of the protein, onto synthetic polymer backbones[61,76].

Furthermore, it is important that downstream cellular signaling can progress following formation of the receptor-ligand complex by understanding the molecular mechanism of the signaling pathway of interest. For example, downstream activity of a number of proteins in the TGFβ superfamily, such as BMP, are believed to require internalization of the receptor following binding of the ligand[77]. Alternatively, clustering of some receptors, such as VEGF, allow for signal potentiation[78,79]. Covalent attachment of the ligand to the scaffold may prevent these things from happening and therefore activation of the pathway would not be substantiated. In these cases you can engineer the attachment of the protein to be temporary, generating the so-called ‘release on demand’ or ‘bioresponsive’ delivery of the growth factor[80]. For example by incorporating a proteolytically degradable sequence into the linking tether between the scaffold and the peptide, such as those that are MMP-sensitive, one can generate a bioresponsive system in which cellular activity mediates bioavailability of the growth factors[81]. Alternatively, external cues can be applied to release the peptide at a desired milestone by adding an exogenous enzyme[82] to degrade the sequence. More recently

an elegant system has been developed in which the growth factor can be released by shining light onto the scaffold by incorporating photolabile peptide sequences into system[80].

3.3 Affinity Binding

To attain a biologically relevant release profile, growth factors can be electrostatically associated to the scaffold via endogenous binding domains between growth factors and the ECM. Electrostatic binding of growth factors can be advantageous because they are designed to mimic interactions between growth factors and the native extracellular matrix. Natural biopolymers, such as fibrin or collagen, are often used to achieve this style of growth factor delivery, as these protein-based polymers naturally present growth factor binding domains. Fibrin gels have also been used to deliver engineered variants of VEGF protein to stimulate angiogenesis[83–85].

To achieve greater and wider engineering design space, synthetic hydrogels have also been developed to contain the functional binding domain of the native ECM. This is frequently accomplished through the chemical addition of negatively charged sulfated polysaccharides. While many negatively charged sulfated polysaccharide can be incorporated into the scaffold to achieve this aim, heparin has become a popular choice due to its linear structure, high affinity binding towards a large variety of growth factors, and ability to preserve the biological activity of the protein. Importantly, heparin also contains an abundance of hydroxyl and carboxylic acid groups allowing it to be easily modified with reactive groups that can be incorporated covalently into hydrogels using chemo-selective chemistry[86]. Release kinetics of the growth factor is then modulated by the affinity of the growth factor to heparin (i.e. strength of the electrostatic interaction) and the amount of heparin incorporated into the scaffold. Heparin-modified hydrogels have now been used to effectively deliver a variety of growth factors for tissue regeneration, such as, angiogenic factors (VEGF, PDGF, FGF), stem cell derived factor, and TGF-β1[87–91]. An additional benefit of the heparin binding system is that it not only enables engineered release of a desired growth factor to the tissue, but it also can bind and retain growth factors synthesized *de novo* from encapsulated cells or surrounding tissues.

3.4 Microparticles and Nanoparticles

Particulate systems, such as mico- or nano- particles, can be engineered to deliver growth factors in a controlled manner for regenerative medicine purposes. These are different from the bioactive scaffolds in that they don’t provide any intrinsic structural support, but their small size enables them to penetrate deep into tissues or be embedded into the scaffolds structures[92]. Growth factor release kinetics from these particles is most often controlled by diffusion. Different sizes of particles will lead to varying delivery times as the size of the carrier particles controls the surface-to-volume ratio[93]. Similarly, pore size of the particle relative to the size of the growth factor can influence release rates from these systems. For example, PDGF and FGF encapsulated within PLGA–polymer nanoparticles has been shown to enhance angiogenesis[94].

Heparin conjugation/or coating techniques have also been applied to these particle systems in order to slow growth factor release. For example heparin (HP)-decorated, hyaluronic acid

(HA)-based nanoparticles were synthesized using an inverse emulsion polymerization technique and it was demonstrated that the presence of heparin enabled a higher BMP-2 loading capacity and more sustained release profile[92]. Similarly, heparin coated methacrylamide microparticles have been effectively used to generate controlled release of BMP-2, VEGF, and FGF2[95].

An integrative approach combining the use of nanoparticulate systems with bioactive hydrogel scaffolds is becoming increasingly popular for the design of growth factor delivery systems. Using the combinatorial approach enables a differential release of growth factors with distinct delivery kinetics. For example, encapsulating microspheres containing PDGF into a scaffold with physically entrapped VEGF resulted in rapid release of VEGF followed by more sustained delivery of PDGF[96]. This technique increased local blood vessel density and was the first report of effective sequential growth factor delivery. Engineering the full temporal complexity of the growth factors needed to induce the various phases of tissue repair will become an increasingly active area in materials development.

4. Role of Stem Cells in Vascularized Bone Regeneration

The therapeutic use of stem cells is a promising component in tissue engineering approaches that are designed to promote angiogenesis and osteogenesis for healing bone defects. Angiogenesis is crucial in bone repair[97,98] and the formation of new blood vessels depends on the ordered interaction of endothelial cells with different types of cells including, macrophages, pericytes, endothelial progenitor cells (EPCs), and MSCs[99–101]. It is clear that multiple stem cell types, immunogenic cues, and cytokines participate in, and are necessary, to initiate angiogenesis and osteogenesis. Because vascularized bone regeneration requires interplay between multiple cell types, recent work has focused on co-culturing of different cell types to facilitate improved healing. There has been greater characterization of, and experimentation with, stem cells from different sources in order to most efficiently promote vascularized bone regeneration.

4.1 Mesenchymal Stem Cell (MSCs)

Mesenchymal Stem Cells (MSCs) are the classic adult skeletal progenitor cell due to their established ability to form bone and cartilage both *in vivo* and *in vitro*. MSCs can be obtained from a number of adult stem cell niche, including bone marrow, adipose tissue[102] and periosteum[103]. A definitive cell surface marker for the MSC has yet to be determined, but these cells are typically identified by their expression of CD90, CD105, CD73, and CD146, and absence of CD45, CD34, CD14, CD11b, CD79a, CD19, HLA-DR.[104,105] Without clear markers for cell sorting, the International Society for Cellular Therapy has proposed a set of basic requirements for a cell to be classified as a MSC. MSCs are defined as a plastic culture adhesive cell with the ability to generate a colony-forming unit and differentiate into bone, cartilage, and adipose tissues[106].

The most common, and best characterized, source of MSCs is from human bone marrow (BM-MSCs) and *in vitro* protocols for their controlled differentiation into bone, and cartilage, and adipose tissues are well-established. More recently, adipose-derived MSCs (ADSCs) have been identified and can also form the bone, cartilage, and adipose lineages.

ADSCs offer a potential advantage over BM-MSCs for clinical translation into regenerative therapies due to their relative high abundance and easy access in the adult. Periosteal stem cells are perhaps the most relevant to bone regeneration since they are the primary source of cells that heal the fracture.[107] While the native function of these cells is critical to healing, these cells are unlikely to play a large role in therapeutic strategies since periosteal stripping could negatively impact normal bone homeostasis, causes donor site morbidity, and these cells are difficult to access and in low quantity relative to BM-MSCs and ADSCs.

It is the prevailing thought that MSCs must be isolated, enriched, and expanded *in vitro* prior to clinical use. This may be especially true in older patients since there is a significant age-dependent decrease in the number and function of MSCs in the elderly population[108]. Newer technologies are looking to create devices that concentrate MSCs in the operating room and some clinical trials have aimed to define the quantity of various MSC populations in different tissues. For example, a recent clinical study, Y Jang *et al* determined that the BM-MSC population was only 0.42% in the bone marrow and 4.28% in the stromal vascular fraction; the ADSC population was considerably more abundant at 0.16% in the bone marrow and 32% in the stromal vascular fraction.[109]

4.2 MSC Osteogenesis

The osteogenic potential of MSCs is well known and consequently these cells have a rich history in use for promoting bone regeneration. At a molecular level osteogenic differentiation is transcriptionally regulated by runt related transcription factor-2 (RUNX2)[110–112] and nuclear localization of p-catenin through canonical WNT signaling[113,114]. Together this leads to downstream activation of the canonical bone genes - osterix, osteopontin, and osteocalcin[110,115] - and suppression of chondrogenesis[116,117]. *In vitro* osteogenesis of MSCs is classically initiated in the plastic adherent cell population by administration of ascorbic acid, β-glycerol phosphate, and dexamethasone or bone morphogenetic protein (BMP). The mechanical microenvironment is also a critical regulator of osteogenesis. Stiffness of the substrate[118], or relative *in vivo* strain environment that the MSCs experience[119], can influence osteogenic potential.

While it is assumed that the various MSC populations are similar, recent studies indicate that MSCs have unique characteristics depending on their source, and this may manifest into important functional differences. For example, despite similar cell surface markers, BM-MSCs and ADSCs demonstrate differentially expressed genes by microarray analysis[120]. Differential expression of genes related to cell proliferation may contribute to the documented increase in proliferative capacity of ADSCs relative to BM-MSCs[120,121]. In contrast, side-by-side comparative studies suggest that BM-MSCs have an improved differentiation potential as they form better osteogenic and chondrogenic systems *in vitro*[122–124]. Definitive comparative studies have not yet been completed and the best source of MSCs for therapeutic application will be an area of continued research as the field develops.

4.3 MSCs as Paracrine Mediators of Tissue Repair

While MSCs are capable of osteogenesis, their ability for *de novo* bone formation relies on encapsulation or recruitment to a suitable scaffold. In the absence of such a scaffold, the paracrine functionality of MSCs dominates their ability for tissue specific differentiation[125–127]. MSCs secrete a number of proteins that play a critical role in regulating inflammation and have a trophic function in stimulating tissue regeneration.

The immunomodulatory role of MSCs plays a critical role both in normal healing and for therapeutic use. During fracture healing, circulating MSCs recruited to the site of injury are likely a major factor in influencing macrophage polarization and regulating the immune microenvironment of the fracture callus[128]. Therapeutically, MSCs are in clinical trials for their ability to modulate immune reactions such as graft versus host rejection[129,130]. Immunomodulation by the MSCs is accomplished by secretion of immunosuppressive and antiinflammatory cytokines, such as interleukin-10[131], nitric oxide[132] and prostaglandins[133]. MSCs can also regulate T-cells in an antigen-independent manner[89] through the suppression of the primary and secondary T-cell responses by inhibiting cell proliferation[134–136].

MSCs also promote a local healing responses by stimulating proliferation and differentiation of resident stem cell populations, reducing fibrosis, and inhibiting adverse apoptosis[137–139]. Some research has been done to determine the secretory molecules produced by mSCs, identifying measurable levels of TGF-β, stem cell factor (SCF), insulin-like growth factor (IGF), epidermal growth factor (EGF), and granulocyte and macrophage colony stimulating factors (G/M-CSF)[134,140].

Taken together, the immunosuppressive and trophic capabilities of MSCs are powerful and characterizing the secretome of MSCs for therapeutic use remains an active area of research. Of particular interest is how MSCs appear to have a lasting therapeutic effect despite very minimal tissue engraftment. Interestingly, current data also suggests that MSCs are immunoprivileged, or at the least immune evasive, enabling the possibility for allogenic use[141]. MSCs do not display major histocompatibility complex (MHC) class II cell surface markers, but only MHC class I markers without the co-stimulator molecules, indicating that they will not illicit an immune response[142].

4.4 Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) may represent another potential adult stem cell population that could be used to promote vascularized bone regeneration with increasing amounts of data providing evidence that EPCs have pro-angiogenic functions *in vivo*[143–145]. EPCs are precursor cells of hematologic origin which reside in the bone marrow and peripheral blood. The existence of EPCs was first documented by Asahara *et al*[146] and subsequently these cells have become functionally defined by their ability to generate the endothelial and smooth muscle cells that give rise to a vascular network during *in vitro* angiogenesis. *In vivo*, EPCs have been shown to be highly effective in mobilizing to areas of ischemic damage and promoting neovascularization[147,148]. The pro-angiogenic effect exerted by EPCs appears dependent on their ability to stimulate endothelial cells growth[101] and interact with mature endothelial cells to support vascular anastomosis[149]. Nevertheless,

the mechanisms by which the EPCs exert beneficial effects on endothelial cell growth is likely multifactorial and might include the transdifferentiation of subpopulations of EPCs into mature endothelial cells[150].

Different experimental approaches for *in vitro* isolation and functionality have defined two EPC populations: the early and late endothelial progenitor cells. Typically, the endothelial cells used for tissue engineering approaches are human outgrowth endothelial cells (OECs), which are otherwise known as late endothelial progenitor cells, and emerge after 14–21 days of culturing mononuclear cells on type I collagen[100,151–153]. An alternative source of endothelial cells is early endothelial progenitor cells (eEPCs) that arise following short-term cultures of peripheral and umbilical cord blood-derived mononuclear cells. eEPCs exhibit a hematopoietic cell phenotype that closely resembles monocytes, whereas OECs are differentiated and mature endothelial cells displaying cobblestone morphology and expressing mature endothelial cell-specific markers, such as VE-cadherin and von Willebrand factor[100,151–154]. OECs behave more like canonical progenitor cells by directly contributing to neovascularization, dividing into new endothelial cells, and forming vascular networks[155].

Another potential source of EPCs has been identified in cardiac tissue as a Sca-1^+/$CD45^-$ progenitor cell. GFP^+ Sca-1^+/$CD45^-$ progenitor cells have been shown to contribute to tissue regeneration in part by undergoing neovascular differentiation characteristic of endothelial cells[156]. Furthermore, when these GFP^+ Sca-1^+/$CD45^-$ progenitor cells are encapsulated in heparin, bspRGD(15) and TGF β1 containing hyaluronic acid based hydrogel, they demonstrated *in vitro* vascular-like network formation and *in vivo* enhanced neovascular response within the hydrogel and significantly anastomosed with the host's blood vessels[88]. Unfortunately, like MSCs, no definite cell surface markers have been identified for the EPCs and they display an overlapping phenotype with other endothelial and haematopoitic cells. Current isolation and enrichment strategies for EPCs focus on the surface markers CD34, CD133, and vascular endothelial growth factor receptor 2[157–159].

In addition to their direct role in angiogenesis, EPCs may also be capable of differentiating into osteogenic cells. EPCs have been shown to be upregulated in response to orthopaedic trauma and mobilization by signals from bone injury sites may contribute to both neovascularization and bone formation during fracture healing[160,161]. The therapeutic application of EPCs in orthopedic trauma has been shown to augment fracture healing and local angiogenesis in segmental defect models in rat femurs[162] and sheep tibia[163,164]. Although EPCs hold promise in regenerative therapies, our understanding of EPCs is still in its infancy. Better characterization of EPCs and determining the role of EPCs during angiogenesis and bone formation requires continued research efforts[165].

4.5 MSC-ESC Interaction during Neovascularization

MSCs promote neovascularization by releasing pro-angiogenic factors such as angiopoietin-1, FGF-2, and VEGF likely through paracrine communication between EPCs and MSCs.[166–168] It has been suggested that MSCs are the major producer of VEGF during vascular bone regeneration.[101,167,169] These MSC secreted pro-angiogenic factors stimulate EPC proliferation and differentiation, which in turn causes EPCs to secrete osteogenic

growth factors that stimulate MSC differentiation.[170,171] While data suggests that BM-MSCs and ADSC secrete comparable levels of angiogenic factors[172], one study found that co-culture of these cells together promoted enhanced angiogenesis by increasing VEGF production and improving osteogenesis.[173] Furthermore, MSC–EPC co-cultures *in vitro* found a more pronounced tube formation in co-cultures compared to single cultures.[174]

It is also suggested that MSCs are the origin of the pericytic cells that wrap around the endothelial cells of capillaries and venules[175]. Within the blood vessels the pericyte and endothelial cell are in direct cell-to-cell contact creating both a physical and paracrine interaction between these cell types. Pericytes and endothelial cells are functionally interdependent and it is believed the role of the pericyte is to stabilize the vascular network during formation[176]. Vascular damage during injury may help to mobilize pericytes/MSCs to the site of damage and into circulation. Furthermore, secreted signals from the vascular endothelial cells may help to promote differentiation of the MSCs. Failure of proper communication between the pericyte and endothelial cell has been linked to numerous human pathologies[177,178].

4.6 Role of Macrophages in Regulating Vascularized Bone Regeneration

Macrophages play an important role in regulating the process of angiogenesis and bone regeneration. Their role is complex, but it is clear that macrophages have polarizing phenotypes that exhibit either so-called proinflammatory (M1 phenotype) or anti-inflammatory (M2 phenotypes) manner. M1 macrophages dominate the early phase of fracture healing, secreting pro-inflammatory cytokines such as IL-6, IL-8 and TNFα, and contribute to the formation of the hematoma and early angiogenesis.[179] The recruitment of inflammatory cells to sites of injury is mediated by four different subfamilies of chemokines (CC, CXC, CX3C and C) based on their biochemical, structural and functional properties[180]. In a Ccr2 −/− mouse model of decreased macrophage recruitment, it was shown that disruption of Ccr2 signaling impaired vascularization, decreased formation of callus, and delayed maturation of cartilage and bone remodeling up to 21 days after injury[181,182]. M2 macrophages contribute to the resolution of inflammation and are important to the progression bone healing. These anti-inflammatory M2 macrophages stimulate cell proliferation, survival, and tumor growth in various pathological states[183–186]. Dysregulation of macrophage polarization, specifically persistence of the M1 phenotype or dampened M2 response, may contribute to age related delays in bone healing.[180–182]

5. Improved Vascularized Bone Regeneration through Endochondral Ossification

As detailed above, therapeutic strategies to promote vascularized bone regeneration have focused on enhancing bone formation and/or stimulating angiogenesis through the application of growth factors and stem cells. Traditionally, these approaches look to form bone directly through the process of intramembranous ossification. Intramembranous ossification involves direct differentiation of MSCs into osteoblasts. This is the embryonic program by which bones in skull develop, and is the mechanism through which rigidly stabilized fractures heal.

In contrast, a second method for bone formation is endochondral ossification where a temporary cartilage matrix is formed prior to being remodeled into bone. Endochondral ossification is the embryonic developmental pathway for long bone formation, enables post-natal bone elongation, and is the process by which the majority of fractures heal. As an indirect pathway for bone formation, this process involves a complex series of highly regulated steps in which MSCs differentiate into chondrocytes, mature to a hypertrophic state, and then stimulate vascular invasion and mineralization before transforming into bone[187,188]. Recently, this pathway has been explored as a novel approach to bone regeneration.

5.1 Shortcomings of Bone Grafting Relying on Intramembranous Bone Formation

The gold standard technique for stimulating bone regeneration remains bone autograft, typically through transplantation of morselized iliac crest or structural grafts taken from the rib or fibula. Outcomes from these procedures are typically good since the transplanted tissue contains living osteocytes, osteoclasts, and vascular cells. Together these cells enable bone remodeling and maintain viability and strength of the graft. However, as discussed in the introduction, the limited availability of autograft bone, along with significant donor site morbidity limit overall effectiveness of this procedure. Bone allograft, demineralized bone matrix, synthetic bone grafting substitutes, and tissue engineering approaches to bone regeneration have all emerged as alternatives to autograft. These products attempt to be either osteoinductive, osteoconductive, or osteogenic – with the goal of stimulating direct bone formation and mineralization.

The intramembranous bone formation approach fails to promote adequate vascular invasion as a result of excess mineralization and/or inability of cells to invade the grafts to enable efficient remodeling of the tissue[189]. The lack of a vascular network hinders the delivery of nutrients and the removal of waste from the center of current bone grafts and tissue engineered constructs[190]. As a result these bone grafts are met with a number of failure mechanisms, such as, loss of mechanical strength over time, poor osseointegration, and osteonecrosis of the graft.

5.2 Endochondral Ossification Promotes Angiogenesis and Osteogenesis

Poor clinical outcomes using traditional intramembranous approaches to bone regeneration have recently inspired researchers to investigate the therapeutic potential of endochondral ossification as a strategy for promoting well-vascularized bone repair. (Figure 2) In these studies cartilage grafts are used to stimulate bone formation. Murine studies using ectopic transplantation of cartilage have demonstrated that growth plate cartilage and chondrogenically primed hMSCs, but not articular cartilage, have the capacity to form bone[191–195]. More recently, clinically relevant rodent models for critical sized bone defects have demonstrated the clinical utility of cartilage to promote endochondral bone regeneration[196,197]. Importantly, these studies demonstrate that the endochondral derived bone is highly vascularized and promotes significantly better osseointegration with the host bone when compared to bone allograft.[196,197] Successes with bone regeneration from a cartilage template suggest that a hypertrophic cartilage scaffold has all the inherent signals

needed for bone tissue formation and vascular invasion and open the potential for whole bone regeneration in addition to segmental bone repair[198,199].

A potential advantage of using cartilage rather than bone grafts for promoting repair is that cartilage is physiologically adapted to survive in avascular conditions. Consequently, cartilage grafts may be better suited for survival in the low vascular conditions, such as traumatic injury or critical-sized defects, which typically require bone grafting[200,201]. In a study by Scotti *et al.*, chondrocytes survived hypoxia and then instructed cells to differentiate into osteoblasts[194].

Tissue engineering studies for endochondral bone repair have also found that re-vascularization can be optimized by scaffold parameters. Specifically, scaffold chemistry[202], porosity[197], and ultrastructural architecture[203,204] can enhance the inherent bioactivity of the chondrocytes and facilitate vascular invasion. Furthermore, angiogenesis in these systems is an intrinsic function of the hypertrophic chondrocytes in the cartilage template that precedes bone formation. These chondrocytes produce VEGF and PlGF to promote angiogenesis and initiate vascular invasion[187,194].

Remodeling of the cartilage template into bone is a complex process that is currently the topic of much debate[187,188,205]. Degradation of the cartilage matrix occurs in both an intrinsic and extrinsic fashion. Hypertrophic chondrocytes secrete MMP-13 to help degrade the collagen II and aggrecan in the cartilage template. The cartilage matrix is then further degraded as the blood vessels invade due to secretion of MMP-9 from the vascular endothelial cells.[190] Dogma held that at this stage the hypertrophic chondrocytes were destined for apoptosis and that new bone forms from osteoblasts invading into the cartilage matrix. However, recent studies using genetic labeling of chondrocytes during bone formation and healing demonstrate that all hypertrophic chondrocytes do not undergo apoptosis, but rather can transdifferentiate into the osteoblasts and osteocytes that directly contribute to new bone formation[194,196,206,207]. Subsequently, degradation of the cartilage matrix, apoptosis, and osteoclast remodeling facilitate formation of bone marrow cavities. While the molecular mechanisms that regulate cartilage to bone transformation are currently not known, therapeutic manipulation of this pathway represents a novel strategy for promoting vascularized bone regeneration.

6. Summary

Current strategies for bone grafting fail to produce an adequately vascularized bone regenerate. In order to improve clinical outcomes from bone grafting procedures, better technologies to control and direct neovascularization within a mineralized construct need to be developed. Engineering the appropriate growth factors, scaffolds, and cell combinations into a comprehensive system for promoting both angiogenesis and osteogenesis remains a challenge and future goal.

Moving forward, a critical challenge in achieving this goal remains strategic delivery of therapeutic proteins. We have learned from recent experience with BMP that supraphysiological dosages of recombinant proteins can result in off-target effects, and even

tumorgenesis[208,209]. Maintaining growth factors locally and designing controlled release schematics that resemble the native biological demand during healing should be a goal of the next round of smart biomaterials. Furthermore, given the complexity of the normal healing response, it is unlikely that a single protein will generate optimal results. The timing of multiple growth factor signaling will likely be necessary to engineer sophisticated cell differentiation events, as would be needed to encourage endochondral ossification from an MSC population. Consequently, improved systems need to be developed to deliver growth factors with distinct kinetics, especially in situations where delivery of one factor should follow another.

Smart scaffold design will play a critical role in delivering these growth factors, encapsulating target cell populations, enabling vascular invasion and tissue remodeling. Bioactive, biomimetic, and bioresponsive scaffolds aim to recapitulate the native ECM in a dynamic synthetic scaffold. As the building blocks of polymer science continue to grow, we can compliment this toolbox with a better understanding of the molecular and cellular mechanisms that regulate bone repair and neovascularization. The importance of modeling the normal developmental pathway was recently underscored by improved vascularized bone formation upon promoting endochondral, rather than intramembranous, ossification. Coupled with improved nanotechnology, bioprinting, and advances in electrospining we may be able to create ultrastructural microenvironments that are optimized for vascularized bone formation[210–212].

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Abbreviations

MSCs	mesenchymal stem cells
VEGF	Vascular endothelial growth factor
PDGF	platelet derived growth factor
TGF-β1 & TGF-β2	transforming growth factor beta 1 and 2
IGF	insulin-like growth factor
FGF-2	Fibroblast growth factor-2
rhPDGF-BB	recombinant human platelet derived growth factor-BB
PlGF	Placental growth factor

Shh	Sonic hedgehog
Ang	angiopoietins
Tie	tyrosine kinase receptors
HA	hyaluronic acid
EPCs	endothelial progenitor cells
OECs	outgrowth endothelial cells
eEPCs	early endothelial progenitor cells
BM-MSCs	bone marrow derived mesenchymal stem cells
ADSCs	adipose-derived MSCs
BMP	Bone morphogenic protein

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Highlights

- Angiogenic growth factors play a very important role in directing neovascularization.
- Delivery methods allowing sustained release can overcome major hurdles that previously prevented the translation of growth factors to the clinic.
- Further research is needed to determine the best source of therapeutic stem cells and the benefits of stem cell co-culture.
- Cartilage grafts have the potential to promote vascularized bone regeneration through the endochondral ossification pathway.

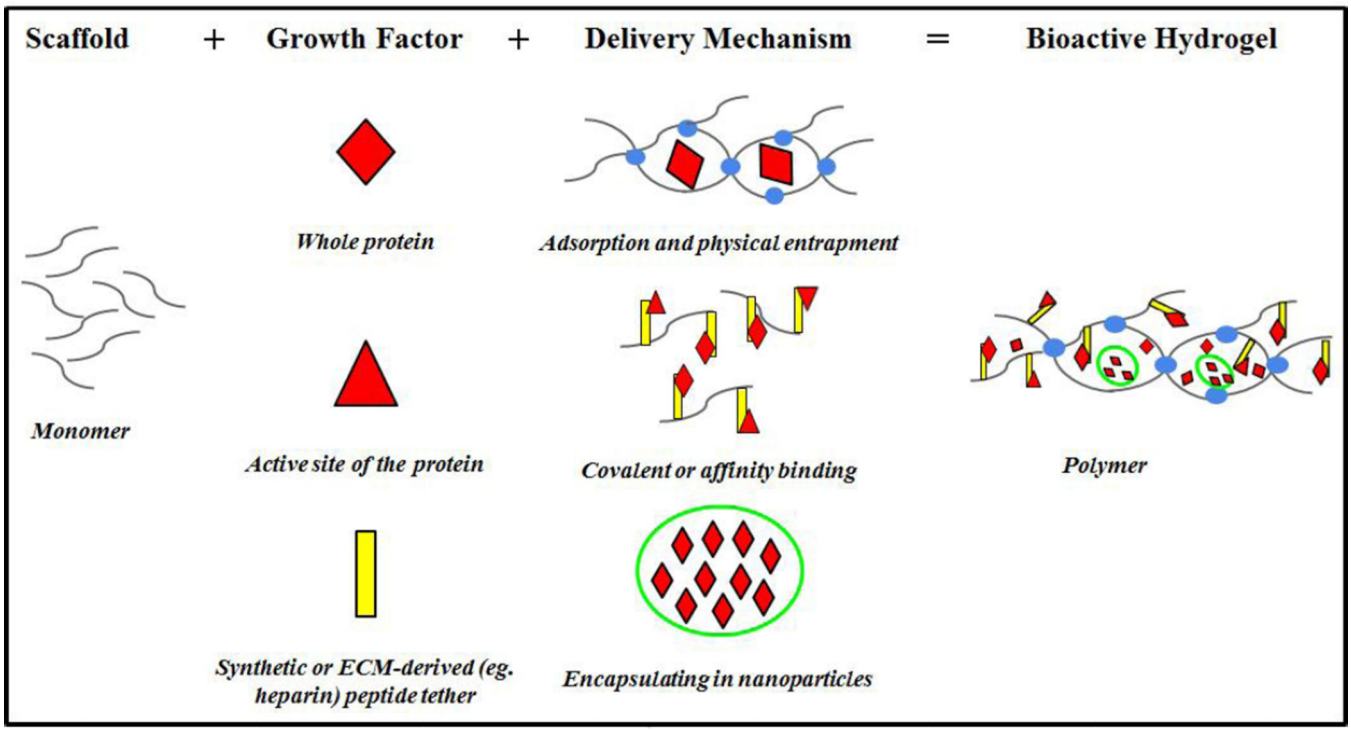

Figure 1. Summary of biomaterial based strategies to deliver growth factors

Techniques to control release growth factors. Thus, when there are several growth factors in the polymer, it is possible to engineer unique temporal release kinetics.

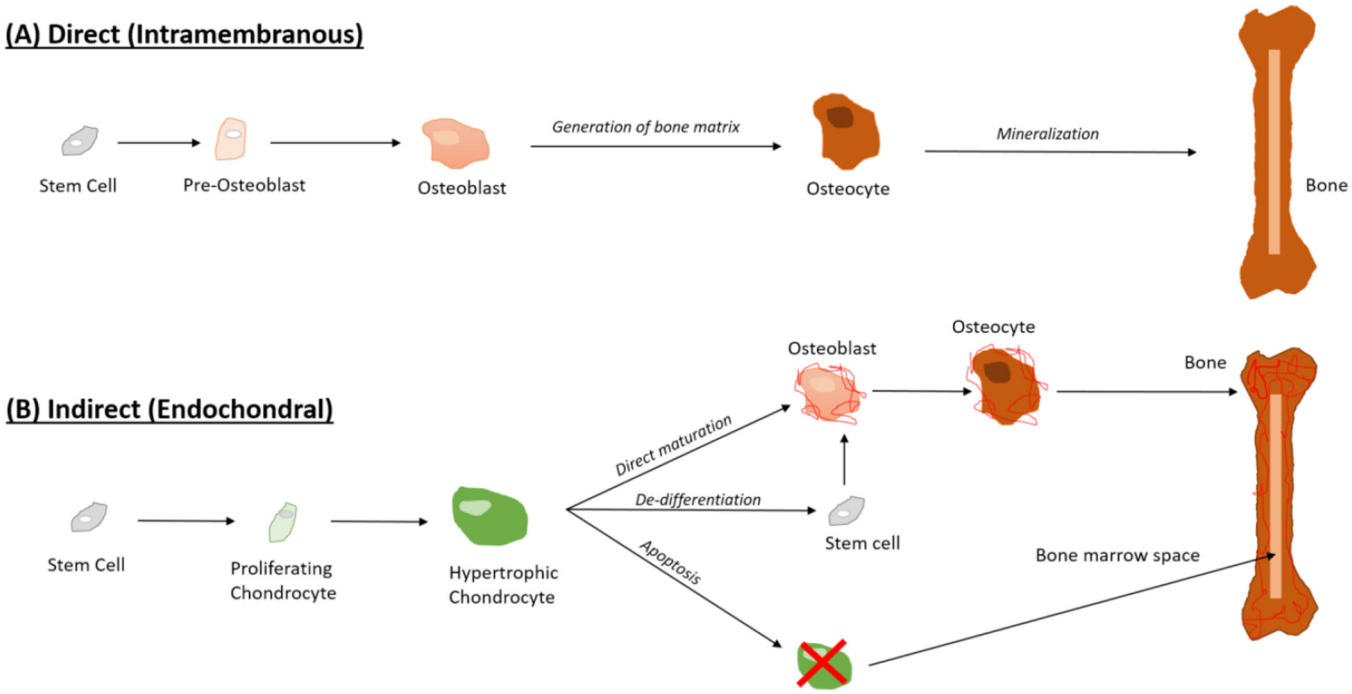

Figure 2. Therapeutic Approaches for Bone Regeneration

(A) Classic techniques in bone regeneration attempt to stimulate intramembranous ossification, with limited success at producing a vascularized bone regenerate. (B) Transformation of chondrocytes into bone may represent a better therapeutic strategy for promoting vascularized bone regeneration as a result of the strong angiogenic action of the hypertrophic chondrocyte during endochondral ossification.

Table 1

Summary of the role of growth factors in angiogenesis and osteogenesis with current status of preclinical and clinical trials

Growth Factor	Role in Angiogenesis		Osteogenic Effects		Preclinical/Clinical trials	Refs
PlGF	**1**	Inflammatory cell recruitment.	Bone formation and remodeling		Semistabilized bone fractures in WT mice	46
	2	Proliferation and differentiation of MSC's into osteoblasts and osteoclasts.				
IGF	**1**	Promote neovascularization	**1**	Regulates chondrocyte maturation	Knockout mice, segmental defects in Sprague-Dawley rats and Swiss alpine sheep	49,50, 54
	2	Chondrocyte hypertrophy	**2**	Mediates skeletal response to PTH		
			3	Modulates bone mechanosensitivity.		
SHH	Proliferation and differentiation of bone marrow-derived MSC's into an osteoblastic lineage.		Osteogenic differentiation marker expression, mineralization and alkaline phosphatase		Adult rats, ischemic hind limb aged mouse model	58,61
VEGF	**1**	Vascular invasion.	Endochondral ossification and intramembranous bone formation		Rat calvarial defects, critical sized bone defects in New Zealand white rabbits	18,24
	2	Stem cell recruitment				
FGF-2	**1**	Angiogenesis.	Increased callus, bone mineral density and mechanical stability		Many including rat segmental defects, rabbit osteotomies and canine tibial defects	32
	2	Proliferation of MSC's and osteoblasts.				
Ang-1	Blood vessel remodeling and maturation		None		Transgenic mice	65
PDGF	**1**	Chemotactic factor for osteoblasts.	Improves bone density, biomechanical strength, and torsional strength. Increases callus size and density.		Osteoporotic rats, rabbit osteotomies, dog periodontal defects, human periodontal defects, diabetic foot ulcers	37,38, 39,40, 41,42, 43
	2	Proliferation of osteoblasts.				
	3	Increases the expression of VEGF on endothelial cells.				

Table 2

Summary of the advantages & disadvantages of different delivery approaches

Delivery Method	Advantages		Disadvantages	
Physical Entrapment	**1**	Control of release kinetics through changing pore size	**1**	Simple burst release
	2	Ability to use biodegradable scaffolds	**2**	Risk of protein denaturation with polymerization
			3	Risk of loss of growth factor bioactivity with prolonged entrapment
Covalent Binding	**1**	Ensures local delivery of protein	**1**	Risk of protein denaturation during fusion
	2	Low quantity of protein required	**2**	Difficulties in engineering the attachment site
	3	Engineered for “on demand” growth factor release		
Electrostatic Binding	**1**	Allows slower release based on molecular affinity	The presentation of growth factors may not be uniform and defined in the polymeric network due to the number of variations in the structures of sulfated polysaccharides	
	2	Ability to bind and retain exogenously added and endogenously secreted growth factors		
Micro/Nanoparticles	**1**	Small size allows for better tissue penetration	**1**	Size of particle can disrupt scaffold structure
	2	Control of release kinetics through diffusion by changing pore and particle size	**2**	Potentially complicated growth factor loading in the particles due to limited structural support
	3	Can be incorporated into a scaffold with other particles for sequential growth factor delivery		