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Bone Regeneration: Current Status and Future Prospects

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Abstract

The ability of bone to heal with practically no scarring is the most extraordinary feature of it. However, perturbations of the fracture site could disrupt the repair process when defects reach a critical size, resulting in non-union. Current therapies include allografting, autografting, applying vascularized grafts, and other bone transport techniques. However, although commonplace in orthopaedic surgery, these treatments have some limitations.

Harvesting autografts is very expensive, typically from the iliac crest, associated with donor-site morbidity due to infection and haematoma and constrained by anatomical limitations. Allografts are limited by the possible risks of introducing infection or disease, while vascularized grafts are prohibitively expensive. So, due to technical difficulties and shortcoming of reconstructive surgery, the need for suitable fillers in large fracture reconstructive surgery is inevitable. Thus, recent tissue engineering approaches have been attempted to create new bone based on stem or precursor cells seeded onto biocompatible materials or scaffolds, with or without appropriate growth factors to improve clinical outcome. This chapter review the clinical necessity for tissue engineered bone, recent approaches attempting to create new bone, the main challenges of them and the novel strategies to overcome these barriers.

Keywords: bone fracture, regenerative medicine, stem cell, scaffold, growth factor, osteogenesis

1. Introduction

Reconstruction and regeneration of significant skeletal defects have amazed mankind for thousands of years. Grafting techniques were employed as early as 2000 BC when Khurits employed a piece of animal bone to reconstruct a small skull defect. In the modern age, Job

van Meekeren, a Dutch surgeon, performed first documented bone graft in 1668. He utilized a xenograft to repair a skull defect in an injured soldier [1]. The understanding of orthopaedic science and bone grafts was further propelled in the seventeenth century by the work of Antoni van Leeuwenhoek who is famously known for his work on microscopy. Also, he primitively explained the microarchitecture of bone, what we now refer to as Haversian canals [2]. Hard-working examination of bone-grafting criteria and outcomes surfaced in the early 1900s by Vittorio Putti who determined the principles of grafting. Putti's work presented a foundation for grafting science in the orthopaedic field. Since then, researchers and surgeons have continued to smooth the science of bone grafting to allow for the most proper surgical intervention with the best outcomes [2, 3]. The current standard treatment is harvesting autologous grafts from other positions in the body (harvested primarily from the patient's iliac crest or other locations, such as the distal femur, proximal tibia, ribs and intramedullary canal) and transplantation into the massive fractures, or the transplantation of allografts, which have many obstacles, such as donor-site morbidity, limited tissue supply, infection, and poor integration [2, 4, 5]. Autografts are clinically approved therapies, which demonstrate the biological characteristics of osteogenesis, osteoconduction, and osteoinduction. Both grafts possess unique advantages and disadvantages; however, autografts gained desirability over allograft in the early 1900s with recognition of the advantage that vascularization provided to the integrity of the graft with the surrounding bone [6]. So, synthetic bone graft substitutes that were developed to overcome the inherent limitations of auto- and allograft represent an alternative strategy. These synthetic substitutes, or matrices, are made from a variety of materials, such as natural and synthetic polymers, ceramics, and composites that are designed to mimic the three-dimensional (3D) characteristics of autograft tissue while maintaining viable cell populations. Matrices also function as delivery vehicles for factors, chemotherapeutic agents, and antibiotics depending on the nature of the injury to be repaired. This junction of matrices, cells, and therapeutic molecules has collectively been termed tissue engineering (TE) [7]. Clinically, a bone regenerative therapeutic to treat patients must provide fundamental criteria, including safety, predictability, and reproducibility, in providing the clinical outcome. Also, as noted earlier, a tissue-regenerative therapy should exhibit four characteristics, including osteogenicity, osteoconductivity, osteoinductivity, and osteopromotivity [8, 9]. Osteogenesis refers to the process by which osteoprogenitor cells mature into osteoblasts, which subsequently mineralize and form bone tissue [9]. During osteoconduction process, bone formations occur on a surface. With respect to biomaterials, osteoconduction is defined by the ability of an implant to support the growth of bone at a defect site three dimensionally. Osteoinduction is the process of recruitment of immature osteoprogenitor cells to the site and the subsequent differentiation of them into osteoblasts under the influence of a diffusible bone morphogenetic factor. Finally, osteopromotion refers to the ability of a substance to enhance osteoinduction without being osteoinductive on its own [1, 9, 10].

2. Bone grafts

Fracture healing is performed based on a delicate balance between biology of fracture repair and biomechanical stability of fracture fixation, which are interrelated. Too many - attempts

have been developing to minimize damage to the blood supply of the fracture blocks during surgery, but the sequential activation of cells and bioactive molecules necessary for fracture healing still remains disrupted. Moreover, a non-union often develops when this sequential activation is interfered. Some approaches suggested to overcome non-unions and some acute fractures include bone grafts and bone graft alternatives—specifically autologous bone grafts, allografts, synthetic bone grafts, and osteoinductive proteins. The ability of grafts to promote healing depends on their osteoconductive, osteoinductive, osteogenic, and osteopromotive qualities [11–13]. Each bone graft type and its alternative own some combination of these qualities. This section is going to compare benefits and potential limitations of available grafting strategies.

The iliac crest bone graft (ICBG), harvested from the anterior and posterior iliac crest, is the gold standard for cancellous autografts in cases in which fracture healing rather than void filling is needed. It is corticocancellous with osteoconductive, osteoinductive, and osteogenic effects. Also, the other benefit of ICBG is the availability of large amounts of bone without structural compromise to the extremity [14]. In a study, Takemoto et al. objected to consider whether there are variations in the expression of bone morphogenetic proteins (BMPs) and their receptors in different bone-graft-harvesting sites. They analysed autogenous marrow aspirates obtained from the iliac crest, the proximal humerus, and the proximal tibia for the mRNA levels of BMPs and their receptors. Their results suggested that ICBG is rich in colony-forming cells, and the number of progenitor cells directly promotes healing [15]. Despite the relative advantages of ICBG, it is not without disadvantages. The limitations, however, have been well documented in the literature and include donor-site morbidity, increased time in the operating room, and an increased length of hospital stay [16, 17]. So, for certain patients with compromised bone or inadequate volume for grafting, bone graft substitutes may be preferable.

Substitutes to bone grafting consist of bone bank allograft, osteoconductive materials, demineralized bone matrix (DBM), and osteoinductive proteins. The orthopaedic association has extensive experience with bone bank allograft, with the first clinical tissue bank opening in 1949 [18]. The main concerns of allografts include the risk of rejection, disease transmission, inconsistent incorporation, and late resorption. An alternative to bone bank allograft is DBM. DBM is made from an allograft with the inorganic materials removed. Researchers demonstrated that DBM implanted intramuscularly resulted in new bone formation [19]. Also, DBM has osteoconductive property but only weak osteoinductive feature. Furthermore, DBM offers an advantage over allografts or synthetic biomaterials that need incorporation by the host before they can support mechanical loads and would diminish the morbidity associated with harvesting autologous bone [20].

Synthetic osteoconductive materials have been widely used for bone graft in orthopaedic practice and include hydroxyapatite (HA), coralline hydroxyapatite, CaSO_4 and CaPO_4 cements, and collagraft [21]. Hydroxyapatite has a porous structure comparable to the cancellous bone and functions as an effective osteoconductive matrix and thus replicates the biological properties of bone extracellular matrix (ECM). The nominal composition of this mixture is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ with an atomic ratio for calcium-to-phosphate of 1.67 [22, 23]. Most

studies have reported the mineralization and remodelling of this material can lead to the formation of mature bone [21]. Coralline hydroxyapatite is a similar substance, in which coral is converted to pure crystalline hydroxyapatite. It has good compressive strength but has low tensile strength and limited remodelling potential. Similar to hydroxyapatite, coralline hydroxyapatite functions strictly osteoconductive, but lacking osteogenic and osteoinductive properties. Calcium-based bone cements are osteoconductive and primarily used for filling metaphyseal defects. They possess sufficient compressive strength but lack resistance to shear and torsional forces and are very costly. They are also associated with resorption, leading to wound drainage [21]. The situations in which osteoinduction is the primary concern, BMPs are available. Detailed insights into BMPs will be provided later.

3. Molecular aspects of fracture healing

Fracture healing is a complex physiological process. Cascade of complex biological events involving intracellular and extracellular molecular signalling for bone induction and conduction remain unknown to a great extent. Indeed, it is a multistep repair process that follows a determined spatial and temporal sequence [24–26]. It was clearly demonstrated that known molecular mechanisms that regulate skeletal tissue formation during embryological development are replicated during the fracture-healing process [27]. Many growth and differentiation factors (GDFs), such as cytokines, hormones, and extracellular matrix, are local and systemic regulatory factors that interact with various cell types, including bone- and cartilage-forming primary cells, or even muscle mesenchymal cells, recruited at the fracture site or from the circulation. Advances in understanding cellular and molecular mechanisms will provide the tools for discovering the fracture-healing process. This section aims to contribute to promoting and inhibiting fracture healing and to prepare awareness of the complexity of involved signalling pathways.

3.1. Biology of fracture healing

The nature of the repair phase is dependent on mechanical conditions in the fracture-healing zone (primary or secondary bone healing) and the anatomical location of the fracture (metaphyseal-epiphyseal trabecular bone healing or diaphyseal callus healing). Indeed, fracture healing is a complex process, resulting in optimal skeletal repair and restoration of skeletal function. However, it is a well-orchestrated, regenerative process, which is initiated in response to injury. Repair process is promoted by the normal pathway of embryonic development repeated with the coordinated participation of several cell types [28]. Depending on several parameters involved in the fracture site, such as growth factors, nutrients, hormones, and oxygen tension, pH, the mechanical stability and the electrical environment, various components present at the injured tissue, such as the cortex, the periosteum, the external soft tissues and the bone marrow, contribute to the healing process [29–31]. Classical histology has divided fracture healing into direct (primary) and indirect (secondary) mode.

Direct strategy (known as primary cortical bone healing) occurs only when there is extremely low interfragmentary movement or if the bony fragments are under compression [32]. Most often compression plates and lag screws provide the required stability for direct healing [33]. Similar to the normal bone-remodelling process, fracture surfaces in contact and under compression are bridged by Haversian systems (or osteons) when such stability is achieved. Indeed, primary process involves a direct attempt by the cortex to regenerate new Haversian systems by the formation of discrete remodelling units known as 'cutting cones', in order to restore mechanical continuity [34]. Osteoclasts digest bone, causing tunnels from one side of the fracture to the other, which provides the in-growth of blood vessels. Subsequently, vascular endothelial cells and perivascular mesenchymal cells prepare the osteoprogenitor cells to differentiate into osteoblasts which create new osteons connecting both fragments [35, 36]. Healing by Haversian systems is slow, and notable time is necessary to gain sufficient strength by healing zone and, therefore, allow removal of load-bearing implants. Also, because it is not associated with a major influx of inflammatory cells, primary bone healing is less affected by systemic inflammation [37].

Another type of fracture healing is *indirect mode* that heals the majority of fractures. This mode of fracture healing occurs by either intramembranous ossification or endochondral ossification with the subsequent formation of a callus [38, 39]. This mode is usually enhanced by motion and inhibited by rigid fixation [38].

Intramembranous ossification forms bone directly without first forming cartilage. Migrated mesenchymal stromal cells that reside in the periosteum directly differentiate into osteoblasts that synthesize and deposit bone matrix. This process results in callus formation, characterized histologically as 'hard callus' [40]. In this type of healing, the bone marrow contribute to bone formation during the early phase of healing, when endothelial cells transform into polymorphic cells that subsequently express an osteoblastic phenotype [12]. Advanced studies have shown that flat bones such as bones from the skull, trabecular bones, and clavicle heal via intramembranous ossification [41].

By contrast, endochondral ossification involves the recruitment, proliferation, and differentiation of undifferentiated mesenchymal cells into a transient cartilaginous matrix, which calcifies into mature bone. This type of fracture healing is advocated to have the following identifiable stages: (1) an initial stage of haematoma formation and inflammation, (2) subsequent angiogenesis and formation of cartilage, (3) cartilage calcification, (4) cartilage removal, (5) bone formation, and (6) ultimately bone remodelling [42]. Also, it is contributed from the adjacent to the fracture periosteum and the external soft tissues, providing an early bridging callus, histologically described as 'soft callus' that stabilizes the fracture fragments [40]. Many studies have shown that diaphyseal fractures heal by endochondral mechanisms, forming a cartilaginous callus intermediate [41].

The classification of fracture healing into direct and indirect forms reflects the histological events that happen during the repair process. However, it is necessary to provide a further understanding of various signalling molecules and elucidate their contribution in the initiation and control of this physiological event at the molecular level.

3.2. Signalling molecules in bone regeneration and fracture repair

Various types of signalling factors influence the fracture healing, and continuous study of these factors can lead to promising new clinical treatments for bone repairing. To date, the delivery of signalling molecules for bone regeneration has been based primarily on factors that directly affect the bone formation pathways (osteoiduction) or that apply to increase the number of bone-forming progenitor cells. Overall, the signalling molecules can be classified into three groups, including the pro-inflammatory cytokines, the transforming growth factor- β (TGF- β) superfamily and other growth factors, and the angiogenic factors [43].

3.2.1. Pro-inflammatory cytokines

Pro-inflammatory cytokines, such as Interleukin-1 (IL-1), IL-6, IL-11, IL-18 and tumour necrosis factor- α (TNF- α), are critical for triggering the repair cascade [44]. They are secreted by macrophages, inflammatory cells, and cells of mesenchymal origin existing in the periosteum [43, 45, 46]. These molecules play key roles in the induction of downstream mediators to the fracture site by exerting a chemotactic effect on other inflammatory cells, augmenting ECM synthesis, stimulating angiogenesis, and recruiting endogenous fibrogenic cells to injury [47]. Furthermore, cytokines were found to regulate endochondral bone formation and remodelling [43, 47]. For example, TNF- α recruits mesenchymal stem cells (MSCs), promotes the induction of apoptosis in hypertrophic chondrocytes during endochondral ossification and incites osteoclastic function. Also, IL-1 mainly provided by osteoblasts and simplifies bone remodelling by stimulating proteases to degrade callus tissue [46]. The absence of TNF- α results in delayed resorption of mineralized cartilage, delayed endochondral bone formation by several weeks, and impaired fracture healing. Several studies have demonstrated that TNF- α signalling is unique to postnatal fracture repair [46].

3.2.2. Growth and differentiation factors

3.2.2.1. Transforming growth factor- β superfamily

It is a large group of regulatory polypeptides that includes bone morphogenetic proteins (BMPs), multiple isoforms of transforming growth factor- β s (TGF- β s), growth and differentiation factors (GDFs), activins (ACTs), inhibins (INHs), and glial-derived neurotrophic factors (GDNFs), as well as some proteins not included in the above families, such as Mullerian-inhibiting substance (MIS), also known as anti-Mullerian hormone (AMH), left-right determination factor (Lefty), and nodal growth differentiation factor (Nodal) (**Figure 1**) [48, 49]. Their isolation from bone extracts and further gene identification was accomplished in the 1980s, based on the previous results by Marshall R. Urist [19]. Transforming growth factor- β family encompasses at least 34 members in the human genome. These molecules originate from high-molecular-weight precursors, which are activated by proteolytic degradation. They can activate serine/threonine kinase membrane receptor on target cells. TGF- β ligand-bound receptor triggers an intracellular signal transmission via a canonical signalling pathway, which ultimately affects gene expression in the nucleus [47].

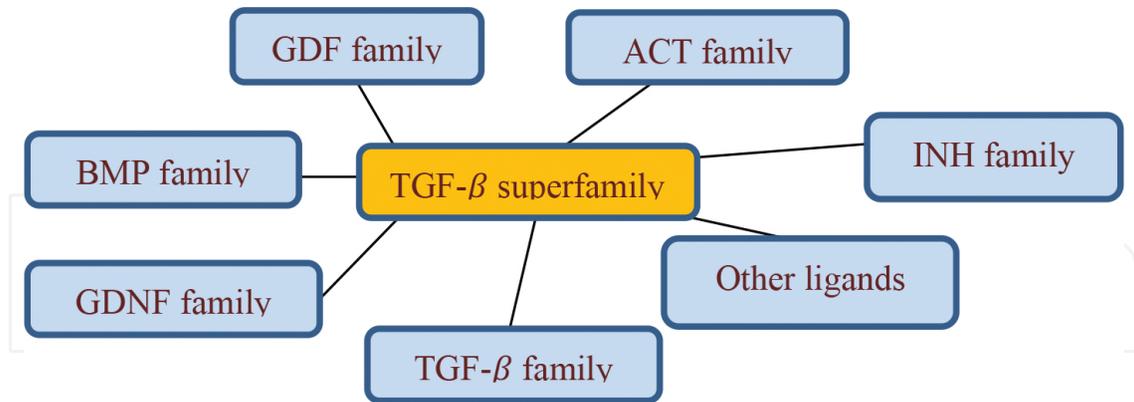


Figure 1. A schematic illustration of TGF- β superfamily. BMPs: bone morphogenetic proteins, TGF- β : transforming growth factor beta, GDF: growth and differentiation factor, GDNF: glial-derived neurotrophic factors, ACT: activin, INH: inhibin, other ligands include Mullerian-inhibiting substance (MIS) or anti-Mullerian hormone (AMH), left-right determination factor (Lefty), and nodal growth differentiation factor (Nodal).

Several members of the subfamilies of these morphogens including bone morphogenetic proteins (BMPs 1–8), growth and differentiation factors (GDF-1, 5, 8, 10) and transforming factor β (TGF- β 1, TGF- β 2, TGF- β 3), have been shown to promote the various stages of intramembranous and endochondral bone ossification during fracture healing (the following parts provide details on the use of them in attempts at bone regeneration) [24]. Of course, it is difficult to determine the physiological role of many of the members of this superfamily because of their functional redundancy.

Bone morphogenetic proteins are secreted signalling molecules that belong to the TGF- β superfamily, acting as potent regulators during embryogenesis and bone and cartilage formation and repair. BMP ligands are divided into at least four separate subfamilies depending on their primary amino acid sequence similarity and functions [50]. The first group consists of BMP-2, BMP-4, and the second group includes BMP-5, BMP-6, and BMP-7. Group three includes GDF-5 (or BMP-14), GDF-6 (or BMP-13) and GDF-7 (or BMP-12), and finally, group four consists of BMP-3 (or osteogenin) and GDF-10 (or BMP-3b) [51, 52]. BMP-1 does not include in this list as a member of the TGF- β superfamily and it may carry out a role in modulating BMP functions by the proteolysis of BMP antagonists/binding proteins, such as chondrin and noggin [47, 53].

BMPs bind to type-II serine/threonine kinase receptors and thus provoke the assembly of type-I and type-II receptors in a hetero-oligomeric complex [54]. Subsequently, the Smad-signalling cascade is triggered into the cell. BMPs are pleiotropic morphogens and carry out an important role in regulating growth, differentiation, and apoptosis of various cell types, including osteoblasts, chondroblasts, epithelial cells, and neural cells [55]. Furthermore, it has been demonstrated that the active signalling molecule is usually formed by homodimerization through a disulphide bond [56]. However, in particular, experimental settings heterodimers have been shown to have enhanced osteoinductive activity regulating more efficiently differentiation and proliferation of mesenchymal cells to osteoblasts *in vitro* and *in vivo* than

the corresponding homodimers (i.e., BMP-2/-5, BMP-4/-7, BMP-2/-6; BMP-2/-7) [57, 58]. In bone, BMPs are produced by different types of cells, including osteoprogenitors, mesenchymal cells, osteoblasts, and chondrocytes. BMPs are able to induce a sequential cascade of events for chondro-osteogenesis, including mesenchymal and osteoprogenitor cells proliferation and differentiation, chemotaxis, angiogenesis, and controlled synthesis of extracellular matrix [53, 55].

Regulatory effect of BMPs depends on the type of the targeted cell, its differentiation stage, the local concentration of the ligand and the interaction with other circulating factors [59].

BMPs are closely related structurally and functionally; however, each has a unique role and different temporal expression pattern during the fracture healing. The researchers demonstrated in several studies that BMPs could have a variety of osteogenic effects, mitogenic capacities, and temporal expressions in the rat and mouse [24, 60, 61].

Cheng et al. prepared a comprehensive analysis of the osteogenic activity of 14 types of BMPs and their results suggested an osteogenic hierarchical model of BMPs. BMP-2, BMP-6, and BMP-9 may act as the most potent to induce osteoblast differentiation of mesenchymal progenitor cells, while most BMPs (except BMP-3 and BMP-13) promote the terminal differentiation of committed osteoblastic precursors and osteoblasts [62]. Furthermore, BMPs are able to stimulate the synthesis and secretion of other bone and angiogenic growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), respectively and also stimulate bone formation by directly activating endothelial cells to stimulate angiogenesis [63].

Recent studies have shown that the expression of the BMP antagonists, most importantly noggin, plays an important role in fracture healing regulation [64]. They could block BMP-2 interaction with its receptor [65].

Transforming growth factor- β family includes five isoforms such as TGF- β 1, TGF- β 2, and TGF- β 3 [66, 67]. The main sources of TGF- β existing during the bone healing are practically all cells involved in healing process, incoming blood platelets, and the surrounding ECM releasing TGF- β following a mechanical injury causing tissue ischaemia and local change in pH, facilitating release of not only of TGF- β , but also other growth factors, such as VEGF, platelet-derived growth factor (PDGF), or BMP-2 [68, 69]. Intracellular signal transduction is exerted via type-I and type-II serine/threonine kinase receptors, activating the Smad cascade (Smad 2 and 3) [70]. TGF- β is a potent chemotactic stimulator of mesenchymal stem cells and it enhances proliferation of MSCs, preosteoblasts, chondrocytes, and osteoblasts. Indeed, its main role is thought to be during processes of proliferation, differentiation, and synthesis of cartilage and bone tissue, collectively mentioned as the bone-healing process [67, 71]. Also, it is able to induce the production of extracellular proteins, such as proteoglycans, fibronectin, collagen, osteonectin, osteopontin, thrombospondin, and alkaline phosphatase [72]. Moreover, TGF- β may trigger signalling for BMP synthesis by the osteoprogenitor cells, while it may inhibit activation, proliferation, and differentiation of osteoclasts and promote their apoptosis [60, 73].

Several studies have shown that TGF- β 2 and possibly TGF- β 3 had stronger effect in fracture-healing process than TGF- β 1, as their expression peak during chondrogenesis. On the other hand, Joyce et al. injected TGF- β 1 and TGF- β 2 subperiosteally to newly born rats, at doses ranging from 20 to 200 ng, and their results showed that subperiosteal MSC starts to proliferate and differentiate at the injection site, promoting chondrogenesis and osteogenesis, and that TGF- β 2 play more important roles than TGF- β 1 [74]. Moreover, Beck et al., designed an experiment concerning local administration of TGF- β 1 at doses ranging from 0.5 to 5 μ g to rabbits with skull defect, caused stimulation, recruitment, and proliferation of osteoblasts at the defect site resulting in healing [75]. Despite different studies demonstrated that TGF- β induces cellular proliferation, its osteoinductive potential is limited by concern for its unforeseen side effects [71].

Platelet-derived growth factors (PDGFs) are homo- or heterodimeric polypeptides in which their A and B chains are linked by disulphide bonds. PDGF receptors exert their effect on cells by activating receptors that have tyrosine kinase activity [76]. PDGF's binding is affected by IL-1, TNF- α , and TGF- β 1 affect [77]. It is synthesized by numerous cell types, including platelets, monocytes, macrophages, osteoblasts, and endothelial cells and is a major mitogen for cells of mesenchymal origin such as osteoblasts, fibroblasts, glial cells, and smooth muscle cells [78–80].

PDGF is released by platelets upon activation during the early callus phase of healing and acts as a potent chemotactic for inflammatory cells and a major proliferative and migratory stimulus for MSCs and osteoblasts. It has been demonstrated that treating with PDGF increased callus density and volume in tibial osteotomies in rabbits [47, 81]. However, its therapeutic potential still remains unclear.

Fibroblast growth factors (FGFs) consist of nine structurally related polypeptides. The acidic and basic FGFs are the most abundant FGFs in normal adult tissue [82]. FGF effect is exerted via binding to tyrosine kinase receptors [82].

FGFs are synthesized by monocytes, macrophages, osteoblasts, mesenchymal cells, and chondrocytes during bone healing. FGFs are able to induce growth and differentiation of a variety of cells, such as fibroblasts, osteoblasts, myocytes, and chondrocytes. They function during the early stages of fracture healing and play a critical role in angiogenesis and mesenchymal cell mitogenesis. α -FGF mainly affects chondrocyte proliferation and is probably crucial for chondrocyte maturation, while β -FGF is produced by osteoblasts and is recognized as a potent mitogen than α -FGF [71]. In a canine tibial osteotomy model, a single injection of FGF-2 resulted in an early increase in callus size [83].

Insulin-like growth factors (IGFs) consist of IGF-I (or somatomedin-C) and IGF-II (or skeletal growth factor) [84]. The sources of IGF-I and IGF-II are the bone matrix, osteoblasts and chondrocytes, and endothelial cells. The concentration of circulating IGF-I is mainly regulated by the growth hormone. Also, it has been demonstrated that the biological actions of IGFs is modulated in a cell-specific manner by IGF-binding proteins (IGFBPs) [71, 85].

IGF-I promotes bone matrix formation such as type-I collagen and non-collagenous matrix proteins by fully differentiated osteoblasts and acts more effective than IGF-II [71, 86]. IGF-II

functions at a later stage of endochondral bone formation and incites type-I collagen production, cellular proliferation cartilage matrix synthesis [87]. The findings from various animal studies assessing the influence of IGF on skeletal repair have reported different results, so further studies are required [88].

3.2.3. Metalloproteinases and angiogenic factors

Conditions of fracture healing establish a demand on the surrounding tissues to increase blood flow so that can induce bone regeneration within the callus [89]. Also, endochondral ossification in normal fracture healing requires the following two processes: (1) molecular mechanisms that regulate the extracellular matrix remodelling and (2) the vascular penetration of new blood vessels into the resorbing matrix [90]. Thus, angiogenesis and matrix degradation are either concurrent or correlated processes during endochondral ossification. The final stages of endochondral ossification and bone remodelling are accomplished by the action of specific matrix metalloproteinases, which degrade the cartilage and bone, allowing the invasion of the blood vessels. Angiogenesis regulation requires the coordination of both separate pathways, including a vascular endothelial growth factor (VEGF)-dependent pathway and an angiopoietin-dependent pathway [91]. Numerous types of studies reported that VEGFs are required mediators of endothelial-cell-specific mitogens and neo-angiogenesis [92]. Whereas angiopoietin 1 and 2 are regulatory vascular morphogenetic molecules related to the formation of larger vessel and development of colateral branches from present vessels [43]. Street et al. showed that exogenous administration of VEGF can induce fracture repair [48]. Also, recent studies have reported that BMPs promote the expression of VEGF by osteoblasts and osteoblast-like cells. However, their contribution in bone repair is still not as well understood.

3.3. Role of mesenchymal stem cells in bone regeneration and fracture repair

Mesenchymal stem cells (MSCs) are non-haematopoietic stromal stem cells capable of extensive replication without differentiation. They have many sources including bone marrow, peripheral circulation, adipose, periosteum, muscle, vessel walls, tendon, umbilical cord blood, skin, and dental tissues. MSCs have the potential to commit and differentiate along several cell lineages giving rise to those cells that form mesenchymal tissues, including cartilage, bone, muscle, ligament, tendon, and marrow stroma and fat [93, 94]. MSCs can migrate to sites of injury and have been used widely in tissue engineering, stem cell transplantation and immunotherapy. There are different sets of molecules interacting with both local cells and circulating cells to coordinate the healing cascade, such as effectors of inflammation (IL-1, IL-6, TNF- α), mitogens (TGF- β , IGF, FGF, and PDGF), morphogens (BMPs), and angiogenic factors (VEGF and angiopoietins). The effects of these molecules on the proliferation and differentiation of MSCs have been widely investigated *in vitro* [47]. The results indicated that these signalling molecules can induce cell proliferation and differentiation, both MSC and other progenitor lineages. The temporal expression of this array of signalling molecules in models of fracture healing has been charted, but explicit data on how this microenvironment can regulate MSC activity is still needed.

4. Tissue engineering strategies for bone regeneration

As it was defined by Laurencin, tissue engineering (TE) is 'the application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissue by using biomaterials, cells, and factors alone or in combination' [95].

Bone tissue engineering (BTE) is a dynamic and complex process that includes migration and recruitment of osteoprogenitor cells, followed by their proliferation, differentiation, matrix formation along with remodelling of the bone. In this section, we consider BTE as three interplaying components: (a) the extracellular matrix/scaffold, (b) the cells that reside in the matrix/scaffold, and (c) the environment that hosts the cells. However, major advances in BTE with scaffolds are achieved through biochemical factors, such as growth factors, genes, proteins, and drugs. Bone scaffolds are typically made of porous-degradable materials that prepare the mechanical support during repair and regeneration of diseased or damaged bone [7]. Also, physical factors, including substrate topography, stiffness, shear stress, and electrical forces, are other stimuli that have been proposed as one of the principal mediators of de novo tissue formation [96]. Box 1 highlights requirements for an ideal scaffold.

4.1. Biomolecule delivery

The strategy of concurrently modulating the chemical environments of the fracture site *in vivo* via controlled delivery/elution of biomolecule agents from an orthopaedic implant represents an elegant method of targeted therapeutics in bone regeneration [97, 98]. This strategy enables higher local concentration (localized delivery) of the bioactive agent to the fracture site, while the favourable bulk properties of the orthopaedic implant are unchanged. It also provides the chance to maximize the local growth-inducing potentials of bioactive agents at a desired rate without any local and systematic toxic effects to the host tissue that is attributed to other routes of delivery such as systemic or non-controllable local delivery. Soluble biochemical molecules that are integrated into scaffolds include proteins/growth factors, such as TGF- β , BMP, VEGF, IGF, and FGF, which have attracted much attention because of their potency in bone tissue repair. As described earlier, these growth factors are able to control osteogenesis, bone tissue regeneration, and ECM formation via recruiting and differentiating MSCs (osteoprogenitor) to specific lineages [99]. Therefore, various growth factors and other biomolecules are of special interest for bone tissue engineering and effective incorporation of them in scaffolds could reduce fracture healing time and thus facilitate in patient recovery [100, 101]. Also, bone is a highly vascularized tissue; therefore, the performance of a scaffold in bone engineering can be affected by its ability to induce new blood vessel formation. Because insufficient vascularization can lead to oxygen and nutrient deficiency, this may result in improper cell integration and cell death [102, 103]. On the other hand, in the *in vivo* conditions, supply of oxygen and nutrients are essential for the survival of growing cells and tissues within scaffolds. So, VEGF is used to induce a complex network of blood vessels throughout a scaffold [104].

Box 1. Requirements for an ideal scaffold

Biocompatibility is one of the primary requirements of bone scaffolds. It is a term that has been defined in many ways. Biocompatibility can be principally defined as the ability of scaffold to support normal cellular activity, such as molecular signalling pathways, without any local and systematic toxic effects to the host tissue [105]. An ideal bone scaffold must act as an osteoconductive substrate such that it permits the bone cells to adhere, proliferate, and form ECM on its surface and pores. Furthermore, the scaffold needs to induce bone formation within the defect through signalling systems and recruiting progenitor cells, a feature known as osteoinduction. Also, an ideal scaffold should be able to serve as a platform for formation of blood vessels in or around the implant during few weeks of implantation to promote nutrients and metabolic waste transportation [106].

Mechanical properties: An ideal bone scaffold should yield a close match to the host bone properties and also convenient load transfer is important. Mechanical properties of bone vary widely from cancellous to cortical bone. Cortical bone exhibits a Young's modulus between 15 and 20 GPa and that of cancellous bone is between 0.1 and 2 GPa. Compressive strength of cortical bone is between 100 and 200 MPa, and between 2 and 20 MPa for cancellous bone. Because of the large variation in mechanical property and geometry, it is difficult to design an 'ideal scaffold' for BTE [106].

Pore size and closed void volumes may concurrently play important roles in scaffold degradation patterns and associated bone healing [107]. It should be approximately 100 μm in diameter for successful cellular infiltration and nutrient and oxygen supply for cell survivability [102]. However, scaffolds with pore sizes between 200 and 350 μm are indicated to be optimum for bone tissue in-growth [108]. Moreover, recent papers have reported that multi-scale porous scaffolds which involve both micro- and macroporosities can act better than only macroporous scaffolds [109]. Unfortunately, porosity can reduce mechanical properties, such as compressive strength, and also increase the complexity for reproducible scaffold making. Researchers have developed porous scaffolds using polymers, ceramics, metals, and composites. Strength of different polymers matches close to the cancellous bone and dense bioceramic materials to that of cortical bone. However, scaffolds manufacturing ceramic-polymer composite are typically weaker than bone. Porous metallic scaffolds provide the mechanical necessities of bone, but fail to meet the required implant-tissue integration and also, there is potential concern regarding metal ion leaching [110].

Bioresorbability is another crucial requirement for scaffolds in BTE [105]. In addition to similar mechanical properties that of the host tissue, an ideal scaffold should be able to degrade with time *in vivo* by cellular and enzymatic activity, preferably at a controlled resorption rate in parallel with the production of new bone matrix. The degradation behaviour of the scaffolds is determined based on their applications; for example, 3–6 months for scaffolds in cranio-maxillofacial applications or 9 months or more for scaffolds in spinal fusion. Recently, design and development of multi-scale porous scaffolds having ideal composition, including related bioresorbability, targeted biomolecules, and mechanical properties are some challenging areas of research [106, 111].

4.2. Stem/progenitor cells applicable to bone tissue engineering

4.2.1. Mesenchymal stem cells

Mesenchymal stem cells have been isolated from a diverse host tissues throughout the adult organism including bone marrow [94] and an array of other postnatal tissues, such as adipose tissue [112], periodontal ligaments [113], synovium [114], blood [115] and the lung [116]. As the ultimate aim of regenerative medicine is to avoid *in vitro* expansion of cells and the associated complications, the adipose-derived stem cell indicates an ideal progenitor cell in bone tissue engineering.

Intriguingly, several studies have reported that 6×10^6 nucleated cells can be isolated from 1 mL bone marrow of which 0.001–0.01% are considered to be stem cells [94]. Contrastingly, adipose tissue aspiration yields 2×10^6 nucleated cells per 1 g, of which 10% are stem cells. Thus, one can easily distinguish the potential clinical implications of this abundant source of MSCs [117, 118]. In a study, researchers compared the *in vivo* osteogenic potential of adipose-derived, bone marrow-derived, and periosteal-derived MSCs in a guided bone regeneration model in pig calvarial defects to identify if there is a more desirable site from which to harvest MSCs for bone tissue engineering. They reported that regardless of the tissue source of MSCs, the speed and pattern of bone healing after cell transplantations into monocortical bone defects were comparable, indicating that the performance of autologous adipose-derived MSCs, periosteal-derived MSC, and bone marrow-derived MSC (BM-MSCs) following *ex vivo* cell expansion was not considerably different for the guided regeneration of bone defects [119].

4.2.2. Endothelial progenitor cells

Vascularization is a vital process for the survival of the implanted cells on the carrier material after implantation. Many studies demonstrated that close spatial and temporal association between blood vessels and bone cells is necessary to maintain skeletal integrity. Several studies have shown that new bone formation in porous scaffolds was considerably increased by the insertion of a vascular pedicle in the scaffold, while others have shown that fracture healing and new bone formation could be prohibited by the administration of angiogenesis inhibitors. Such that previous reports illustrated that the rate of delayed union or non-union of fracture can be as high as 46% in fracture patients with concomitant vascular injuries [120]. Because adequate vascularization making it possible to stem cells reach the site of tissue repair and allows the delivery of nutrients, oxygen, and morphogens and the removal of waste [121–124].

In 1997, Asahara and colleagues identified endothelial progenitor cells (EPCs) in the peripheral blood and reported their ability to initiate neovascularization [125]. EPC derived from purified hematopoietic progenitor cells, express endothelial-associated markers (i.e., cluster of differentiation molecule, CD34) and display endothelial phenotypical characteristics. They can enhance neovascularization by incorporation and differentiation, and by the secretion of angiogenic factors affecting resident endothelium [126].

The major role of EPCs in the ability of EPCs to proliferate and differentiate into endothelial cells and new vessel formation present them as an ideal therapeutic strategy for recovery of

the ischemic environment of a critical-sized bone defect in bone tissue engineering. Furthermore, a research group reported that the frequency of EPCs increased in the bone marrow and peripheral blood in the early stages of fracture repair and further illustrated incorporation of EPCs into developing blood vessels at the site of bone injury. Further histological results demonstrated that neovascularization did not exclusively involve the EPC population; however, supporting the hypothesis that paracrine signalling from EPCs may also contribute to neovascularization at the ischemic site [127].

4.2.3. Induced pluripotent stem cells

Induced pluripotent stem (iPS) cells, a discovery that resulted in a Nobel Prize in 2012, are somatic cells from embryonic or adult fibroblasts that are reprogrammed with defined classical transcription factors (Oct4, Sox2, Klf4, and c-Myc) [121, 128]. By forcing expression of these transcription factors, iPS cells retain the capacities of embryonic stem cells, including self-renewal and pluripotentiality to differentiate into all three germ layers [129]. Using these biological properties, iPS cells with an incorporation of gene therapy will be able to not only treat degenerative syndromes and genetic disorders but also appear as a promising candidate for autologous cell transplantation in bone defects. [129, 130]. Also, iPS cells, without the challenges of immunological rejection and ethical controversy, are preferable to embryonic stem cells and seem to be a potential alternative stem cell source for bone tissue engineering.

5. Conclusion

Bone regeneration strategies can make convenient, efficacious alternative therapies for orthopaedic usages and is attractive on a several aspects including: (1) *in vitro* tissue engineering for transplantation would reduce the necessity for donor tissue as required skeletal cells could be expanded in the laboratory prior to implantation; (2) using scaffolds with similar mechanical characteristics to bone that could integrate with the surrounding native tissue has the potential to alleviate the rate of implant failure and the need for revision surgery; and (3) treatment of damaged tissue at an early stage with mesenchymal stem cells could decrease or even cure the disease, reducing the need for lifelong treatment and improving the quality of life of the patient. Clinical applications include for the support of bone stock, in maxillo-facial surgery as well fracture and non-union fractures [131]. However, it is clear that a single approach is not able to support many of the bone tissue requirements, and refined approaches targeted to a specific application site/problem will be needed.

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