Application of Tissue Engineering in Skeleton: A Review on Recent Trends and Advances

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ABSTRACT. Due to the biological properties of bone and cartilage, tissue engineering, a new solution with no limit on supply, has gained much attention and many investigations over the past few years. In this review, three basic elements of tissue engineering in skeleton are discussed. Take planting trees as an analogy, scaffolds are the soil providing support, growth factors are fertilizers and pesticides which create a suitable environment together with scaffolds, and cells are the seed whose health and proliferation are the ultimate goals. Both stem cells and terminal differentiated cells like chondrocytes are investigated. Growth factors promoting differentiation, osteogenesis and vasculature are also included. Moreover, many studies focus on manufacturing scaffolds with perfect biological and mechanical properties, in which various structures and components are researched. Besides, 3D bioprinting are utilized for accurate control. Furthermore, studies on engineering and science, in vivo trials, and more available resources have been researched.

1. Introduction
Bone deficits could be attributed to accidents, iatrogenic factors (surgical removal of a malignant bone tissue, radical treatment of a certain disease), congenital abnormalities such as hemivertebra, fusion of carpus, and degeneration of skeleton because of old age and general disease like Paget’s disease. With a limited self-healing capability, osteochondral lesions, especially large deficits in bone and joint, require surgical intervention in functional rehabilitation[1]. Bone grafting ranges the second most frequent transplantation around the world, right after blood transfusion[2]. However, like all other transplantation, patients are confronted with long waiting list and possible severe immune response, inflammation and disease transmission when an allogenic bone transplantation is needed[3][4]. As to an autologous graft, bone and skin flap from healthy areas are harvested as the replacement for those taken in surgery, providing both contour and blood supply[3]. Though regarded as the ‘gold standard’ in clinical practice, this procedure leads to additional damage, blood loss, post-operative pain and morbidity to donor sites[1][2][5]. Xenografts are also considered as bone substitute due to easy sources and cheap price, but not widely used because of immune rejection and other contrasting drawbacks[2][5]. Therefore, more attention are cast on osteochondral tissue engineering.

Tissue engineering refers to an attempt to create functional tissue mimics in a laboratory, and its ultimate goal is to replace biological tissues that fail due to diseases, injury, or congenital abnormalities, therefore maintaining or repairing the function. To ensure good prognosis, an ideal bone substitute should be osteoconductive(able to conduct bone tissues), osteoinductive(able to induce bone growth), resorbable, sterilizable and affordable to most patients[2][5]. Four main factors are included in the process of tissue engineering: 1) donor cells; 2) scaffolds; 3)biomolecules that help to make donor cells productive; 4)physical and mechanical forces for cells.
Cells can be harvested from the targeted organ, cell lineage in the laboratory, precursor stem cells, or from the patient’s healthy bone tissue[3][6][7]. Mesenchymal stem cells (MSCs) are multipotent cells with good ability to divide and differentiate into osteoblasts that are required in constructing bones. It is also reported that periosteum-based cells with the same surface marker as MSCs, CD21, also presents good bioconductivity and bioinductivity[7]. Nasal cartilage was also reported, not only in reconstruction after nasal surgery, but also in its accommodation in post-inflammatory areas with hypoxemia and various cytokines[4]. A scaffold is the supporting structure on which donor cells proliferate and differentiate, while providing mechanical connections between mimics and healthy tissues around and acting as the local environment for various cellular signaling pathways. Made by biomaterial and to be implanted into the body, a scaffold should be mechanically stable, biocompatible, biodegradable and elicit no adverse inflammatory or autoimmune response[2][8]. For practical reasons, a scaffold should be easily synthesized, leading to comparatively lower cost and shorter waiting time. Scaffolds are composed of proteins or inorganic molecules that further form the structure as a whole. When implanted into the body, the scaffold, together with proliferative biomolecules, acts as a part of the microenvironment for donor cells[2]. With the property of osteoinductivity, the scaffold recruit cells from the surrounding areas while resorbing automatically, which in the long term, fully regenerate the tissue mimicking the native one. In order to accomplish that goal, mechanical and biological properties of scaffolds, together with the delivery of growth factors are crucial to the success of bone remodeling[1][2], and biodegradability helps the replacement. It has been commonly acknowledged that porous scaffolds provide good room for cell proliferation, but the size and distribution of pores are still in research[1]. The need for suitable water absorption rate, degradation rate, mode of releasing growth factors and sterilization boost the interest of scientists, contributing trails in the constituent ratio, manufacturing method and micro framework of scaffolds[1][4][8]. Meantime, it is reported that hollow cylinder shape and channels inside increase blood and oxygen perfusion, angiogenesis and delivery of stem cells and growth factors[9]. Biomimetic materials include calcium phosphate(such as hydroxyapatite, tricalcium phosphate), calcium sulfate, bioactive glasses and polymers(such as chitosan)[5][6][7].

In this paper, we aim to provide a contemporary and systematic review of tissue engineering in bone regeneration, mainly focusing on grafting materials and scaffolds which are necessary in this new technique. By bibliography retrieval and essay analysis, the basic elements and methods of bone tissue engineering are studied and presented. Moreover, challenges in this field, as well as future prospects are discussed. In the end, a brief summary of this review is presented.

2. Cells and growth factors

2.1 Cells

Stem cells are a group of cells that can differentiate into many types of mature cells while keeping producing stem cells as well, and this mechanism is called obligate asymmetric replication. Various stem cells are first considered as to regenerate bone tissue. Among them, mesenchymal stem cells (MSCs) are multipotent stem cells that further generate bone cells, cartilage cells and adipose cells. With a high capacity of differentiation and the ability of immunomodulation, MSCs are widely exploited in tissue rehabilitation. For instance, when capsulated in bioactive matrix containing molecules like vascular endothelial growth factors (VEGF), MSCs can differentiate into early vascular cells (VECs) which later forms mature and stable vessels[10]. Without doubt, bone marrow is richest in MSCs within the body, but other resources are also researched, and among them, adipose tissue is highly valued[6]. Recently, it is also reported that MSCs can deliver osteoinductive genes and ‘in vivo’ inoculate in a bone deficit[2].

Periosteum is a multi-layered tissue that forms the outside structure of a bone. The inner layer is osteogenic, while the outer provides mechanical support with a substratum rich in blood vessels and nerves[7][11]. Having surface markers such as CD73, CD90, CD105 which overlap some on the surface of MSCs, periosteum-derived cells (PDCs) were researched in bone regeneration, and the
results were quite promising, though largely depends on the quantity of healthy periosteum cells[7]. Decellularized periosteum incorporated with chitosan, combining the properties of biocompatibility and flexibility, was studied as a naval material to fill in the deficit of bone and induce ossification process[11]. Fulco and colleagues did a research utilizing nasal chondrocytes[3]. In their experiment, cartilage was first harvested, then chondrocytes were isolated from samples. After incubation and expansion, they seeded the chondrocytes on a porous membrane, where later on, engineered cartilage formed. This novel experiment provided another alternative besides stem cell in tissue engineering. Scotti et al. did a comparison between articular and nasal cartilage on their ability to tolerate micro environments after injury, which lacks oxygen and is rich in cytokines like interleukin-1β[12]. As a result, human nasal chondrocyte (HNC) retained a better tissue-forming capability. Above two researches are examples utilizing differentiated cells as a biomaterial for tissue engineering, and nasal chondrocyte show promising potential for future investigation. The function of osteoblasts and osteoclasts is also associated with scaffolds. Calcium phosphate-made scaffolds can prolong the life span of osteoblasts and regulate the formation of osteoclasts[8][13].

| Classification of cells in bone tissue engineering. |
|-----------------|-----------------|-----------------|
| **Cell types** | **Microenvironment** | **Products** |
| Undifferentiated | hMSCs | Co-cultured with epithelial cells Fibronectin-containing collagen gels | Vascularized bone |
| | BMSCs | PCL Collagen Nano-hydroxyapatite | Periosteal bone repair |
| | *In-vivo | Chitosan-DP globules | Initial stage of bone healing |
| Differentiated | Nasal C. | **Chondro-Gide** | Engineered cartilage |
| | Articular C. | Pellets or collagen-based scaffolds | |

hMSC: human mesenchymal stem cell; Nasal C.: nasal cartilage; Articular C.: articular cartilage; PDC: periosteum-derived cells; DP: decellularized periosteum; *: this experiment was conducted in-vivo, and the effective cells are healthy bone tissue surrounding the defect; BMSC: bone marrow stromal cells; PCL: polycaprolactone; **: a CE-marked, licensed membrane.

2.2 Growth factors
Growth factors are biomolecules associated with cell proliferation, differentiation, stem cell recruitment, morphogenesis, metabolism and wound-healing through different signaling pathways. They are divided into different families according to their functions or characteristics. Among them, bone morphogenic proteins (BMPs) is considered the most important category, especially BMP-2,4,6,7[2][5][6]. These reasons are included: 1) BMPs induces cell differentiation from pluripotent stem cells to osteogenic and chondrogenic cells; 2) promotes revascularization; 3)increases the activity of alkaline phosphatase. However, it was reported that BMP has a paradoxical inhibitory effects on spine surgery at high concentration, and the treatment effect on cervical spine remains contradictory. Apart from BMPs, products or derivatives of which were also reported to function in bone regeneration. Bone formation peptide-1 (BFP-1), a peptide derived from BMP-7, has the similar ability of osteoinduction with a longer half-life and slower local clearance[16]. Besides, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF) also promotes activation and recruitment of stem cells, which later facilitate reconstruction of bone tissue and revascularization[2][5]. Scaffolds incorporating bioactive factors are verified to have an increased ability of bone formation[14]. As will be mentioned below, the function of growth factors can be influenced by biomaterials those are used as scaffolds. For instance, phosphate increases the expression of BMPs[8][13].
### Table 2 Growth factors in bone tissue engineering.

| Categories | Effects                                                                 | Mechanisms               |
|------------|------------------------------------------------------------------------|--------------------------|
| BMPs       | BMP-2: spine fusion healing fracture                                    | Osteoinduction           |
|            | Incorporation with scaffolds                                            | Promoting angiogenesis   |
|            | BMP-7: tibial non union                                                | Stimulating ALP activity |
| BFP        | Alginate-BFP1 hydrogels                                                | Increasing ALP and calcium deposition |
| VEGF       | Bridging of defects                                                    | Angiogenesis              |
| FGF        | Fibers encapsulated with FGF                                           | Increasing ALP and calcium levels |

BMP: bone morphogenic proteins; BFP: bone formation peptide, a product of BMP; PDGF: platelet-derived growth factor; IGF: insulin-like growth factor; VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; TGF: transforming growth factor; ALP: alkaline phosphate;

3. Scaffolds

Scaffolds in bone tissue engineering is, in fact, an artificial structure supporting the formation of regenerated tissue. In order to get better and faster rehabilitation, scaffolds should be both biologically and mechanically qualified. Among biological features, a scaffold is required to be non-toxic, biocompatible, resorbable, bioactive, and cause no or slight immune response. Due to its use as a framework, a scaffold should be shapable, highly porous with larger contact area for cells to grow, and mechanically stable to sustain strength in articulation. Only by meeting these standards, can a scaffold mimic the regular microenvironment where osteocytes grow. Besides, acting as a microenvironment for surrounding cells, scaffolds are also vehicles for cells, biomolecules, drugs and even genes which further improves its bioactivity of osteoinduction and osteoconstruction in bone deficit.

3.1 Biomaterials composing scaffolds

Several inorganic elements influence the regeneration of bone tissue. Calcium is a mineral necessary for living organisms, and in human bone tissue, calcium forms the majority of matrix, induces bone growth precursor cells, regulates the function and affects life span of osteoblastic cells; phosphate adjusts the differentiation of osteoblasts and increases the amount of BMPs; magnesium promotes vascularization; silicon induces bone regeneration and the ingrowth of blood vessels as well[8][9]. According to Ca/P ratio, crystal structure, stability and solubility, calcium phosphates are divided into categories like hydroxyapatite(HAP), tricalcium phosphate(TCP) and whitlockite(WH), and due to the high porosity, they can be utilized in scaffold to improve mechanical properties[8]. Wenjie et al. made a combination of calcium, silicon, magnesium ions which showed promising osteogenesis and blood vessel ingrowth both in vitro and in vivo[9]. Calcium sulfate hydrate(CSH), a biomimetic material already used in bone augmentation and bone substitute, is also a candidate for scaffold. CSH can react with water to form calcium sulfate dehydrate(CSD) and obtain enhanced mechanical properties at 37°C in 100% humidity[15]. Above inorganic materials have separative drawbacks, for example CSH presents weak acidic microenvironment, high soluble calcium phosphate easily changes the local pH and ion concentration, but such drawbacks could be made up by measures like binding with other bioactive materials[8][15]. Mesoporous bioactive glass(MBG) is recently researched to perform well in bone-forming bioactivity, and it also has a stable rate of ion release, drug delivery and compressive strength. Binding CSH and MBG gets a combination of both biological and mechanical properties, which was tested promising both in vitro and in vivo[15]. Having unstable linkage between backbones, stable degradation rate and good biocompatibility, polymers attract much attention as a candidate for tissue engineering scaffolds[2]. Polymers can be divided into natural and synthetic ones. Chitosan is a copolymer abundant in crab shell, shrimp and other natural resources. With the surface positively charged, cells and growth factors like BMP-2 are attracted which facilitates faster and better tissue regeneration[7]. Natural polymers like chitosan are easily available and have excellent bioactivity but lacks mechanical strength[8], while synthetic ones release molecules steadily through the degradation of covalent bond. Synthetic polymers extensively investigated include poly(lactic-acid)(PLA),

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poly(glycolic-acid)(PGA) and poly(caprolactone)(PCL), moreover, copolymerization which combine multiple constitutions into a new one is also utilized in scaffold manufacturing[2].

### 3.2 Structure of scaffolds

Larger surface area exposes the scaffold more to body fluid which contains proteins needed for tissue regeneration, and provides better efficacy for the delivery of stem cells and growth factors, which therefore improves the prognosis of transplantation. Small granular structure is an easy method to enlarge contact area and provide interconnection inside the framework[7]. Porosity is another choice to enlarge surface area, and study showed that in calcium phosphate scaffold, the biological effects have reached the best with a pore size of 50-200um and an increasing number of pores per unit area[8]. Innovative concepts of pores on scaffolds include a gradient pore size, a progressively increasing pore distribution, a gradually increased calcium phosphate concentration from the upper to the lower part, and cellular experiments showed good bone marrow stromal cells (BMSCs) attachment and biological activities[1]. A hollow structure also increases the surface area. In the BRT-H termed scaffold in Wenjie and colleagues’ research, hollow pipes are put paralleled inside a layer, and two adjacent layers are placed vertically. This internal structure effectively increased surface area, strengthened mechanical properties, fastened the scaffold degradation, and induced angiogenesis which offers more oxygen and blood to the site[2]. A layer-by-layer bottom-up strategy was raised to construct a biomimetic periosteum with high regenerative capacity, making the replacement of the injured one possible[11]. With the development of computerized printing, three dimensional structures can be precisely controlled using 3D printing, thus enhancing the mechanical stability and reducing the cost of scaffolds[9][17].

### 3.3 Delivery of cells, biomolecules, and drugs

In a new report on scaffolds, electrosprun was utilized to attract osteoprogenitor cells which proliferated and differentiated as on periosteum, later, cells migrated off to fit in the three dimensional deficit[6]. The delivery of biomolecules also largely increases the efficacy of tissue transplantation. Various delivery methods includes: surface adsorbed protein release, pumps, bolus injection and biodegradable materials which release biomolecules in a controlled way[2]. Sometimes, a delivery strategy could be a must for a successful bone tissue transplantation. Take BMP-2 for example. As mentioned in 2.2, BMP-2 is a growth factor with high osteoinductivity, but a short biological half life limits its adoption. With a scaffold that delivers it in an appropriate speed and amount, the engineered bone can have a better function. Since tissue regeneration is a complex process involving several types of biomolecules and cells, researches on co-delivering multiple growth factors are conducted, showing better osteogenesis synergistically or additively[6]. Besides biological macromolecules like proteins, bioactive ions are capable of facilitating revascularization and bone regeneration locally. Sterilization is of great significance to post injury recovery and after transplantation, so materials deliver antibiotics comes to the minds of researchers. One of the most commonly investigated is poly(lactic acid-co-
glycolic acid)(PLGA) binding antibiotics which scatter around with the degradation of polymer itself[2].

4. In-vivo Trials

With the continuous development of science and engineering, more and more biomaterials and manufacturing techniques emerge and bring new solutions to bone tissue engineering. However, the ultimate goal of research is to apply new technologies to human bone deficit, whether accidental or iatrogenic. Therefore, large amounts of trials are conducted in vivo, making preparations for clinical application on large scale. Some of them are presented below. In the field of donor cells, Lancet once published an article reporting an in-human trials as regard to nasal cartilage tissue engineering[3]. In this article, a small piece of nasal septal cartilage was harvested from patients who had surgeries of non-melanoma skin cancer on the alar lobule. After incubation, isolation, expansion and other cytological treatment, nasal chondrocytes were seeded on a porous membrane with porcine-originated collagen following a two-week culture with substances supporting matrix deposition and assembly. Eventually, graft cartilage was obtained and implanted to the patient. It is noteworthy that MSCs have cross-communication with other cells like endothelial cells(ECs), when co-cultured in fibronectin-containing collagen gels, MSCs presents a similar biological activity of perivascular cells which further facilitate the regeneration of vessels, in vitro and in vivo[10]. Growth factors conjugated scaffolds presents promising osteogenesis in animal experiments as well. For example, a group of Korean scientists fabricated BFP1-conjugated hybrid alginate scaffolds, and found that on this scaffold, BFP1 has a dose-dependent effect on stem cell growth and bone regeneration[16]. As to scaffolds, the widely existed copolymer chitosan is considered as a possible resource for bone tissue engineering. When incorporating with decellularized periosteum and implanted in vivo, the chitosan fiber swell, thus providing more chance for cells and nutrients to attach onto the scaffold[7]. The combination of CSH and MBG also combines their separate advantages, thus forming a scaffold with both biological and mechanical properties. In vivo trial was conducted on Sprague-Dawley(SD) rats, CSH/MBG scaffolds were implanted in calvarium following intramuscular antibiotic injection, and CT scan after eight weeks showed enhanced bone formation in defects[15]. A rabbit radius segmental defect model was designed to investigate the BRT-H scaffold, which showed a synergistic effect in early angiogenesis of hollow structure and calcium, silicon, magnesium ions, with blood vessels found inside the hollow pipes of scaffolds[9].

5. Conclusion

Injuries in bone and cartilage are common in practicing medicine. In order to restore the structure and function, avoid the side effects of allograft transplantation, and address the problems of long waiting list, bone tissue engineering comes as a new solution. After seeding proliferative cells in an appropriate environment composed of stable scaffolds and osteogenic growth factors, scientists obtain an engineered tissue growing as a substitute, the desired tissues regenerate and the defected organ is expected to restore. Experiments on tissue engineering has been conducted not only on bone and cartilage, but also on other tissues like trachea, blood vessel and bladder. What’s more, there is a wider cell resources, and adipose tissue, peripheral blood and healthy surrounding tissue could be possible alternatives. To improve the survival of implanted tissue, techniques like preprocessing and 3D bioprinting are adopted. Unique procedures offer new prospects to specific fields, but could also set limitations vice versa. When celebrating the overwhelming accomplishments, even more challenges do exist and require new insights. Firstly, in terms of technology, many cell types and biomaterials have been investigated to suit each kind of bone deficit. Therefore, a corresponding relation could be established according to different demands. Then, integration with the original bone, vascularization and even nerve fiber regeneration should be facilitated to maintain and recover the function better and longer. Secondly, long term follow-up evaluation is a must before bone tissue engineering put into wide use. Specially, if bone tissue engineering is applied to joint injury, long term activity and stability should be carefully evaluated. Thirdly, similar to all other new therapy, it is also of great need to lower
the expenses and fasten official approval to be widely used in clinical practice. Lastly, although long waiting list seems unnecessary in the era of tissue engineering, a cell banking system might be helpful for better management. To sum up, based on the three elements (cells, growth factors, and scaffolds), this review covers recent accomplishments in bone tissue engineering. Although research results portrayed a promising future, more large sample clinical trials are needed to further assess the feasibility and polish the techniques before bone tissue engineering is widely put into practice.

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