

PERSPECTIVES

Tissue Engineering of Bone and Cartilage

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Abstract

In vertebrates, the skeleton has pivotal roles in mobility, locomotion, calcium homeostasis, hematopoiesis and protection. Therefore, irreversible skeletal defects often incur considerable morbidity. Tissue engineering has drawn attention as a promising strategy for the treatment of irreversible tissue defects. There are three pillars important for tissue engineering: cell sources, signaling factors, and scaffolds. Since the late 1990s, substantial progress has been made in tissue engineering of bone and cartilage along with advances in stem cell biology, bone and cartilage biology, and materials science. In particular, autologous cell implantation combined with biodegradable scaffolds have been extensively researched. This *Perspective* aims to review recent advances and major obstacles currently faced by the field of tissue engineering of bone and cartilage. It includes discussion of each pillar of tissue engineering, with a focus on several preclinical and clinical studies that are milestones in this field, and suggests future perspectives and directions. *IBMS BoneKEy*. 2009 November;6(11):405-419.

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Introduction

The skeleton provides mechanical support to soft tissues and provides levers for muscle action, and its ability to articulate in various opposing directions is fundamental to mobility and locomotion. It also has important roles in maintaining blood calcium levels, supporting hematopoiesis and housing the brain and the spinal cord (1). Consequently, skeletal defects often incur considerable morbidity. Conventional medical strategies focus on removing causes of diseases and, when it comes to the repair of tissue defects elicited by the diseases, mostly rely on natural healing abilities of tissues, failing to cure irreversible tissue defects. In bone and cartilage, irreversible tissue defects are caused by aging, trauma, disease, tumors, and developmental abnormalities. In particular, osteoporosis and osteoarthritis are notable in terms of their frequency, morbidity, mortality, and medical costs. Osteoporosis is a skeletal disease characterized by decreased bone strength predisposing

individuals to an increased risk of fracture. It occurs in women, the elderly, the immunocompromised, and patients with arthritis, autoimmune diseases, and HIV (2). Osteoarthritis is a chronic degenerative joint disorder induced by accumulated mechanical stress, accompanying articular cartilage destruction and osteophyte formation, and the disease is a major cause of disability in the elderly (3). As the baby boomer generation ages and as lifespan increases, osteoporosis and osteoarthritis are now reaching epidemic proportions.

Bone grafts and prosthetic implant devices are current strategies to repair irreversible bone defects. Those strategies achieve bone repair by eliciting two distinct activities: osteoconductivity and osteoinductivity. The former is an activity that facilitates bone growth on an implant's surface through the migration of preosteoblasts and osteoblasts from an intact bone. The latter is an activity that newly induces osteogenesis through the recruitment of immature cells and their differentiation to osteoblasts. Autograft is

superior to the other techniques in function and engraftment because it uses bone tissue derived from the same individual containing live cells and growth factors. Autograft has both osteoconductivity and osteoinductivity, and is speedily fused and integrated to the bone of the implantation site. However, because this process requires highly invasive bone collection surgery from healthy sites, donor site morbidity often occurs, limiting the quantity of autograft (4). Although allograft is usually collected from cadavers and thus is free from the invasiveness to the recipient and less restricted in quantity, it runs a biological risk of contamination by pathogens as well as an ethical risk associated with illegal body trading (5). In addition, because allograft is usually heat-treated and kept frozen in order to reduce immunological reactions, no live cells are present, and growth factors are inactivated to some extent. Therefore, it has a lower activity of bone repair than autograft.

Compared to bone defects, the treatment of cartilage defects is more challenging. Several strategies have been reported: autografts of periosteum and perichondrium, cartilage transplantation, and mechanical penetration of subchondral bone for bone marrow entry into the defect site. However, these approaches fail to provide reproducible results or complete repair of the defects (6). In addition, common to the procedures for both bone and cartilage defects, grafts must be manually carved to fit to deformities during surgery. This process is often time-consuming and laborious and is associated with low precision (7). In short, grafts have shortcomings concerning both quantity (availability of suitable graft material) and quality (donor site problems, graft rejection and disease transmission). Prosthetic implants overcome some problems associated with grafts, but have shortcomings concerning biocompatibility, function, and longevity.

Bone and cartilage regenerative medicine, by using the technique of tissue engineering, attempts to provide solutions to such problems. There are three components important for tissue engineering: cell

sources, signaling factors, and scaffolds (Fig. 1). To bring tissue engineering into reality, it is crucial to sufficiently advance and combine the three pillars (8). It is also important to justify and optimize the use of each pillar. This *Perspective* reviews current research on tissue engineering of bone and cartilage, focusing on important advances as well as major obstacles in the field. We also highlight important translational studies and discuss future perspectives.

Cell Sources for Bone and Cartilage Tissue Engineering

In bone tissue engineering, autologous cell transplantation of mesenchymal stromal cells (MSCs) derived from bone marrow (bone marrow MSCs) has been widely used in combination with various biodegradable scaffolds. It has been reported that, when infused into children with osteogenesis imperfecta, bone marrow MSCs induce new lamellar bone formation and an increase in total body mineral content with an increased number of osteoblasts (9;10). The use of bone marrow in the treatment of non- or delayed union has been described in several clinical studies (11). An uncontrolled study has described the successful use of autologous MSCs in three patients with large bone defects, in combination with porous ceramic scaffolds (12).

Regarding cartilage repair, the implantation of autologous MSCs into human knees for the treatment of cartilage defects has been reported (13;14), with several studies describing that MSCs isolated from synovium (synovial MSCs) showed higher chondrogenic capacity than MSCs isolated from other tissues. An *in vitro* study indicated that human synovial MSCs had higher capacity to differentiate into chondrocytes than MSCs derived from any other tissues including bone marrow, periosteum, muscle, and adipose tissue (15). In a rat cartilage defect model, synovial MSCs were shown to induce cartilage repair more potently than muscle- or adipose tissue-derived ones (16). Moreover, it has been reported that synovial MSCs expanded more *in vitro* than bone marrow MSCs in autologous human serum (17). These data suggest that synovial MSCs may be a more

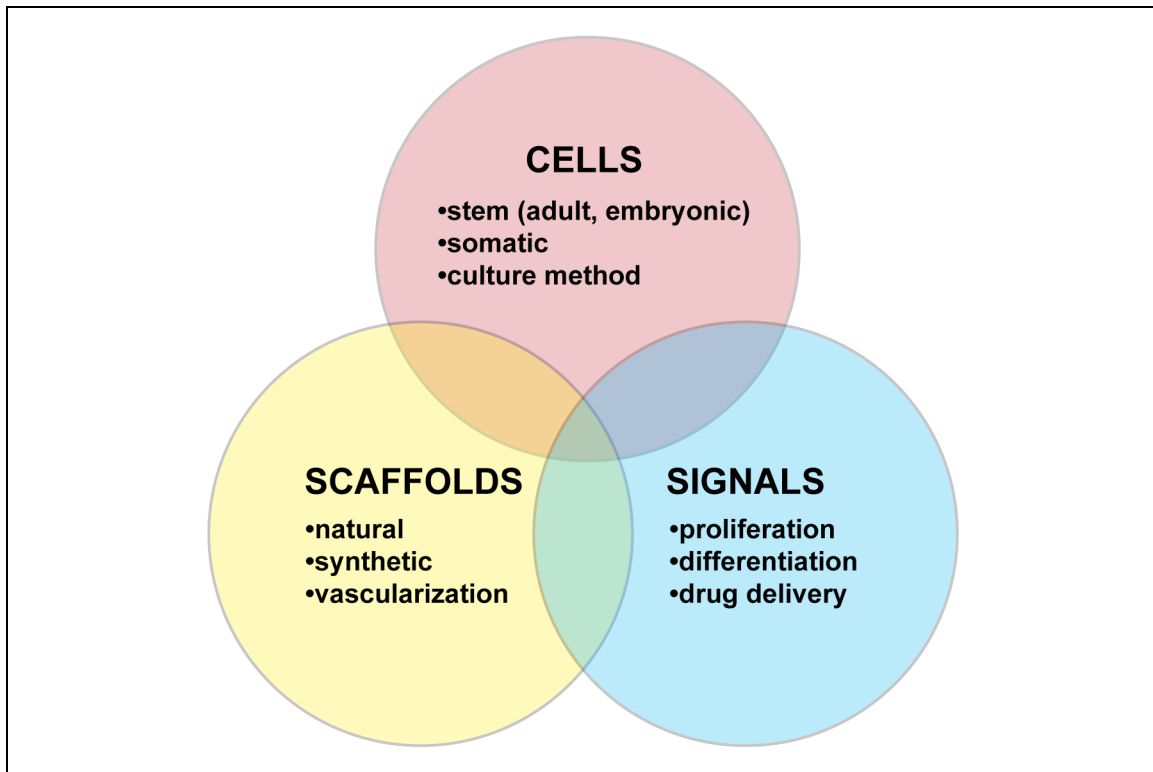


Fig. 1. Schema of the three pillars of tissue engineering. To bring tissue engineering into reality, it is crucial to sufficiently advance and combine the three. Currently, there is increasing recognition of the importance of cell culture methods, vascularization of scaffolds and drug delivery of signaling molecules.

potent cell source for cartilage repair than bone marrow MSCs. However, bone marrow can be harvested more easily than synovium, which is why bone marrow MSCs have been used more widely (18). The United States National Institutes of Health website, www.ClinicalTrials.gov, reports that several clinical trials are ongoing to evaluate the safety and/or efficacy of autologous MSC transplants in bone and cartilage defects.

However, MSCs still have substantial technical limitations both in terms of quantity and differentiation capacity. From 10 ml of bone marrow fluid or adipose tissue, only 10³ to 10⁶ cells can be isolated (19;20). To treat clinical bone defects, ~10⁹ cells may be required (19), but it is difficult to expand MSCs by several rounds of passages without affecting their differentiation capacity (21).

On the other hand, embryonic stem (ES) cells proliferate practically indefinitely and possess totipotency (an ability to give rise to all cell types of the embryo) (22). The cells,

isolated from the inner cell mass of blastocysts, were initially established from mouse embryos in the 1980s and later from human embryos. *In vitro* and *in vivo* studies have shown that ES cells can differentiate into osteoblasts and chondrocytes under certain conditions (23-25). However, four major problems remain to be solved: low efficiency of differentiation protocols, teratoma formation by residual undifferentiated cells, immunological reactions, and ethical issues accompanying the use of human embryos.

Induced pluripotent stem (iPS) cells may solve the immunological and ethical problems of ES cells to some extent, but the problems of low differentiation efficiency and teratoma still remain. Although the successful differentiation of iPS cells into osteoblasts has been reported (26), it is still necessary to avoid contamination of any undifferentiated cells for clinical use, which will be quite a tough hurdle. On the other hand, studies on the generation of iPS cells shed light on how adult non-stem cells can be reprogrammed. An interesting recent

finding is that the modulation of chromatin structure is important for reprogramming (27). Cells change their transcriptional program dramatically according to their behavior, and in this process, epigenetic regulation has an important role. In fact, overcoming epigenetic barriers that are involved in the silencing of reprogramming-related genes – histone deacetylation, histone methylation and DNA methylation – was shown to improve the efficiency of transcription factor-induced reprogramming (28-30). In the future, we may be able to enhance the efficiency of osteogenic and chondrogenic differentiation as well as reprogramming by modulating epigenetic regulation.

One solution to overcome problems associated with the use of stem cells is to utilize abundant autologous adult cells such as skin fibroblasts. We and others have examined whether skin fibroblasts could be a cell source for bone and cartilage regeneration. Using animal models, other investigators have described *in vivo* bone regeneration using dermal fibroblasts that were transduced with adenoviruses expressing bone morphogenetic protein (BMP)-7 and BMP-2, respectively (31;32). We have shown the efficacy of optimized osteogenic signals for *in vivo* bone regeneration using mouse dermal fibroblasts (33) and the induction of chondrocyte markers in human skin fibroblasts *in vitro* (34).

Osteogenic Signaling Factors

Substantial progress has been made towards a basic understanding of major osteogenic signaling molecules and genes such as BMPs, Hedgehogs (Hhs), Runx2, Wnts, and insulin-like growth factors (IGFs) (35-39). Among those molecules, the application of recombinant human (rh) BMP-2 and BMP-7 has been intensively studied for the treatment of fracture repair of the tibia and spine fusion (11). A randomized, prospective, multi-institution study of the treatment of tibial non-unions using rhBMP-7, also known as osteogenic protein (OP)-1, has been reported (40). Clinical outcomes of rhBMP-7 treatment are comparable to those of autologous bone graft with no adverse

events, leading the study's authors to conclude that rhBMP-7 is a safe and effective alternative to the bone graft. A prospective, randomized, controlled, single-blind study to evaluate the safety and the efficacy of rhBMP-2 in the treatment of open tibial fractures has also been reported (41). Patients receiving standard care (intramedullary nail fixation and standard soft tissue management) were compared to patients receiving standard care and rhBMP-2 implants (0.75 mg/mL or 1.5 mg/mL rhBMP-2 with absorbable collagen sponges). The 1.5 mg/mL rhBMP-2 group had a reduction in the risk of failure, significantly fewer invasive interventions, significantly faster fracture-healing, and fewer infections than did the control patients. Regarding the use of BMPs in spine fusion, the use of rhBMP-2 in posterolateral lumbar spine fusion (42) and rhBMP-7 in noninstrumented posterolateral spinal fusions (43) has been evaluated and successful results have been obtained. There are other promising results on the efficacy of BMPs in clinical settings, and recently, the use of BMPs in clinical applications was reviewed (11). However, a large amount of BMP is required for successful outcomes and BMP-containing devices fail in a certain percentage of cases, raising concerns over costs and safety (44-46). The reasons for this may be related to a lack of controlled and sustained BMP delivery, its short biological half-life, and the inability of its presentation to mimic the biological condition (47).

Preclinical studies also suggest that Runx2 is useful for bone regeneration if it is applied to stem cells, osteoblast lineage cells, or cell populations containing either kind (48-51). In the above studies, the possibility was not excluded that other signaling molecules, including their combination with BMP, might exert a stronger effect on bone regeneration because neither BMP nor Runx2 was selected through comprehensive screening. In addition, although most of these individual molecules are endogenously expressed in various tissues, the region where osteogenesis occurs is restricted. Based on these data, we have hypothesized that individual factors are not potent enough and that ideal signaling may be achieved by a new factor or combination of factors.

Through screening cDNA libraries and the combination of known osteogenic signaling pathways (BMP, Hh, Runx2, Wnt, and IGF-1), we previously identified BMP signaling and Runx2 as a potent combination for osteogenic differentiation. Rapid bone regeneration was induced by transplantation of a monolayer sheet of fibroblasts transduced with the combination (33).

The use of small chemical compounds is another strategy to induce osteogenic differentiation by activating osteogenic signaling pathways. Despite recent successes with drugs inhibiting bone resorption, there are a limited number of reports on such anabolic agents that effectively increase bone formation. Statins (52), isoflavone derivatives (53;54), and TAK-778 (55) were reported to stimulate osteogenic differentiation, but their osteogenic activity was shown only in specific cell types including osteoblastic cells and stem cells. We have identified a couple of osteogenic small compounds including 4-(4-methoxyphenyl)pyrido[4',3':4,5]thieno[2,3-b]pyridine-2-carboxamide (TH) (56), icariin isolated from the herb *Epimedium pubescens* (57), and an isoflavone derivative, glabrisoflavone (58).

Chondrogenic Signaling Factors

A number of factors have been shown to be vital for chondrogenesis. These factors include the sex-determining region Y-type high mobility group box (SOX) family of transcription factors (59), IGF-1 (60), fibroblast growth factor 2 (FGF-2) (61), Hhs (62), BMP-2 (63), transforming growth factor- β (TGF- β) (64), and Wnts (62).

The use of three factors, TGF- β 3, BMP-6 and IGF-1, in pellet cultures of human bone marrow cells for chondrogenic induction has been reported (65). TGF- β 1 was shown *in vivo* to induce the differentiation of MSCs to form ectopic cartilage and to repair a full-thickness cartilage defect by improving chondrocyte integration into the endogenous tissue (66). Regarding FGFs, FGF18 stimulated repair of damaged cartilage (67), and it has been reported that the implantation of a fibrin sealant incorporating

FGF-2 successfully induced healing of the surface with hyaline cartilage and concomitant repair of the subchondral bone in cartilage defects in rabbits' knees (68). The efficacy of IGF-1 on cartilage repair was also shown in a horse cartilage defect model encompassing sub-chondral bone. IGF-1 was able to induce migration of chondrocytes, and the combination of IGF-1 and chondrocytes improved the consistency of the repair tissue (69).

We previously identified the combination of SOX5, SOX6, and SOX9 (the SOX trio) as a potent one for the induction of permanent cartilage (34). The SOX trio successfully induced chondrocyte differentiation in all cell types tested, including ES cells, MSCs, and human skin fibroblasts, and the induction occurred regardless of the culture system used. Contrary to conventional chondrogenic techniques, the SOX trio suppressed hypertrophic and osteogenic differentiation at the same time.

For the clinical application of autologous chondrocytes to cartilage regeneration, investigators have optimized the combination of growth factors to expand human chondrocytes and to re-differentiate de-differentiated chondrocytes in culture (70;71). They have concluded that the combination of FGF-2 with insulin or IGF-I may be useful for promotion of chondrocyte proliferation. Regarding redifferentiation, the combination of BMP-2, insulin, and triiodothyronine (T3) was found to be the most effective one causing redifferentiation of the dedifferentiated cells after repeated passaging.

Scaffolds for Bone Tissue Engineering

Primarily three biomaterials (metals, polymers, and ceramics) have been used in bone tissue engineering. Titanium is a traditional inert biomaterial for implants and is characterized by a minimal immune response, which is the biggest advantage of this material. Some studies have shown that titanium fiber meshes or titanium with zinc-containing hydroxyapatite enhance the osteogenic activity or the proliferation of seeded cells (72-74). However, the difficulty in performing histological analyses is a

serious drawback in further investigating the biological activity of this material (75).

Biodegradable synthetic polymers applied to bone tissue engineering include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ϵ -caprolactone), and poly(lactic-co-glycolide) (PLGA) copolymers (76-84). Although Poly(ϵ -caprolactone) carries biocompatibility and processability, it is less suitable for long-term applications because of its high hydrophobicity and low degradability *in vivo* (85). Cellular adhesion to PLGA is significantly higher than on PLA surfaces. PLGA was shown to support osteoblasts' proliferation and their differentiation, which was demonstrated by high alkaline phosphatase activity and deposition of a mineralized matrix (86;87). PLGA has also been utilized for encapsulation and release of several growth factors including TGF- β , BMPs, IGFs, VEGF, and NGF (88). Several approaches have been followed including the use of variable-sized PLGA microspheres with growth factors and subsequent embedding of them in other polymer matrices with variable degradation rates (88). PLGA/hydroxyapatite and PLGA/calcium phosphate hybrids (89;90) have also been utilized for bone tissue engineering. In addition, the development of PLA-p-dioxanone-PGA as a carrier of rhBMP-2 has been reported (91).

Among natural polymers, bovine type I collagen has been used as a promising biomaterial. Several type I collagen-based materials are commercially available including CollapatII® (Biomet Inc.), Healos® (Depuy Spine Inc.), Collagraft® (Nuecoll Inc., Zimmer Inc.) and Biostite (Vebas S.r.l.) (92). Given that collagens are dominant and an important matrix component in bone tissues, it makes sense that the scaffold has biocompatibility as well as activity to facilitate osteogenesis or cell proliferation. However, two major concerns, disease transmission from other species and its poor mechanical properties, remain to be solved.

Hydroxyapatite- and beta-tricalcium phosphate-based scaffolds are widely used in bone tissue engineering, and several different bioceramics have been developed

in order to improve their properties (93-96). In particular, calcium phosphates are the most popular materials for artificial bones (97;98). As approximately 70% of bone in the body is made of calcium phosphates (99), their biocompatibility and biosafety were, in a sense, already tested in the living body. Calcium phosphates are naturally osteoconductive (99) and metabolized and degraded by the endogenous system for bone remodeling, although the speed of degradation varies depending on particle size and form. Thus, artificial bones made of calcium phosphates are superior to autograft and allograft in terms of biosafety, quantity (unlimited), and invasiveness (low). Furthermore, they are made from limestone and mineral phosphates, thus being free from contamination by pathogens and free of donor site problems that occur with bone collection (100). However, the artificial bones used thus far in clinical settings usually require a post-fabrication sintering process to increase their mechanical properties, which causes contraction in size and often decreases biodegradability, as well as shape adjustment during surgery (100-103).

Therefore, novel artificial bones that have better shape compatibility to deformities, appropriate mechanical strength without post-fabrication sintering, and biodegradability are needed. We have focused on the vital role of the dimensional compatibility of the scaffolds. By controlling the 3D shape of the scaffolds, there has been significant improvement in the performance of the artificial bones, which have good dimensional compatibility, resultant reduction in operation time and corresponding invasiveness, and resultant speedy union with host bone tissues (104;105). 3D shape control is vital to the performance of the scaffolds, and it is worth attempting to control the 3D shape of the scaffold by optimizing the design and fabrication method before resorting to expensive and high-risk growth factors and cells.

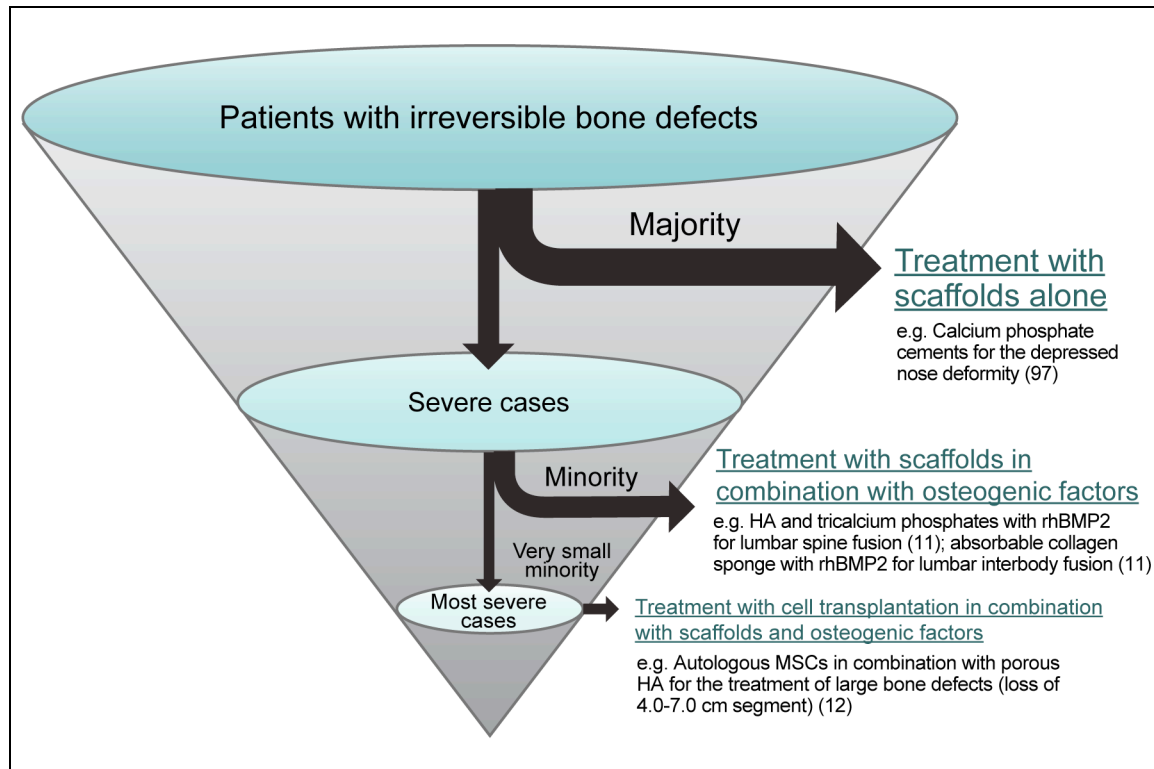


Fig. 2. Schema of proposed flow chart of decision-making; bone tissue engineering is used as an example. The majority of patients with irreversible bone defects can be treated with high-performance scaffolds alone. A minority will be treated with scaffolds in combination with osteogenic factors to induce host cell regenerating abilities. The rest, representing a very small fraction, will truly require cell transplantation in combination with scaffolds and osteogenic factors. It is crucial to justify and optimize the use of each pillar for individual cases. Representative clinical applications are shown, corresponding to each step.

Scaffolds for Cartilage Tissue Engineering

Scaffolds for cartilage tissue engineering are classified into three groups: protein-based, polysaccharide-based, and synthetic biomaterials. Among protein-based scaffolds, bilayer collagen type I and III membranes are clinically available for autologous chondrocyte implantation (ACI), including MACI® (Matrix-induced ACI, Verigen, Leverkusen, Germany), Maix® (Matricel, Hezoeath, Germany) and Chondro-gide® (Geistlich Biomaterials, Wolhusen, Switzerland) (6). Atelocollagen® (Koken Co. Ltd, Tokyo, Japan) is a type I collagen gel, from which telopeptide causing antigenicity is removed. The material has an advantage in generating a 3D structure of the implant. However, when implanted, the atelocollagen-cell composites should be covered with periosteum not to be detached from implantation sites (106). In addition, fibrin glue (Tissucol®, BAXTER, Austria) has

been used for cartilage repair, and its 1-year clinical results have been reported (107).

Polysaccharide-based materials include alginate, chitosan, cellulose, and hyaluronic acid (6). Hyalograft® C is a combination of a hyaluronic acid-based matrix HYAFF-11® (Fidia Advanced Biopolymers, Abano Terme, Italy), and autologous chondrocytes. The efficacy of Hyalograft® C in clinical settings has been reported (108).

Bio-Seed®-C (BioTissue Technologies, Freiburg, Germany) is a synthetic material that is clinically available. In the use of the material, a porous 3D scaffold consisting of PGA, PLA and polydioxanone is combined with autologous chondrocytes embedded within fibrin gel (109). Bio-Seed1-C was reported to induce the formation of hyaline cartilage and a significant clinical improvement of joint function. Poly(ethylene glycol) (PEG) is a synthetic polymer used to create synthetic-based hydrogels. Two

groups have reported that chondrocytes remained viable and synthesized cartilage-specific ECM, even when they were encapsulated in a PEG hydrogel under a compressive modulus (*i.e.*, 260-900 kPa) similar to that of human cartilage (*i.e.*, 790±360 kPa) (110;111).

Conclusions and Future Perspectives

In the late 1990s, investigators applauded the isolation, expansion, and characterization of human multipotent MSCs (112) with enthusiasm. Since then, both researchers and physicians working on bone and cartilage defects have explored suitable ways to apply the cells to bone and cartilage regeneration. As a result, a large number of preclinical and clinical studies were performed with several promising results, but also provided us with an important indication: stem cells are not necessarily a panacea. No one disputes that stem cells are a promising cell source for tissue regeneration. However, in reality, the field still struggles with finding ways to utilize stem cells more safely and effectively. It is probably time to stop and think whether the use of stem cells is really required for all cases with irreversible skeletal defects. In some cases with bone defects, for example, it appears possible to repair such defects without cell transplantation by acting on host cells and inducing host tissues' regeneration abilities. Therefore, it seems advisable to start by considering simple strategies using scaffolds and then some signaling factors before resorting to complicated strategies using cells (Fig. 2). We believe that in tissue engineering, using stem cells is not an end, but a means that must be justified and optimized for individual cases.

To advance this field more steadily and rapidly than ever before, we should keep our eyes on progress in every field related to tissue engineering – medicine, biology, engineering, pharmaceutical science, medical economics, and medical ethics – and attempt to build multi-disciplinary collaboration, which will open a new avenue for the realization of tissue engineering-based therapies.

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