# Chapter 25: Stem cells for osteochondral regeneration

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**Abstract** Stem cell research plays a central role in the future of medicine, which is mainly dependent on the advances on regenerative medicine (RM), specifically in the disciplines of tissue engineering (TE) and cellular therapeutics. All RM strategies depend upon the harnessing, stimulation or guidance of endogenous developmental or repair processes in which cells have an important role. Among the most clinically challenging disorders, cartilage degeneration, which also affects subchondral bone becoming an osteochondral (OC) defect, is one of the most demanding. Although primary cells have been clinically applied, stem cells are currently seen as the promising tool of RM related research because of its availability, *in vitro* proliferation ability, pluri- or multipotency and immunosuppressive features. Being the OC unit a transition from bone to cartilage, mesenchymal stem cells (MSCs) are the main focus for OC regeneration. Though, promising alternatives, which can also be obtained from the patient or at banks and have great differentiation potential towards a wide range of specific cell types, have been reported. Still, ethical concerns and tumorigenic risk are currently under discussion and assessment. In this book chapter, we revise the existing stem cells-based approaches for engineering bone and cartilage, focusing on cell therapy and TE. Furthermore, 3D OC composites based on cell co-cultures are described. Finally, future directions and challenges still to be faced are critically discussed.

## 25.1 Introduction

Osteochondral (OC) lesion is an injury or defect of the articular cartilage extended to the subchondral bone.(1) A disorder affecting the OC interface usually results in osteoarthritis (OA), which is not just associated to disability, but also to other conditions, such as neuropathic pain, depression, and sleep disorders.(2,3) Some assessments of disease burden suggest that OA is even an important cause of premature death.(4,5)

Alterations in the tissues and cells surrounding articular joint of osteoarthritic patients such as the synovium are now-a-days considered a predictive factor of the disease progression.(6) The activity and phenotype of the cell population's resident within the synovium are crucial to keep a healthy joint. However, might also accelerate the OA symptoms when unstable, indicating that synovial inflammation further than being a feature in early disease, might be the initiator of degenerative cascades that lead to tissue destruction.(7) On the other hand, the synovium is a natural origin of repair responses involving the endogenous progenitor cells present in the tissue.(8) Still, full-thickness articular cartilage damage does not resolve spontaneously.

Clinically applied operative techniques for OC repair are usually based on bone marrow (BM) stimulation for promotion of tissue restoration by recruitment of stromal cells. These treatments provide acceptable clinical results over midterm follow-up periods but often fail in the long term, resulting in fibrous tissue covering the lesion(9,10), which then leads to scar tissue and biomechanical insufficiency(11,12). The most used techniques like abrasion arthroplasty, subchondral drilling, microfracture, autologous matrix-induced chondrogenesis,(13) and mosaicoplasty, also frequently face drawbacks. Formation of fibrocartilage rather than hyaline cartilage (14,15), limited donor site availability or morbidity and poor integration(16,17) are among the most common marks of unsuccessful regeneration.

Cartilage repair has evolved at a rapid pace from marrow stimulation techniques to articular chondrocyte transplantation (ACI). Taking this into consideration, the field started using techniques that apply specialized cells together with invading stems cells resulting from marrow stimulation. ACI and its later evolution, matrix-induced autologous chondrocyte implantation (MACI), (18,19) offered great promise when compared to other approaches with 80% of patients showing improved results at 10 years.(20) However, despite the improvements, hyaline-like cartilage repair keep experiencing complications not only as the failure of graft integration in the host tissue but also periosteal hypertrophy and delamination.(21,22) In addition, it has

also been reported that chondrocytes may lose their phenotype during expansion *in vitro*.(23,24) Recent reports are now projecting the future focused on stem cells based strategies for cartilage repair.(25)

There is, therefore, a growing interest in exploring stem cells features to regenerate OC lesions either through the direct injection of the cells into the blood stream or tissues, or the combination of the cells with supporting scaffolds. Stem cell-based strategies are therefore seen as the possibility to improve the limitations experienced with primary chondrocytes in ACI therapies. Unlike primary differentiated cells, stem cells have the ability to divide and specialize into specific cell types, depending on the differentiation potential and source. Pluripotent stem cells (PSCs) are often harvested from embryonic niches and can develop into any type of cell derived from the three germ layers whereas multipotent stem cells are generally isolated from adult tissues and have limited differentiation ability.(25) Mesenchymal stem cells (MSCs), not hampered by availability and donor site morbidity, are a form of multipotent cells that may offer an alternative to cartilage repair techniques.(25) *In vitro* generated chondral or OC grafts using stem cells are currently in a queue for FDA approval for clinical studies.(26) However, the use of stem cell therapies into the clinic is a form of translational research that still is associated with regulatory issues that are currently under discussion under different perspectives worldwide.(27,28)

Current investigation using MSCs from bone marrow (BM), adipose or embryonic tissues for cell therapies and TE approaches on bone and cartilage regeneration are overviewed in this book chapter. Specific issues that are slowing down the fast progress observed over the last 30 years are also analyzed. Since stem cells niche has been proved to be an important factor when considering regeneration of bone and cartilage disorders, the works applying stem cells from varied origins are described. Furthermore, 3D co-culture systems are revised as the basis to discuss the future of stem cells-based approaches for osteochondral regeneration.

## 25.2 Progenitor cells, stem cell sources and cell recruitment on endogenous skeletogenesis

Skeletogenesis is the process encompassing the formation of the several components of the skeleton such as bones, cartilage and joints. These components are the result of the action of specialized cells such as chondrocytes and osteoblasts that give rise respectively to cartilage and bone. During embryonic development, each of these cells derive from skeletogenic mesenchymal progenitors of mesodermal or ectodermal origin, depending on the skeletal site.(29) These progenitors home at

prospective skeletal formation sites and condense in structures that then give rise to the different skeletal components(30). In the case of bone, two processes are possible for ossification of the skeleton: endochondral and intramembranous ossification(30). The former consists on the production by chondrocytes of a cartilaginous template that is later mineralized and is the way through which most bone tissue is formed. In intramembranous ossification, through which flat bones are formed, bone is directly produced by osteoblasts without an intermediate cartilage anlage.

The coordination of all these events is made through direct cell-cell communication and by the action of several key signaling molecules that regulate the cell recruitment and the associated patterning of the skeleton. Among the most significant signaling molecules are fibroblast growth factors (FGF)(31), bone morphogenetic proteins (BMPs)(32), sonic hedgehog (SHH)(33), and Notch(34) and Wnt(35) ligands. The coordinated action of these molecules will determine the location of skeletogenic mesenchymal cells and orchestrate the timing of condensation. At this stage, skeletogenic mesenchymal progenitor cells express both Sox9(36) and Runx2(37) transcription factors which are determinant for the differentiation into the chondrogenic and osteogenic lineages, respectively. The commitment to either one is achieved by the action of signaling pathways such as Wnt and Indian hedgehog (Ihh) that mostly down or upregulate Sox9 and Runx2(30).

Critically, mesenchymal progenitors are present in adult vertebrates as what are known as MSCs. It is not clear if MSCs directly derive from the same skeletogenic mesenchymal progenitors responsible for tissue development during embryogenesis. MSCs are multipotent stem cells that can differentiate in specific lineages of tissues such as bone, cartilage, fat, muscle, etc, and present no immunogenicity after transplantation.(38) These cells are present in almost all tissues of the human body(39) and therefore it is hypothesized that they have the ultimate involvement in tissue repair(40). In the skeletal system, MSCs have a direct role in injury repair and tissue regeneration, where some of the embryonic events of skeletal formation are recapitulated. MSCs are recruited to the injury site and undergo differentiation by the action of signaling molecules such as BMPs, FGF, TGF-$\beta$ and SDF-1(41). Such signaling molecules can be released from the matrix or secreted by other cells present at the injury site. The low oxygen tension typically found at the injury site due to blood vessel disruption is also a major factor in MSC recruitment and differentiation(42). Under such type of stimuli, MSCs not only home to the injury site but also engage in proliferation and differentiation, ultimately repairing or regenerating the injured tissue.

MSCs have been isolated and cultured in vitro and were found to possess clinically relevant proliferation ability(43). This is important since typically, fully differentiated cells such as osteoblasts present decreased proliferation rates in vitro, which undermines their potential to be used in, for e.g., a Tissue Engineering strategy. Furthermore, MSCs have been found to positively modulate the response of immune system cells, which besides being important on itself for injury resolution, also suggests these cells might be tolerated in allogeneic approaches(44). It is then clear that MSCs are a very attractive source of cells to induce post-natal skeletogenesis in TE strategies. Being present on multiple tissues means that several potential sources of MSCs for therapeutic strategies have been projected(45). A suitable source of MSCs should allow easy accessibility, minimally invasive harvesting and yield usable numbers of cells. BM is the most explored source of MSC in a myriad to TE strategies (46). However, the isolation of these cells encompasses a significant degree of morbidity to the patient and therefore alternative sources, namely perinatal tissues such as umbilical cord(47), teeth(48), and adipose tissue(49) have been sought. The latter, in particular, has been the focus of increasing attention since fat can be obtained in relatively non-invasive procedures and adipose-derived stem cells (ASCs) can be isolated from discarded fat residues from plastic surgeries. While these cells appear to have more tendency for adipogenic regeneration, several works demonstrate their feasibility for bone(50) and cartilage(51) TE. Recently, it has been hypothesized that the perivascular niche might also be a suitable source of cells for bone and cartilage TE(52). In fact, some researchers even claim that MSCs and pericytes might be one and the same entity(53) although this is still quite controversial at the moment. Nevertheless, it is unquestionable that MSCs have been the prime choice for developing TE strategies for bone and cartilage tissues.

Other examples of stem cells that can be considered for post-natal skeletogenic strategies are embryonic stem cells (ESCs)(54). These cells are pluripotent, ie, they have a broader differentiation potential than MSCs and similar, if not better, proliferation ability. However, they bring attached several ethical constraints since they are derived from human embryos. Furthermore, they have a significant tumorigenic risk associated. Due to these concerns, great promise has been placed in induced pluripotent stem cells (iPSCs) as proposed by Yamanaka and others(55). These cells can be obtained by introducing specific reprogramming factors into terminally differentiated somatic cells. This avoids the need of embryo destruction and yields pluripotent stem cells from a specific patient, allowing in principle the development of personalized therapies(56).

While all these cells have been used to obtain bone and cartilage cells for tissue engineering, the question as to which is the best cell source for post-natal skeletogenesis is still open.

## 25.3 Stem cells for engineering bone

As stated above, BM-MSCs have been the top choice as a MSC source for bone TE. This is mainly due to their high potential for osteogenic differentiation, which is probably related with their tissue of origin(57). These cells have been combined with 3D scaffolds to deliver a myriad of bone tissue engineering strategies that ultimately resulted in the formation of bone tissue after transplantation (46,58–60). However, these cells present several limitations. The isolation procedure is painful for the patient as it normally consists of an invasive procedure to aspirate the marrow in the iliac crest. Other methods exist where BM is accessed from patients undergoing hip or knee replacement (61). These are however exceptions to the rule. Furthermore, the yield of stem cells obtained from BM is low, estimated at 0.001% of colony forming unit-cells per nucleated cells (38). This means that these cells need to be heavily expanded to reach therapeutic numbers, which ultimately results in reduced differentiation capacity and therapeutic efficiency (62). Finally, the potency of each population of BM-MSC greatly depends on the donor's age and general condition, which further complicates their applicability (63). The quantity of MSCs declines with age; approximately 1 MSC per 10 000 marrow cells are found in a newborn, whereas 1 MSC per 400 000 marrow cells is found in a 50-year-old adult (64,65).

Adipose-derived stem cells (ASCs) are an increasingly used cell source for bone TE. Through a relatively simple and painless procedure, adipose tissue can be harvested from a patient in generous amounts and ASCs can be isolated from the stromal vascular fraction (SVF) by employing straightforward enzymatic protocols (66). Importantly, the frequency of stem cells in adipose tissue is 100 to 1000 times bigger than for BM, which coupled with the relatively large volumes of adipose tissue that can be harvested, makes this cell source even more appealing (67). Furthermore, the potential of these cells to undergo osteogenic differentiation is robust although a higher tendency for adipogenic differentiation has been found (68). The increasing number of bone TE strategies using these cells attempt to capitalize on

these advantages (52,69–71). It is interesting to note that the SVF from which ASCs are isolated also contains angiogenic cell populations such as endothelial cells, endothelial progenitor cells and pericytes (72). These constitute a perfect cocktail of cells to create functional vasculature in bone TE constructs, therefore addressing one of the main current obstacles in TE (73,74). The possibility of using the whole fraction both for osteogenesis and angiogenesis is extremely exciting and will allow from one simple procedure to obtain patient-specific cell population for a complete bone TE strategy.

Other adult MSCs sources have been considered for bone TE. Dental pulp MSCs (DP-MSCs) are isolated from the pulp of definitive or deciduous teeth (75). While accessibility and ease of isolation are strong points in favor of DP-MSCs, the fact is that only a small number of cells can be isolated from each tooth. The ensuing need of comprehensive cell expansion until therapeutic numbers are achieved is negative due to potential impact in cell potency. Nevertheless, these cells have been extensively tested and characterized (76). Several works describe their similarity with BM-MSCs in term of cell markers and multilineage differentiation potential while having superior proliferative ability (75,76). Accordingly, these cells are being increasingly proposed for bone TE applications (77–80). Cells from perinatal tissues such as umbilical cord blood, umbilical cord and amniotic membrane and fluid have been found to have all the hallmarks of MSCs, while retaining some traits of embryonic cells such as robust proliferation ability and differentiation pluripotency (81–83). Furthermore, such cells present low tumorigenic risk and appear to retain the immune privileged nature of MSCs (84). The volume of bone TE research using perinatal cells trails behind that of other stem cells (85,86) and the coming years will reveal if this is a cell source that will be embraced by tissue engineers, particularly in the case of bone tissue.

ESCs, as stated before, are pluripotent stem cells isolated from embryonic tissues that can differentiate in cells from every germ layer. Osteogenic cells have been obtained from ESCs using well developed protocols (54). Therefore, many bone TE strategies have been proposed using cells derived from ESCs (54,87–90). However, several constraints are associated with the use of these cells. Since they are isolated from the inner cell mass of blastocysts they encompass the destruction of the latter which raises a series of ethical issues. Furthermore, these cells have a high tumorigenic potential in vivo which is obviously a very serious safety concern for clinical applications. This tumorigenic risk can be partially mitigated using a combination of culture and cell sorting techniques (91). However, the ethical concerns are unavoidable. In order to overcome this issue, iPSCs were developed and famously proposed by Yamanaka and colleagues (55). These cells are pluripotent stem cells

vastly similar to ESCs but they are derived from adult cells. The technique to achieve this derivation is based on the delivery of pluripotency-associated factors such as cMyc, Oct4, Sox2 and Klf4 to fully de-differentiate cells in order to effectively reprogram those cells into pluripotent cells. This strategy avoids the ethical issues of ESCs while adding a very significant benefit: the ability to produce patient-specific pluripotent stem cells (56). This is very important since it allows the combination of the advantages of adult stem cell sources such as BM-MSCs and ASCs, with the pluripotency of ESCs. While some concerns exist related with potential tumorigenicity of the original reprogramming factors used in iPSC protocols, other protocols have been put forward using less worrisome factors (92). Therefore, numerous bone TE strategies using iPSCs have been proposed by first inducing differentiation to the mesenchymal and finally to the osteogenic lineages (93–96). However, the safety of these cells is still a major issue and more clinical research is needed to confirm these cells potential for bone TE.

## 25.4 Stem cells for engineering cartilage

To date, only few studies directly compared MSCs and chondrocytes for cartilage repair. Interestingly, no significant differences were found on the histological scores.(97,98) Moreover, the efficacy of MSCs *in vitro* pre-differentiated into chondrocytes or undifferentiated, was equally superior in comparison to the untreated condition, but without significant differences among themselves.(98) Clinically, the intra-articular injection of MSCs favorably influenced the progression of lesions larger than 109 $mm^2$ associated with subchondral cysts, in patients older than 50 years, thus hampering the development of an associated degenerative disease.(1) Therefore, many uncertainties are still to be clarified regarding the mode of action and the efficacy of MSCs to engineer cartilage tissue.

MSCs were proposed as a valid option for the treatment of cartilage defects because of their ability to differentiate into chondrocytes, among other cell lineages.(99–101) These cells are also immune-privileged (102–104) and have the ability to modulate inflammatory cytokines including interferon-gamma, tumor necrosis factor-$\alpha$ and interleukin-1$\alpha$ and -1$\beta$.(105) While these features might be of major therapeutic interest when inflammatory conditions, such as OA, are associated to cartilage degeneration, it also reinforces the possibility of following allogeneic approaches as demonstrated for BM-MSCs (106,107) and ASCs (108) in different animal models. In fact, the need for cartilage repair is greatly associated to age, thus autologous approaches are significantly compromised by its retrograde effect on the cell's intrinsic properties and regenerative capability.(109)

While adipose tissue could be considered an alternative source, ASCs have less chondrogenic potential than BM-MSCs.(110) The infrapatellar fat pad also known as Hoffa's body, an extra-synovial tissue placed in the knee under the patella (111) that is commonly harvested and discarded in arthroscopic surgeries (112), was shown to possess a higher percentage of stromal cells than subcutaneous fat.(113,114) In addition, the regenerative capacity of the cells obtained from Hoffa's body in an OA model was demonstrated (115) thus reinforcing the positioning of this source for cartilage engineering.

As an alternative to the above mentioned sources of MSCs, peripheral blood (116), periosteum (117) and synovium (118) have been explored to engineer cartilage in particular. However, there are no reports showing clear evidences of superiority of one over the other.

Interestingly enough, the debate on the possibility of following an allogeneic approach come up again when considering Wharton's jelly-derived MSCs (WJ-MSCs). The umbilical cord, which is discarded at birth, provides an unlimited source of cells which are believed to be more primitive, proliferative and to have broader multipotency than adult MSCs (119,120). WJ-MSCs express markers of both MSCs and ESCs, but in opposition to ESCs these cells do not induce teratoma formation (121). In addition, the composition of Wharton's jelly extracellular matrix, rich in aggrecan and type II collagen, is very similar to that of cartilage (122). hWJ-MSCs also express growth factors, chemokines, and cytokines at levels similar to those of cartilage cells (123). Altogether, these indications might be sufficiently supportive to consider Wharton's jelly as an ethically noncontroversial source of MSCs to engineer and regenerate cartilage.

At a different stage of development but with promising expectations, a multipotent subpopulation of muscle-derived stem cells (MDSCs) isolated from mouse skeletal muscle was genetically engineered to express BMP-4 as a way to enhance chondrogenesis (124). MDSCs transduced with retroviral vectors CLBMP-4, CLLacZ, can potentially be used to locally secrete BMP-4 for cartilage repair.

As mentioned before, one of the great promises of iPSCs is the possibility to generate autologous cells from adult somatic fully differentiated cells that can be expanded and then differentiated into the cells of interest. iPSCs derived from human chondrocytes biopsied from osteoarthritic knees (125), and from fetal human neural stem cells (126) were successfully differentiated into the chondrogenic lineage. Despite this, the lack of expression of pluripotency associated markers is not fully achieved until sometime in culture (127) which raises some concerns regarding the

control of cell phenotype and cell fate. In alternative, some studies have been supporting the generation of intermediate MSC-like cells and the purification of chondrogenic progenitors, such as neural crest cells (NCCs) (128), to eliminate residual PSCs. NCCs which have an ectoderm origin, are known to give rise to many craniofacial tissues including bone and cartilage, (129) but NCC-derived cells have been also detected in the BM of limb tubular bones (130). MSC-like cells derived from intermediate NCCs upon chondrogenic differentiation exhibited X collagen upregulation, features of hypertrophic chondrogenesis (131). Still, further investigation on this cells potential for bone and cartilage regeneration is needed.

While the use of pluripotent stem cells is still hampered, alternative approaches that take advantage of stem cells intrinsic signaling moieties such as extracellular vesicles/exosomes are starting to be explored. As the efficacy of many MSC-based therapies has been attributed to paracrine secretion, exosomes have been posed as a 'cell-free' strategy (132). A weekly intra-articular injection in rat OC defects led to the formation of cartilage similar to hyaline cartilage and underlying subchondral bone resembling that of age-matched native control.

Despite an extensive preclinical research and promising clinical results, there are yet some drawbacks related to the harvesting and culture of stem cells to be addressed. Cell seeding number, serum conditions, or even the plastic surface of the expansion systems, can affect cell phenotype. A forced selection happens during cell expansion, which can affect for example MSCs properties by their technical preparation (149–151). Cell culture was not a concern for long time, being poorly controlled over most of the published studies. As a specific example, chondrogenic differentiation requires a 3D environment, but MSCs are commonly expanded on 2D plastic surfaces. Furthermore, as two-stage procedures involving cell culture are expensive and cumbersome, there is an increasing push towards a single stage stem cell treatment, which focuses again on autologous strategies. In this situation, there is some supportive pre-clinical data (133–136), but a direct comparison between fresh MSCs concentrates and MSCs expanded *in vitro* is not available (25). The schematic represented in figure 1 summarizes the use of stem cell strategies for regenerative approaches.

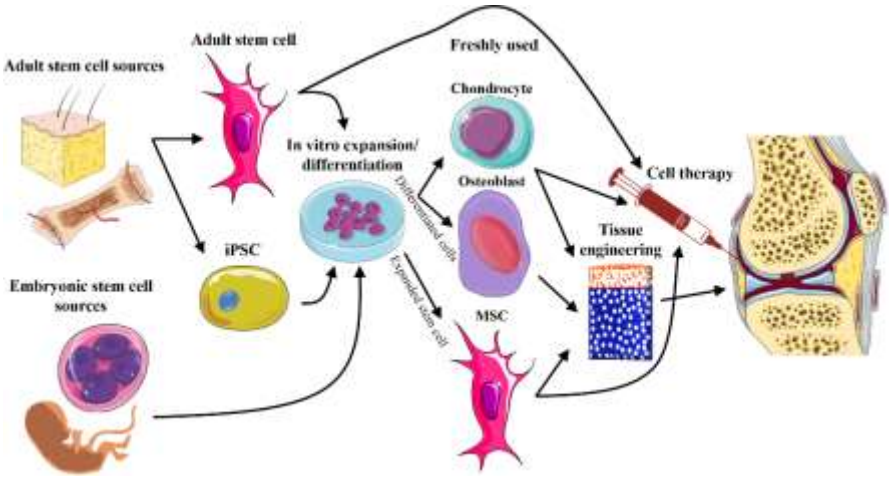


**Figure 1.** Stem cell-based strategies for bone and cartilage engineering. Cell isolation have been relying on several sources, from adult to embryonic tissues. After isolation, cells can be freshly used or *in vitro* expanded. During *in vitro* phase, stem cells can be directly used taking advantage of their stemness, or differentiated. When applied, these cells can be directly injected at the defect site using carriers or not.

## 25.5 Engineering Osteochondral Composites using stem cells

A persistent challenge in the field of RM has been the ability to engineer complex tissues comprised of multiple cell types and organizational features. This is the case of the OC unit in which a particular gradient interface integrates the cartilage and the bone parts in which chondrocytes and osteoblasts can be recognized as characteristic cells, respectively. Furthermore, OC tissue also represents a gradient of ECM constituents through it. The control over spatial patterning of tissue development represents in this way the main challenge for the advance towards engineering functional OC grafts. This control has been approached following different strategies such as heterogeneous 3-D structures providing physical cues for stem cells differentiation (137), which are represented in figure 2. In addition to several materials engineering strategies, the spatial controlled delivery of differentiation factors as GFs (138) and gene vectors (139), and the use of parallel culture medium flow for osteo- and chondrogenic conditioning by custom-made dual-chamber bioreactors (140) or microfluidic devices (141), have been applied.

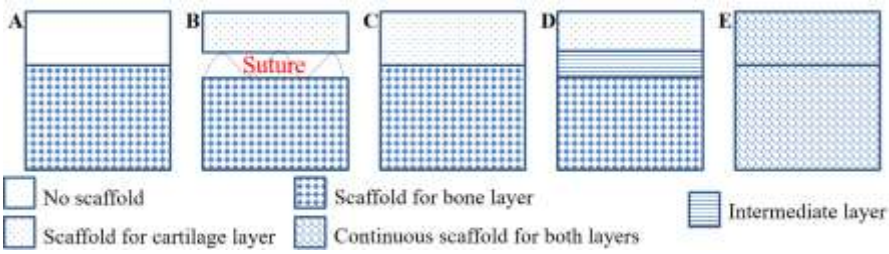


**Figure 2.** Strategies based on materials engineering strategies to be coordinated with cellular strategies for OC TE. (A) Scaffold-free cartilage layer combined with bony-scaffold; (B) Two separated constructs sutured together, usually involving individual osteo- and chondrogenic pre-maturation; (C) Two different integrated scaffolds merged together; (D) Tri-layer structure composed of two different scaffolds for bone and cartilage and an intermediate layer in the interface; (E) Continuous scaffold for both bone and cartilage layers, which can be different in material properties or cell type.

In any of the previous cases, to build a heterogeneous construct, cells either from different lineages or differently differentiated (ending up in two different phenotypes) are applied at the seeding moment. In coordination with the previous strategies, different cell types from primary osteoblasts co-cultured with primary chondrocytes (142); primary chondrocytes co-cultured with stem cells (143); or co-differentiation of stem cells towards osteo- and chondrogenesis (144), have been tested envisioning the enhancement of OC phenotype by the synergistic action of the different factors produced by each cell type. Envisioning these strategies, both pre-differentiation (145) and *in situ* differentiation (146) of the stem cells were tested. The full set of previous materials and cells combinations is summarized in figure 3.

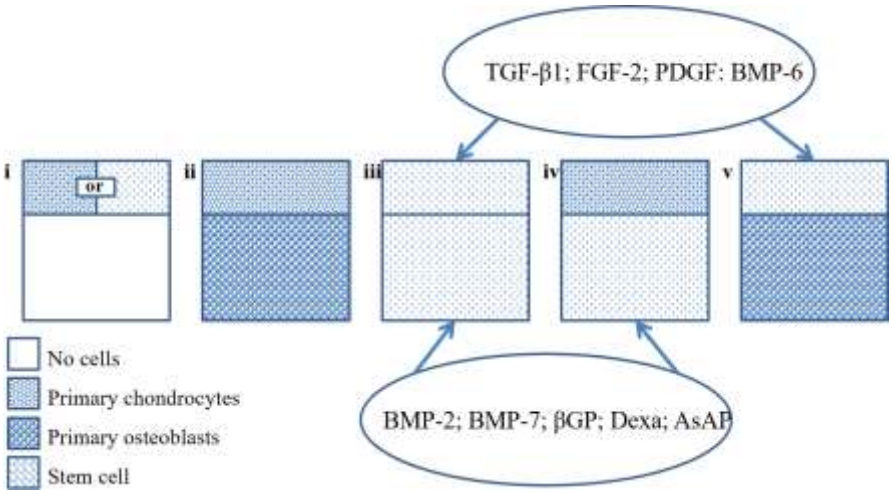


**Figure 3.** Strategies based cellular approached coordinated with scaffolds for OC TE. (i) cell-free approach for bone region combined with primary chondrocytes or stem cells for cartilage layer; (ii) primary chondrocytes co-cultured with primary osteoblasts; (iii) single stem cell population for co-differentiation; (iv) primary chondrocytes co-cultured with stem cells for cartilage and bone, respectively; (v) stem cells co-cultured with primary osteoblasts for cartilage and bone, respectively.

The challenge of controlling osteogenic and chondrogenic differentiations in a single system has been addressed with the independent pre-maturation of the bone and cartilage parts. A hydrogel phase made of chitosan and a cancellous bone part were seeded with hASCs for chondrogenesis and osteogenesis, respectively (147). After 2 weeks of differentiation period, constructs were sutured together and cultured for further 2 weeks under static or dynamic conditions. The dynamic culture improved the resulting interface connection and enhanced homogeneous nutrient transfer. However, this technique keeps lacking integrity and a robust interface integration between cartilage and bone tissues.

In a functional OC unit, the interface needs to recapitulate the transition between bone and cartilage without compromising a strong integration between the layers. A tri-layer structure has been proposed as a way to improve layer's integrity and to have a better control over cell phenotype by avoiding cell migration from one region to the other due to the interface layer. Like the sutured approach, primary chondrocytes embedded in a RGD-modified peptide amphiphilic nanofibrous hydrogel, and MSCs seeded in a silk scaffold, were respectively and independently cultured in chondrogenic and osteogenic media. After 2 weeks the structures were combined with a soft silk scaffold as an interface layer and cultured under an OC cocktail

medium without the addition of GFs (148). The presence of chondrocytes in the co-cultures significantly increased the osteogenic differentiation potential of the MSCs. On the other hand, the effect of hMSCs on chondrogenic phenotype was less. A similar tri-layer approach was compared with the respective independent part. A layer of pure gelatin was assembled with layers composed of varied amounts of gelatin and hydroxyapatite. Gelatin was also used as a glue to stick all the layers together. The multi-layered strategy showed an increased expression of bone markers but not of the chondrogenic ones, in relation to the respective single constructs. Authors reported that each layer enhanced cell phenotype but difficulties associated to the simultaneous use of very different media must be also highlighted (149).

This issue has been addressed with the use of microfluidics and bioreactors with special interfaced designs. Based on a microfluidic approach, Goldman and Barabino (141) described a process for the formation of integrated cartilaginous and bony tissues by culturing a single cell source, bovine BM-MSC, within a single structure. Two independent microfluidic networks supplying inductive media independently for osteo- and chondrogenesis were designed to achieve co-differentiation as shown by the differential gene expression of chondrogenic and osteogenic markers through the 3-D constructs. As an alternative, Kuiper et al (140) used a custom-made bioreactor to perform a co-culture of primary osteoblasts, seeded in a tri-calcium phosphate and poly-L-lactic acid composite, and chondrocytes encapsulated in Extracel® hydrogel. The cell-laden hydrogel was cast on top of the composite in a dual-chamber perfusion bioreactor system and compared to a static condition. The authors demonstrated that the dual-chamber bioreactor positively influenced the co-culture of chondrocytes and osteoblasts in the scaffolds in terms of gene expression and matrix deposition markers for bone and cartilage.

In addition to the different biochemical requirements to achieve concomitant osteo-osteogenic and chondrogenic differentiation within an OC construct, the spatial control of cells distribution in between layers is also critical. This has been addressed by playing with the architectures of the supporting structures. An integrated scaffold made of one layer with monodispersed porosity of 38 um pore size and coated with hydroxyapatite particles, and of a second layer with 200 um pores coated with hyaluronan, was proposed to recreate respectively the bone and cartilage parts of an OC construct. After seeded with MSCs and chondrocytes differentiated from MSCs, respectively, constructs were cultured over 4 weeks *in vitro* in the absence of soluble GFs. Concurrent deposition of ECM typical to each cell type was promoted in standard base medium (150). A similar approach used pre-differentiated rat BM-MSCs into the chondrogenic and osteogenic lineages respectively

to populate a sponge composed of a hyaluronan derivative, and of calcium phosphate ceramic. Both layers were joined together with fibrin sealant to form an OC graft. After 6 weeks of *in vivo* implantation the heterogeneous structures were integrated in the host OC tissue. However, fibrocartilage but not hyaline cartilage was identified in the sponge. Although collagen type II was predominant in the neocartilage, collagen type I was found in both bone and cartilage regions (151). In a different approach, the control over 3D OC differentiation has been targeted by a defined local displaying of GFs or genetic vectors. Brunger et al (152) hypothesized that by genetically modifying MSCs in a spatially restricted fashion would differentially determine cell fate and ECM deposition. To prove this, the authors tested whether two interfaced tissues and an OC composite could be derived from a single stem cell type (153). The localized differentiation factors, such as BMP-2 or RUNX2 and TGFb-3, drove concomitant mineral and GAG production in a spatially-defined fashion, showing the potential of this strategy to be further explored.

Although there are several approaches to recreate the OC tissue *in vitro* promoting the control over cell phenotypes under co-culture systems, the exact phenotype changes of subchondral bone during inflammation and OC repair is still poorly understood. In addition, inconsistent outcomes have been reported. Studies focus on GFs and stem cell sources effectiveness *in vitro*, and implantation of autologous chondrocytes and MSCs *in vivo*, creating mismatching conclusions (16). The current state-of-the-art of 3D engineered grafts is characterized by tailoring stem cells phenotype, such as advanced scaffold strategies with multiple layers, which were eventually combined with GFs or bioreactor designs to provide chemical and physical stimuli. However, there is a gap on vascularized constructs, which will demand one extra level of complexity. A vascularized bone and non-vascularized cartilage is required. Specific pathways and incorporation of GFs have to be explored for connecting the graft to the host vasculature, and co-cultures of stem cells with endothelial cells have to be optimized under a whole and integrated bone and cartilage-like 3D construct.

## 25.6 Conclusion and future directions

Over the last years, great progress has been made to validate cell therapy and TE strategies for OC regeneration. Approaches that mimic physiological stimuli, support cell proliferation and warrant adequate differentiation, thus avoiding hypertrophic cartilage maturation and osteogenic induction in cartilage region are a demand. Knowing and taking into consideration the principles of skeletogenesis is

believed to be fundamental. Although MSCs are known to be key players, alternatives to BM are required mainly because this is an age-dependent and limited cell source. For example, infrapatellar fat pad is an interesting source of stem cells to be applied on knee OC disorders, since Hoffa's body has to be harvested for surgical arthroscopy in most of the cases. ASCs are much more available than BM- MSCs, but the coming years will reveal if iPSC and perinatal stem cells like WJ-MSCs, can be used to replace adult multipotent stem cells, overcoming the current ethical and tumorigenic issues, and becoming the cell sources under the focus of the tissue engineers for OC repair. Having this in mind, the ideal stem cell source might be dependent on the target tissue and the right strategy dependent for example, on donors age. However, further investigations relating to optimal cell strategy, such as, autologous *versus* allogeneic, freshly-isolated *versus* expanded, stem cells *versus* differentiated cells, as well as the use of pluripotent stem cells in a consistent way and long-term efficacy, are the main challenges still remaining.

Beyond the stem cell source, another issue that is a matter of controversy is the dose of cells that should be used. Huge ranges of cell densities have been applied in the different phases of investigation. In *in vitro* studies aiming for cell therapy, numbers vary from few thousands to several millions of cells per milliliter. Preclinical animal studies have been applying from a thousand to a billion of cells per milliliter, depending on the animal model, while in clinical trials, the reported densities range from 1.2 million cells/mL to 24 million cells/mL, which is, in fact, a more concise range, but still too broad. Although some studies state that higher cell number leads to a better repair (25), the most appropriate cell dose remains unclear as the huge reported range of cell densities evidences. In fact, cell saturation is also linked to limited cell survival (154). Thus, there is certainly a maximum cell number to aid repair (155). This uncertainty has inevitably direct implications in the costs of each process, but even bigger consequences on the costs associated to false positive results at the level of clinical trials (156).

Moreover, *in vitro* models have been changing from 2D to 3D, increasing the number of varying parameters affecting stem cell differentiation and asking for further optimization on cell densities. Studies focusing on the analysis of the cell phenotype after *in vitro* expansion, the cell density for cell therapy or for engineering a graft, the long-term viability of a graft after implantation, and the maintenance of a stable cartilaginous phenotype, are required. The frequently varying outcomes of cartilage repair from cell implantation approaches are likely the consequence of different cell sources and differentiation strategies, GF liberation patterns, scaffold properties, model-specific defect environments, and, when applied, the animal model itself.

Regarding the strategies applied for OC unit development, the most promising strategies seem to rely on gradient structures supporting one stem cell type population under two different culture medium for its maturation. This has been requiring the design and use of specific bioreactors. Some efforts have been made to create grafts using these, while *in vitro* models could be developed using microfluidic approaches. In any case, this concept was just born 5 years ago, and several improvements are required. Independently of the generation of OC TE constructs, either by the individual or concurrent development of the bone and cartilage counterparts, the vascularization of the engineered bone is under developed. Co-cultures can be introduced for bone vascularization processes, and SVF fraction is one of the most promising cell cocktail sources that should be further explored. In addition to the development of a vascularized subchondral bone, the mimicking of OA inflammatory state is still to be achieved.

Concluding, the current methods for engineering physiologically relevant bone, cartilage, and OC composites from stem cells ask for a better understanding of the complex *in vivo* healing environment, its maturation, and homeostasis. For the future, patient-specific approaches have to be the focus and big data can foster optimal variants selection for this goal. So, the availability of compound libraries and high throughput screening technologies will play a key role in the selection process of the most appropriate approach in view of a stable clinical outcome.

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