Adult and Embryonic Stem Cells in Cartilage Repair

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Abstract: Injured cartilage tissue has a limited capacity to heal. There is increasing need for the development of biologically based approaches for cartilage repair. The ultimate aim is to repair the damaged cartilage tissue with new functional tissue using living cells (alone or in combination with suitable scaffolds) that will integrate with the patient's remaining tissue and yield a regenerated functional joint, which could continue to repair itself and maintain tissue homeostasis, and remain functional throughout the life of the patient. Cell therapy approaches represent a novel strategy in the treatment of cartilage diseases. Among the different types of stem/progenitor cells that are currently being evaluated, the benefits and limitations of approaches using embryonic and adult stem cells will ultimately depend on factors related to efficacy and safety. To achieve these goals a clear and profound understanding of the molecular determinants of chondrocyte differentiation and cartilage tissue formation is essential, including the specific effect of trophic factors guiding the chondrogenic differentiation process, their relationship with the tissue microenvironment, and how they translate to an epigenetically stable and homogeneous functional tissue. Despite the increasing progress in the application of human embryonic stem cells for cartilage repair, ethical and safety concerns (primarily teratoma and tumor formation) remain to be resolved. Adult stem cells have demonstrated the greatest promise and bone marrow-derived stromal cell subpopulations represent the most widely studied cell types. Developmentally immature marrow stromal cells have been isolated by several groups which appear capable of meeting most, if not all, of the criteria needed for a successful approach. Here we will review the use of embryonic and adult stem cells for cartilage tissue engineering.

Keywords: Stem cells, self-renewal, chondrogenesis, cartilage reparative medicine.

INTRODUCTION

Diseases of articular joints represent a major medical, social and economic burden on society and this will inevitably increase as the proportion of the elderly in the population increases [1]. The limited ability of native cartilages to heal effectively has created the need for the development of musculoskeletal cartilage repair strategies. Cartilage diseases, such as inflammatory joint disorder, and injuries consistently result in chronic pain and decreased quality of life, thus repair or regeneration of cartilage is in constant need and a challenge to biomedical science. Cell therapy approaches represent a new strategy in the treatment of cartilage diseases. In inflammatory joint disorders, the tight osteochondral homeostatic regulation is profoundly dysregulated, with proinflammatory cytokines acting as an important catalyst of a disturbed homeostasis. Thus, cell therapy and tissue engineering strategies must take into account means of contributing to normalize this disturbance. Due to the wide prevalence of their disease and injury, fibrocartilages (like the knee meniscus) and hyaline articular cartilage are the main focus of tissue engineering research efforts. The replacement of damaged and diseased joints, such as hips and knees, with plastic and metal articulations is highly successful [2]. However, in younger patients where the lifetime of the patient is likely to exceed well beyond the lifetime of the prosthesis, this approach is inadequate. Thus, there is immense need for the development of biologically based repair approaches, to repair the damaged tissues with new functional tissue using living cells (alone or in combination with suitable scaffolds) that will integrate with the patient's remaining tissue and yield a regenerated functional joint, which could continue to repair itself and maintain tissue homeostasis, and remain functional throughout the life of the patient.

The ultimate challenge of repairing and regenerating joints damaged by osteoarthritis is enormous and should not be underestimated. Progress towards achieving this goal is being made by focusing on a comparatively simpler and more defined clinical problems, the defects in articular cartilage that arise as a result of joint trauma and sports injury. In this context, the problem is limited to repairing a defined area of articular cartilage in a healthy joint and it does not involve repairing other tissues. The different strategies to engineer diverse types of cartilages share major obstacles including the detailed characterization of the molecular determinants of chondrocyte differentiation and establishment of a functional phenotype, identifying a readily available cell source that can be used in patients, and developing a delivery system that will allow tissue integration and functional recovery.

Both stem/progenitor cells as well as functionally mature chondrocytes (naïve and genetically modified) have been used in cell therapy and tissue engineering approaches. There are at least three different types of stem/progenitor cells that are currently being evaluated. They can be classified in terms of their sources: prenatal, such as embryonic stem cells and fetal stem cells; those obtained at term, such as those isolated from umbilical cord blood [3, 4], placenta, and amniotic fluid [5];

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and post-natal, generally referred as adult stem cells, and include cells derived from bone marrow (mesenchymal stem cells, marrow-isolated adult multilineage inducible [MIAMI] cells, multipotent adult progenitor cells [MAPCs]) [6-14], adipose tissue [15-17], skin [18], cartilage [19, 20], muscle [21], and other tissues. We will focus our discussion on a brief review of the molecular determinants of chondrogenic differentiation, the response of adult and embryonic stem cells to molecular developmental cues, and the advantages and disadvantages these cells for cartilage repair strategies.

Determinants of Chondrogenic Differentiation and Cartilage Repair Capacity

Chondrocyte differentiation is a multi-step process characterized by coordinated changes in gene expression and cell morphology. One of the main functions of cartilage is to provide the intermediate template for endochondral bone formation. The initial step of cartilage formation requires the recruitment of marrow stromal cells to future sites of skeletal development, followed by cellular condensation, and the differentiation of stromal cells to the chondrogenic lineage, a process termed chondrogenesis. Chondrocytes can continue to differentiate to hypertrophy forming the cartilage intermediate template, called anlagen [22], which is later replaced by bone. The chondrocytes remaining on either end of the mineralized bone are organized into growth plates and are responsible for longitudinal growth of long bones. Culture systems have been developed that promote chondrogenesis. The three-dimensional micromass system is a widely used culture model providing the sufficiently high cell density required for chondrogenesis. Furthermore, this method reduces the interactions of cells with cell culture plastic while promoting the cell-cell interactions and morphological changes that resemble *in vivo* chondrogenesis [22, 23]. Chondrogenesis involves stem cell differentiation through the coordinated effects of growth/differentiation trophic factors, extracellular matrix (ECM) components, and epigenetic factors.

Trophic Factors

Chondrogenic differentiation of undifferentiated progenitor cells has been induced *in vitro* most often by growth factors of the transforming growth factor (TGF) family, primarily by TGF-β1, TGF- β3, bone morphogenetic protein (BMP)-2, and BMP-4, or by insulin-like growth factor (IGF)-I, alone [24-35], or in combination [36]. Biochemical agents that have roles in chondrogenesis and collagen production, such as dexamethasone; insulin, transferrin, and selenious acid (ITS); ascorbic acid; and L-proline [25, 37-39] have been used to supplement the differentiation culture medium. In addition, blockage of Notch signaling is associated with significantly decreased cell proliferation and expression of Notch markers (including HES5) decreases during cartilage differentiation [40]. Interestingly, Jag-1 mediated Notch signaling activation in human mesenchymal stromal cells (hMSCs) was necessary to initiate chondrogenesis, but must be switched off for chondrogenesis to proceed [41]. For the most part, these studies have demonstrated chondrogenic differentiation through analysis of gene expression by polymerase chain reaction [25, 26, 28, 30], characterization of cartilage matrix by histology [25, 26, 28, 30], or identification of cell surface and cell associated matrix markers by flow cytometry [30]. Although these characterizations help to determine whether cells exhibit a chondrocytic (or fibrochondrocytic) phenotype, a functional approach to using any cell source for engineering purposes should incorporate quantitative evaluations of the biochemical and biomechanical properties of the generated tissue [42].

Microenvironment: Extracellular Matrix, Oxygen Tension, Biomechanical Forces

The extracellular matrix (ECM) of adult articular cartilage is not just an inert framework for holding chondrocytes and for cushioning the ends of the bones. Rather the ECM provides signals to the chondrocyte which regulate a number of important cellular functions. Progenitor cells within the condensation regions and in cell aggregates express cell adhesion molecules such as N-cadherin [23] and neural cell adhesion molecule (N-CAM) [43]. MSCs undergoing chondrogenesis acquire a characteristic spherical cell morphology and initiate the chondrogenic differentiation program with the expression of the transcription factors Sox9 [44], Sox5, and Sox6, which regulate the genes encoding the ECM molecules Collagen II and Aggrecan [45, 46]. The ECM produced by differentiated chondrocytes is essential for maintaining and regulating the chondrocyte phenotype and, during hypertrophy, for the formation of the future bone. Collagen II provides tensile strength to the cartilaginous ECM and contributes to the establishment of temporal and spatial organization of other matrix components such as the main proteoglycan, Aggrecan. Sulfated glycosaminoglycans (GAGs) modify aggrecan extensively contributing to the formation large aggregates in cartilage which attracts numerous water molecules [47, 48]. Aggrecan and other proteoglycans provide a cushioning role of the matrix, but also act to immobilize and store growth factors and thereby function as molecular organizers of the ECM and cartilage in general [49].

The integrins are the major family of ECM receptors in the cell which can transmit information from the matrix to the cell. Integrin binding of ECM ligands results in the activation of signaling pathways which play a key role in the regulation of stem cell survival, proliferation, chondrogenic differentiation, and matrix remodeling. Integrin signaling is coordinated with both the organization of the cytoskeleton and with signaling initiated by activation of growth factor and cytokine receptors. At least 20 different integrin heterodimers have been described resulting from the combination of 9 types of β subunits (designated β1 . . . β9) with 14 types of α subunits. Initial studies were performed to characterize which integrins were expressed by adult articular chondrocytes and which cartilage ECM proteins served as chondrocyte integrin ligands. Work from several laboratories has revealed that chondrocytes express at least the α1β1, α2β1, α3β1, α5β1, α6β1, α10β1, αvβ3, and αvβ5 integrins [50-55]. Using immunohistochemical staining of adult human articular cartilage, Salter *et al*. [54] noted that the α5β1 integrin was a prominent chondrocyte integrin with variable and weaker expression of α1β1 and α3β1. Using a combination of immunofluorescence, immuno-precipitation, and FACS analysis, Woods *et al*. [55] demonstrated that adult human chondrocytes express α1β1,

α5β1, and αvβ5 integrins accompanied by weak expression of α3β1 and αvβ3. Interestingly, an increase in integrin expression *in situ* in osteoarthritic (OA) cartilage compared to normal cartilage has been noticed [53]. The α1β1, α2β1 and α10β1 integrins can all serve as receptors for type II collagen [50, 52, 53, 56, 57]. The α1β1 integrin also mediates adhesion of chondrocytes to type VI collagen [57] and to cartilage matrix protein (matrilin-1) [58]. In addition to collagen, α2β1 can mediate binding to chondroadherin [59]. The α5β1 integrin serves as the primary chondrocyte fibronectin (FN) receptor [53] while αV-containing integrins bind to vitronectin and osteopontin [60] and may serve as alternative FN receptors.

Recent gene expression analysis of hMSCs undergoing chondrogenesis in micromass cultures over a 21-day period identified molecules already known to be involved in attachment and cell migration, including syndecans, glypicans, gelsolin, decorin, fibronectin, and type II, IX and XI collagens. Importantly, the expression of molecules that were not previously associated with MSCs or chondrocytes were also detected, namely metalloproteases (MMP-7 and MMP-28), molecules of the connective tissue growth factor (CTGF); cef10/cyr61 and nov (CCN) family (CCN3 and CCN4), chemokines and their receptors chemokine CXC motif ligand (CXCL1), Fms-related tyrosine kinase 3 ligand (FlT3L), chemokine CC motif receptor (CCR3 and CCR4), molecules with A Disintegrin And Metalloproteinase domain (ADAM8, ADAM9, ADAM19, ADAM23, A Disintegrin And Metalloproteinase with thrombospondin type 1 motif ADAMTS-4 and ADAMTS-5), cadherins (4 and 13) and integrins (α4, α7 and β5) [61]. Accumulating data suggests that crosstalk between ECM components of the microenvironment and MSCs within the cartilage dramatically contributes to the differentiation of MSCs into chondrocytes.

Cartilage is an avascular tissue, and chondrocytes *in vivo* experience a severely hypoxic microenvironment. Increasing evidence of a central role of hypoxia and the transcription factor hypoxia-inducible factor (HIF)-1α in cartilaginous tissues has been demonstrated [62]. Growth plates with functionally inactivated HIF-1α display great defects in their central areas caused by massive cell death, indicating that HIF-1α is absolutely necessary for chondrocytes to survive extremely low oxygen tensions. HIF-1α is a highly conserved transcription factor that has key functions in controlling energy generation, cell survival and matrix synthesis by articular and growth-plate chondrocytes. Other factors, such as proinflammatory mediators and mechanical load, have been shown to increase HIF-1α activity in articular chondrocytes independently of hypoxia. Low oxygen tensions and HIF-1α are important factors in articular chondrocyte behavior during cartilage homeostasis and osteoarthritis [63]. In mouse osteo/chondroprogenitor stromal cell lines stimulated to chondrogenic differentiation (BMP-7) hypoxia dramatically enhanced the expression of Sox 9 and its downstream targets aggrecan and Col2α followed by the accumulation of mucopolysacharide. Hypoxia increased nuclear accumulation of HIF-1α which mediated the activation of the Sox9 promoter. This data suggests that a hypoxic microenvironment contributes to the differentiation of mesenchymal cells along a chondrocyte pathway after chondrogenic stimulation in part by activating Sox-9 *via* a HIF-1α-dependent mechanism [64].

Cartilage provides a means of cushioning joints and protecting them to the stress of mechanical loading. Chondrocytes and chondroprogenitors are constantly exposed to a microenvironment loaded with mechanical forces. The effects of mechanical loading on chondrocyte differentiation and homeostasis have been extensively studied using agarose and cartilage explant cultures. Compressive loading has been shown to modulate cartilage-specific macromolecule biosynthesis and pericellular matrix deposition of mature chondrocytes [65-70]. Additionally, static and dynamic compressive loadings promote chondrogenic differentiation of embryonic limb-bud mesenchymal cells [71-73]. In addition, cyclic hydrostatic pressure enhanced the extracellular matrix deposition of human bone marrow-derived MSCs (hBM-MSCs), which underwent chondrogenesis in pellet cultures [74]. However, the effects of physical stimuli associated with the mechanical environment of articular cartilage on chondrogenic differentiation of BM-MSCs still remain unclear. Cyclic compressive loading can promote the chondrogenesis of rabbit BM-MSCs by inducing the synthesis of TGF-β1, which in turn can stimulate the BM-MSCs to differentiate into chondrocytes [75]. It is clear that a microenvironment loaded with mechanical forces contributes to the differentiation program of stem cells towards the chondrogenic fate.

Epigenetic Factors (Chromatin Modifications, MicroRNA)

Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequence. Epigenetic changes are crucial for the development and differentiation of the various cell types in an organism. Epigenetic processes can involve chemical modifications to DNA or to the proteins that are closely associated with DNA (the histones, which form the cores of chromatin packaging), and a prominent role for RNA is also emerging. During development, cells start in a pluripotent state, from which they can differentiate into many cell types, and progressively develop a narrower potential. Their gene-expression programs become more defined, restricted and, potentially, "locked in". Pluripotent stem cells express genes that encode a set of core transcription factors, while genes that are required later in development are repressed by histone marks, which confer short-term, and therefore flexible, epigenetic silencing. By contrast, the methylation of DNA confers long-term epigenetic silencing of particular sequences in somatic cells. Long-term silencing can be reprogrammed by demethylation of DNA. Development is, by definition, epigenetic. Differences in the programs of gene expression that result in the development of different organs and tissues occur without changes to the sequence of our DNA (with one or two exceptions).

As described earlier, during endochondral ossification chondrocytes lay down a cartilaginous template rich in particular ECM molecules and undergo a program of proliferation, maturation and hypertrophy. Chondrocytes in the articular cartilage are constrained from completing this program, allowing the maintenance of a low friction load-bearing cartilaginous surface that facilitates joint movement [76]. Histone deacetylases (HDACs) are usually transcriptional co-repressors that modulate cell growth, differentiation and

apoptosis [77]. HDAC4-null mice display a skeletal phenotype, with early onset chondrocyte hypertrophy and premature ossification [78]. HDAC4 is expressed in pre-hypertrophic chondrocytes and is postulated to regulate hypertrophy by inhibiting the action of Runx2, a key transcription factor required for both chondrocyte hypertrophy and osteoblast differentiation [76]. This establishes HDAC4 as a critical regulator of chondrocyte hypertrophy and skeletogenesis.

In addition to transcription factors and histone deacetylases, microRNAs (miRNAs) have emerged as a new class of gene expression regulators. These miRNAs are 20–24 nucleotide non-coding RNA molecules that post-transcriptionally regulate gene expression. They are generated from precursor RNA molecules with hairpin structure in which partially the double-stranded stem is cleaved by the enzyme Dicer thus releasing the mature miRNA [79]. One of the mature miRNA strands forms an effector ribonucleoprotein complex termed RISC (RNA induced silencing complex) which guides the miRNAs to specific mRNAs [79]. Depending on the degree of sequence complementarity RISC either cleaves or blocks translation of the target mRNA. Most miRNA targets in animals are not cleaved but translationally suppressed due to the mismatches between miRNAs and target sites. Expression analysis of miRNA expression in different tissues indicates that miR-140 was expressed only in zebrafish cartilagenous tissue [80]. Assessment of the expression pattern of miR-140 in the developing mouse embryo on dissected tissues demonstrated that miR-140 was first detected at 11.5 days post-conception (E11.5) and throughout subsequent stages of development and it was detected specifically in cartilagenous tissues of the developing limbs, ribs, vertebrate, sternum and the skull [81]. Furthermore using a luciferase reporter assay and western blot analysis it was established that HDAC4 is a miR-140 target, suggesting a potential role of miR-140 in the formation and/or maintenance of chondrocytes from progenitor cells *via* a mechanism involving HDAC4.

There is a need for much more precise molecular methods of detecting and characterizing the miRNA and epigenetic changes that accompany cell differentiation and distinguishing them from the modulation of phenotype caused by changes in the physical and biochemical signals to which the cell responds. Furthermore, we need to further investigate how miRNA regulation and epigenetic control may modulate chondrogenic signal transduction during aging and how they may contribute to cartilage degeneration.

Sources of Cells for Cartilage Repair

As earlier described, although chondrogenic differentiation has been described for umbilical cord blood [3, 4, 82] and amniotic fluid [83]–derived pluripotent cells, we will focus our discussion on adult and embryonic stem cells for cartilage repair.

Embryonic Stem Cells

Although not as popular as adult stem cells for strategies in cartilage repair, these cells have tremendous potential for regenerative medicine efforts [84]. However, there are numerous challenges with the use of human embryonic stem cells (hESCs), primarily teratoma formation and ethical concerns. Upon ES cells transplantation into the knee joint and a subcutaneous space of mice with severe combined immunodeficiency teratomas formed in the joints grew and destroyed the joints in both areas [85]. The teratomas were proved to have been derived from the transplanted ES cells. Interestingly, when the ESCs where transplanted within a scaffold and subjected to mechanical stress, no teratoma formation was observed [86]. Further optimization of donor ES cells to differentiate as well as inhibit tumor growth may help to mitigate the concerns of potential teratoma formation. Recent studies with hESCs [27, 30] provide important knowledge in formulating novel strategies for using stem cells in cartilage tissue engineering and repair.

Use of human embryos, however, faces ethical controversies that hinder the applications of human ES cells. In addition, it is difficult to generate patient- or disease-specific ES cells, which are required for their effective application. One way to circumvent these issues is to induce pluripotent status in somatic cells by direct reprogramming. Recently, human ESC-like cells have been generated by forced expression of 4 different factors into human somatic cells [87, 88]. Thomson and colleagues [87] introduced four genes encoding the transcription factors Oct-4, Sox-2, Nanong, and Lin-28. In contrast, Yamanaka and colleagues generated induced pluripotent stem (iPS) cells, capable of germline transmission, from adult human dermal fibroblasts with the four factors: Oct3/4, Sox2, Klf4, and c-Myc [88]. Human iPS cells were similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. Furthermore, these cells could differentiate into cell types of the three germ layers *in vitro* and in teratomas. These findings demonstrate that iPS cells can be generated from adult human fibroblasts. However, these cells generated tumors in animal models, thus further improvement of gene delivery techniques are needed in order to prevent mutation leading to tumor formation (such as retroviral integration).

Another significant advance in the field of embryonic stem cell research has been made recently by developing human ES cell lines from a single cell harvested from the blastomere, without destroying the embryo or affecting its development [89, 90]. While these research findings are exciting, the techniques are still in their early developmental stage [91]. The formation of three-dimensional tissue by differentiation of such ES cells has not yet been demonstrated. The long-term impact to the human embryo development after the 'donation' of the single ES cell is still unknown.

More scientific data are needed for the acceptance hESCs in approaches of cartilage regeneration.

Adult Stem Cells

In contrast to ES cells, adult stem cells transplanted in pre-clinical animal models have shown no evidence of tumor formation, not even, teratomas. This may be due to fact that ESCs and adult stem cells are under different epigenetic control and miRNA regulatory profile. Nevertheless, these properties, combined with the possibility of autologous transplantation, demonstrate significant advantages over embryonic stem cells in many proposed clinical applications at

the current time. There are several types of adult somatic stem cells with different potentials to differentiate toward functionally mature cells. Adult somatic cells with the potential to differentiate into mesenchymal lineages such as cartilage, bone, ligament, tendon, fat, and other connective tissues have be referred here as mesenchymal stromal cells [MSCs] despite the different nomenclature found in the literature to refer to dissimilar cells with similar properties. MSCs obtained from different adult tissue sources are found to have dissimilar capacities to differentiate to mature cells. Adipose derived MSCs have a higher adipogenic potential while cells from the periosteum exhibit a superior osteogenic and chondrogenic ability**.** In addition, periosteal MSCs exhibit high osteogenic potential while also exhibiting chondrogenic and myogenic capacity. Though skeletal muscle derived MSCs are known for their relatively low potential for chondrogenesis they do possess multi-differentiation capacity. Synovial membrane forms the lining of the chondyle surface and it is the most proximal vascularized tissue to cartilage. MSCs derived from the synovial membrane and synovial fluid show high chondrogenic potential which is comparable to that of bone marrow derived MSCs. It is assumed that these cells originate from the bone marrow and migrate to the synovium *via* vasculature. Interestingly, some studies have also indicated the presence of MSC - like progenitor cells in the surface zone of normal and osteoarthritic adult human articular cartilage as well as in immature bovine articular [20] cartilage [92]. This observation is intriguing because it shows lack of regeneration of diseased articular cartilage in apparent presence of chondrogenic cells. A probable explanation is that the MSCs found in cartilage are actually recruited from the synovial membrane as a reparative response to damage. This could also explain the detection of a higher number of MSCs in OA cartilage compared to healthy cartilage. However, the increased frequency of progenitor cells in OA-cartilage could also result from proliferation of resident progenitor cells [19]. These observations incite the presumption that the mere presence of MSCs at the site of injury is not sufficient for induction of repair processes. More likely, MSCs require cues from the microenvironment to differentiate towards chondrocytes. This issue still needs to be addressed in greater detail.

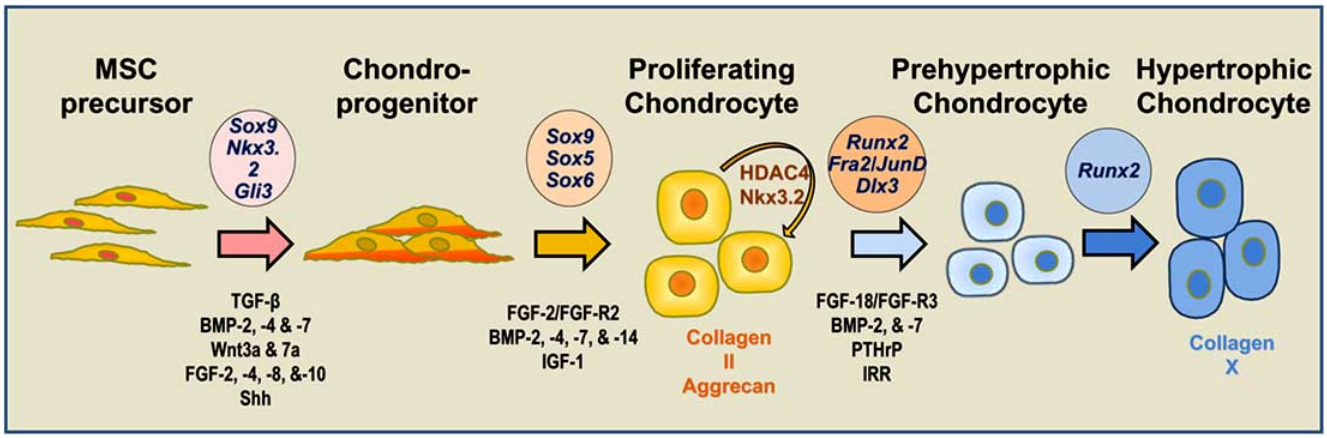
Adipose Derived Adult Stem (ADAS) Cells

Adipose Derived Adult Stem (ADAS) cells are MSC-like cells isolated from adipose tissue by collagenase digestion [16, 17, 93]. These cells have been scrutinized for their osteo/chondrogenic potential, and appear to have a decreased chondrogenic potential compared to bone marrow-derived MSCs; suggesting that the original location of the tissue appears to play a significant role in determining their differentiation potential. ADAS have been shown to have *in vitro* and *in vivo* chondrogenic potential. Cartilage-like tissue was observed in pellet cultures and cell-loaded carriers (agarose, alginate & gelatin sponge) after treating ADAS with TGF-β and dexamethasone for 2 weeks. Cartilage formation was also observed *in vivo* after implanting the cells in an alginate carrier into subcutaneous pockets in immunodeficient mice, and repairing articular cartilage in a rabbit model [94-96]. Defects repaired with these cells were filled with hyaline cartilage that integrated with the surrounding host tissue. However, when ADAS cells and MSCs isolated from the same patient were compared, no significant differences were observed for yield of adherent stromal cells, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction efficiency [97]. These results suggest that adipose tissue is an abundant source of ADAS cells, which have a potential like bone marrow-MSCs for use in tissue-engineering applications and as gene delivery vehicles. However, compared to bone marrow harvesting, liposuction is not a minimally invasive procedure. Some individuals, in particular conditioned athletes, may not have enough adipose tissue for the isolation of sufficient number of stem cells for therapeutic purposes.

Bone Marrow Stromal Stem Cells

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) are by far the most widely studied MSCs. The first report of the existence of stromal cells in the bone marrow dates to 1867 by Cohnheim [98]. Later in the mid-1970's Friedenstein and colleagues performed a series of detailed *in vitro* and *in vivo* studies describing the isolation and properties of fibroblast-like cells, termed marrow stromal cells, which adhered to the surface of the cultured dish in whole bone marrow samples [99-101]. These cells proliferated, formed colonies, and showed osteochondrogenic capacity, even after passaging the cells in culture over many population doublings. Later on Caplan and colleagues called the process of marrow stromal cell proliferation and differentiation mesengenesis and termed the cells mesenchymal stem cells [102-105]. However, marrow stromal cells and mesenchymal stem cells represent a heterogeneous population of cells with different properties. Prockop and colleagues have isolated several subpopulations of marrow stromal cells with different self-renewal, differentiation, and engraftment capacity [6, 106-108]. Thus, the methods used to isolating the cells and the culture conditions can have an immediate impact on the proliferative and differentiation capacity of marrow MSCs. Using a unique isolation, expansion, and culture method we have isolated a population of developmentally immature marrow MSCs, reminiscent of ES cells, termed marrow-isolated adult multilineage inducible (MIAMI) cells, characterized by a defined molecular profile and a broad differentiation potential [10-12]. Using a different strategy Verfaillie and colleagues have isolated and characterized a similar immature stem cell population with a strong self-renewal and differentiation capacity and have termed them multipotent adult progenitor cells (MAPCs) [13, 14]. BM-MSC subpopulations, considered to be of mesodermal origin, have been found to differentiate, under appropriate conditions, to cells types of tissues derived from other germ layers [10, 14].

Studies on the chondrogenic differentiation of BM-MSCs have serve as the basis to further characterize *in vitro* the cytokines that regulate chondrogenesis, the signaling pathways activated, and the transcription factors that are involved in this process originally identified using developmental biology approaches. These studies have contributed to provide further support for the, and help clarify the complex, roles of sonic hedgehog (Shh), FGF (2, 4, 8, and 10), TGFβ superfamily (1, 3



The trophic factors are indicated below the arrows. The transcription factors mediating the progression are circled above the arrows. Factors promoting the maintenance of cells at the proliferating chondrocyte stage (HDAC4 & Nkx3.2) are described above the cells. Synthesized ECM proteins characteristic of proliferating and hypertrophic chondrocytes are described bellow the cells. Most factors are described in the text.
BMP: Bone Morphogenetic Protein; FGF: Fibroblast Growth Factor; FGF-R: FGF Receptor; IGF: Insulin-like Growth Factor; IRR: Insulin Receptor-related Receptor; PTHrP: Parathyroid Hormone related Peptide; Shh: Sonic Hedgehog; TGF: Transforming Growth Factor.

Fig. (1). Schematic representation of the factors regulating the sequential events during chondrogenesis.

& BMP-2, 4, 7, and 14), Wnt (3a and 7a) during chondrogenic differentiation and FGF-18 and PTHrP during chondrocyte hypertrophy. These cytokines in synchrony contribute to the regulation of the key transcription factors Nkx3.2 (Bapx 1), Gli3, Sox 9, Sox 5 and Sox 6 during chondrogenic determination and of Runx2, Dlx3 and Fra2/JunD during hypertrophy (Fig. **1**) [elegantly reviewed by Djouad *et al.* 2006 [61]]. In this context, maintenance of chondrocyte proliferation and inhibition of hypertrophy appears to be mediated by the sustained action of Nkx3.2 to prevent hypertrophy *via* a mechanisms leading to the inhibition of Runx2 [109, 110]. Thus, BM-MSCs, and in particular the more primitive subpopulations, appear to be the most suitable cell source for cartilage repair and tissue engineering approaches, because these cells can be carefully and sequentially directed to progress toward the chondrogenic pathway in a controlled fashion, without the risk of tumor formation, by providing the right molecular cues at the right time. Furthermore, these cells could be molecularly controlled to promote or prevent hypertrophy depending on the therapeutic role or tissue engineer need. BM-MSCs have been the most widely used cells in cartilage repair; alone or in combination with scaffolds such as collagen sponges, hyaluronic acid hydrogels, and other biomaterials, in several animal models.

Articular cartilage is structurally a complex tissue whose function depends partly on the mechanical support of subchondral bone [111]. Thus, functional restoration of articular cartilage will depend on the mechanical and structural support of subchondral bone tissue. Since BM-MSCs are capable of forming cartilage and bone tissue, it is conceivable that they could be the single cell source used to repair osteochondral structures. Engineering osteochondral grafts by combining the most developmentally immature autologous BM-MSCs into different integrated biomaterial carriers with specific bio-directive factors, one directing osteogenesis and one chondrogenesis (without hypertrophy), could provide an approach for repairing articular cartilage. The resulting tissue engineered autologous osteochondral graft could potentially substitute for, and outlast, prostheses currently used for the treatment of severely diseased joints.

Clearly, further studies of the biology of adult stem cells, in particular immature BM-MSCs, will help determine the right conditions (cytokines, microenvironment, biomechanical forces, scaffolds, etc.) that will help achieve these goals. In addition, safety issues, such as potential disease transmission due to the use of fetal bovine serum use for MSC culture and expansion, and potential tumor formation, have to be taken into account.

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