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Emergence of Chondrogenic Progenitor Stem Cells in Transplantation Biology—Prospects and Drawbacks

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ABSTRACT

Avascular tissues such as a cartilage contains a unique type of cell called as the chondrocyte. We, however, have not understood the origin of the chondrocyte population and how this population is maintained in the normal tissue. In spite of being considered to be a simple tissue, scientist had always faced difficulties to engineer this tissue. This is because different structural regions of the articular cartilage were never understood. In addition to this, the limited self-repair potential of cartilage tissue and lack of effective therapeutic options for the treatment of damaged cartilage has remained an unsolved problem. Mesenchymal stem cell based therapy may provide a solution for cartilage regeneration. This is due to their ability to differentiate into chondrogenic lineage when appropriate conditions are provided. An ideal cell source, a three-dimensional cell culture, a suitable scaffold material that accomplishes all the necessary properties and bioactive factors in specific amounts are required to induce chondrocyte differentiation and proliferation. Cartilage tissue engineering is a promising and rapidly expanding area of research that assures cartilage regeneration. However, many unsolved questions concerning the mechanism of engraftment of chondrocytes following transplantation in vivo, biological safety after transplantation and the retention of these cells for lifetime remain to be addressed that is possible only through years of extensive research. Further studies are therefore required to estimate the long-term sustainability of these cells in the native tissue, to identify well suited delivery materials and to have a thorough understanding of the mechanism of interaction between the chondrocytes and extracellular matrix and time is not far when this cell based therapy will provide a comprehensive cure to cartilage disease. J. Cell. Biochem. 113: 397–403, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: CHONDROCYTES; SCAFFOLDS; ENGRAFTMENT; GROWTH FACTORS; CARTILAGE REPAIR; PROLIFERATION

Stem cells have recently evoked interest as a promising alternative cell source for treating articular cartilage defects. Due to the controversy that is associated with the use of embryonic stem cells, there was an urgent need to establish a viable alternative to embryonic stem cells that is characterized by its clonogenicity, multipotency and its migratory activity and above all they must be associated with a non-controversial source. A suitable alternative such as the stromal stem cells also called as mesenchymal stem cells (MSCs) is associated with the mesodermal lineage and it is known to differentiate into numerous cell types including adipocytes, osteocytes [Pittenger et al., 1999], myocytes [Negishi et al., 2000], astrocytes, and neurons [Woodbury et al., 2000]. Apart from these lineages, MSC's has also shown to have the potential to differentiate into chondrogenic lineage [Kafienah et al., 2006, 2007] and hence they are termed as chondrogenic stem cells (CSCs). The process by which these multipotent cells differentiate into chondrocytes is called as chondrogenesis.

MSCs are shown to be present in various tissues such as bone marrow [Friedenstein et al., 1970; Castro-Malaspina et al., 1980], adipose tissue [Zuk et al., 2001], synovial membrane [De Bari et al., 2001], trabecular bone [Noth et al., 2002], and other tissues. The high proliferation capacity, make them attractive as a distinguished cell substitute for chondrocytes in cartilage regeneration [Baksh et al., 2004]. MSCs can fulfill the requirements demanded by cells for tissue engineering of cartilage, as they can be conveniently manipulated in vitro to differentiate to chondrocytes for these purposes. MSC's were first isolated from the bone marrow and hence the name marrow stromal cells was coined [Bernardo et al., 2009]. However, due to the painful procedure involved in its isolation, risk-containing sampling procedure and limitations in the number of cells in older individuals made the isolation of stem cells from other sources an attractive alternative. An alternative source, however, is the Wharton's Jelly that is isolated from the Umbilical Cord Tissue [Nekanti et al., 2010]. These cells are naïve in nature as

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they originate from the extra embryonic tissue and thus considered as an attractive alternative. Adipose tissue is particularly considered to be an equally attractive source for MSC to bone marrow, as it is easily accessible in large quantities and adipose-derived MSCs show a proliferation and multilineage capacity comparable to sources that are isolated from other established sources [De Ugarte et al., 2003; Winter et al., 2003]. MSC from synovial membrane [De Bari et al., 2001], muscle [Bosch et al., 2000], periosteum [Nakahara et al., 1991], and many other mesenchymal tissues are in experimental use in the field of regenerative medicine. In this review we will discuss the current knowledge of MSCs and its differentiation into chondrocytes. We shall focus on the attempts undertaken in the isolation strategies, characteristic features, and its role in Transplantation Biology. Though this discussion attempts to identify key areas, further detailed research in the potentials of these cells in the areas of regenerative medicine is required.

ROLE OF SCAFFOLDS AND 3D CULTURE IN CHONDROGENIC INDUCTION

In order to induce chondrogenesis, a three-dimensional culture together with a proper scaffold material is required which can be comparable to the cartilage formation in physiological conditions. It has been reported that cells are not retained in the tissue if they are directly administered into the damaged site [Archer et al., 1990]. Therefore, cells are grown in a scaffold that helps to retain them after transplantation. Several publications have reported the chondrogenic differentiation potential of MSCs in vitro [Winter et al., 2003] and in vivo [Breinan et al., 2001; Madry et al., 2002]. Human umbilical cord-derived MSCs (hUCMSCs) cultured in a scaffold made of synthetic polymers polyglycolic acid (PGA) and poly-L-lactic acid (PLA) also differentiate into chondrogenic lineage and upregulates chondrocyte specific genes like collagen type I and II, aggrecan, etc. [Wang et al., 2010]. Similar results were obtained by others [Guilak et al., 2004] for demonstrating the potential of adipose tissue-derived stem cells differentiating into chondrogenic phenotype that synthesized collagens and proteoglycans (Fig. 1).

THE ORIGIN OF CHONDROCYTES DURING EMBRYONIC DEVELOPMENT

Stem cells are known to be classified based on the origin. They may be either of the embryonic or adult origin. Adult stem cells are thought to be present in a specific area of the tissue called a ''stem cell niche.'' In an organism, adult stem cells remain in a state of inactivity for long periods unless they are prompted to divide by some external or internal signals. Multipotent stem cells that are mainly of the hematopoietic or mesodermal lineage are found in many areas of the body such as bone marrow, umbilical cord tissue, adipose tissue, dental pulp, etc. These cells upon stimulation give rise to specialized cells which has created tremendous interest among researchers.

During the embryonic stages of development, MSCs is responsible in the differentiation into two different kinds of cartilage: permanent and transient [Kronenberg, 2003]. The permanent hyaline cartilage arises from MSCs that form the distal ends of the developing bones. After initial condensation, the stem cells differentiate towards stable chondrocytes that thereafter synthesize the typical hyaline extracellular matrix of articular cartilage. In addition to permanent cartilage, a second form also develops from MSCs: the transient cartilage. Prior to skeletal bone formation, chondrocytes originating from MSCs build up a transitional cartilaginous model of the skeleton that is later replaced by mineralized bone in a process called endochondral ossification. After the cartilaginous scaffold is formed, chondrocytes in the middle of the diaphysis cease to proliferate and become hypertrophic, implicating that they enlarge in size and start expressing the hypertrophy marker molecule collagen type X. After further differentiation, hypertrophic chondrocytes start calcifying the surrounding matrix and either transdifferentiate towards osteoblasts or undergo apoptosis [Adams and Shapiro, 2002]. When using MSCs for articular cartilage repair, the great challenge during chondrogenesis is to generate chondrocytes comparable to articular cartilage-derived chondrocytes that do not undergo hypertrophy as a terminal differentiation stage.

EPIGENETIC REGULATION OF CHONDROGENIC STEM CELLS

Chondrogenesis is believed to be a multistep process consisting of several stages: MSC condensation, chondrocyte proliferation, differentiation, maturation, and programmed cell death [Goldring et al., 2006]. The whole process of chondrocyte differentiation is controlled by several signaling molecules, bioactive factors, and transcription factors. These molecules have been reported to phosphorylate the transcription factor cAMP response element (CREB) which induces chondrogenic specific gene expression. Sox9 considered as a fundamental transcription factor plays a pivotal role in promoting chondrogenesis. It is thought to enhance the MSC condensation and stimulate chondrocyte differentiation [Akiyama, 2008]. Sox9 interacts with Sox9 binding sequences on promoters of chondrocyte specific genes like collagen typeII (Col2a1), aggrecan and cartilage oligomeric matrix protein (COMP) and initiates transcription [Bell et al., 1997; Bridgewater and Lefebvre, 1998; Kou and Ikegawa, 2004; Liu et al., 2007] The post-translational modification of Sox-9 gene alters it's functional activity and hence affects the Sox9-dependent transcription in chondrogenesis [Akiyama, 2008; Wegner, 2010]. Protein-kinase A mediated phosphorylation of Sox9 facilitates the Sox9-dependent transcription [Huang et al., 2000]. On the contrary, the transcriptional activity of Sox9 retards with PIAS1 mediated sumoylation [Oh et al., 2007]. Sox5 and Sox6 are members of Sox gene family and are also believed to promote chondrogenic differentiation together with Sox9. This indicates that the transciptional activity of Sox9 is controlled by many factors during the process of chondrogenesis. Other chondrogenic related genes like Runx2, transcription factor Scleraxis (Scx), and chondromodulin-I also contributes in the process of chondrogenesis. This process also involves many chromatin factors other than the transcription factors indicating

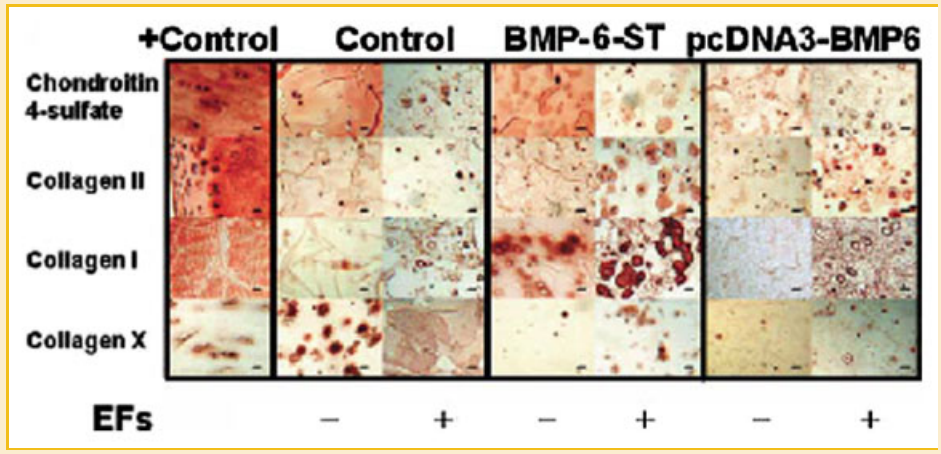

Fig. 1. Representative immunohistochemistry results for chondroitin-4-sulphate and types I, II, and X collagen for a typical experiment with ASCs encapsulated in alginate after 4 weeks in in vitro culture. Positive control: porcine cartilage for C-4-S, collagen II and collagen X; porcine ligament for collagen I. Control: incomplete chondrogenic medium supplemented with 10% FBS. Treated: incomplete chondrogenic medium supplemented with short-term exposure to BMP-6, in addition to continous exposure to rhEGF, ehFGF, and 10% FBS [Estes et al., 2010]. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

a crucial role played by them as well in chondrogenic differentiation.

Chondrogenic induction is initiated by the addition of growth factors like transforming growth factor-β(TGF-β) and bone morphogenetic protein (BMP-6) to the culture media. Many downstream pathways including Smad2, Smad3, and mitogen-activated protein kinase (MAPK) are thought to be activated after TGF-β binds to its receptor [Liu, 2003]. Furumatsu et al. [2005] showed that Smad3 pathway associates with Sox9 to induce chondrocyte differentiation. Other pathways like MAPK pathway also induces the expression of Sox9 and Col2a1 [Murakami et al., 2000; Stanton et al., 2003; Tuli et al., 2003]. It has been demonstrated that growth factors like fibroblast growth factor 1 and 2, insulin-like growth factor-1 (IGF-1) enhances the transcriptional activation of Sox9 [Shakibaei et al., 2006]. These findings suggest that that the process of chondrogenesis is regulated by several growth factors and transcription factors. However, it had remained a controversy that only specific genetic pathways are activated in the process though all cells contain the same genes. This has led to the discovery of another revolutionary field "epigenetics" although many unsolved questions need to be addressed.

The term epigenetics refers to mitotic or meiotic heritable changes in both phenotype and gene expression caused by a mechanism that is not coded in the DNA sequence. It also includes the modification of the histone protein core that includes acetylation, phosphorylation, methylation, and ubiquitation. Epigenetics involves two major modifications: (1) Post-translational modification of the histone proteins by certain enzymes like HAT, histone deacetylases (HDAC), and histone methyl transferases. (2) Methylation of DNA at CpG sites. DNA is wrapped around the histone protein core (consisting of two copies each of the core histone H2A, H2B, H3, and H4) and the structure is called as "nucleosome." Post-translational modifications like acetylation, phosphorylation, ubiquitination, and methylation occur at histone-N-terminals which are known as "histone tails" (Fig. 2). A balance between histone acetylation and deacetylation to regulate the epigenetic transcription is shown in Figure 3. HAT complexes such as coactivator p300 assists in the acetylation of histone protein while HDAC acts as a corepressor and is thought to deacetylate the histones resulting in reduced gene expression.

Highly methylated regions tend to be less transcriptionally active though the exact mechanism is not fully clear. It is believed that methylated areas prevent the binding of transcription factors to the DNA and hinder the process of chondrogenesis. Furumatsu et al. [2005] demonstrated that CREB posseses HAT activity and promotes gene expression after growth factors and other necessary bioactive molecules are added to the culture media. These studies reveals epigenetic activation of chondrogenic specific genes by histone acetylation but further studies are required that focus on methylation–demethylation of DNA. Other mechanisms that determine the epigenetic regulation in cartilage repair are still to be resolved.

LOCATION, CHARACTERISTIC FEATURES, AND ZONAL DISTRIBUTION OF THE MESENCHYMAL PROGENITOR CELLS (MPC's) IN THE NORMAL CARTILAGE

During embryonic skeletogenesis, there is a degradation of the hyaline cartilage. During osteogenesis, some of the remnant cartilage tissues serve as growth site between the ossified epiphyses (ends) and diaphysis (shaft) of the long bones until the completion of the longitudinal bone growth post-natally. In the adult, remnant hyaline cartilage serves as the principal skeletal tissue in the nose, trachea, bronchi, larynx. Remnant hyaline is also found within the rib cage (costal cartilage) and on the articular surfaces of diarthrodial joints [articular cartilage; Ross et al., 2003]. However, in the process of organogenesis of the vertebrate embryo, cells from three distinct mesenchymal lineages (sclerotome, somatopleure, and neural crest) independently undergo cartilage differentiation, or chondrogenesis, to form the multiple hyaline cartilages of the

Fig. 2. Histone modifications: Epigentics include modification of the histone protein core that includes accetylation, phosphorylation, methylation, and ubiquitation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

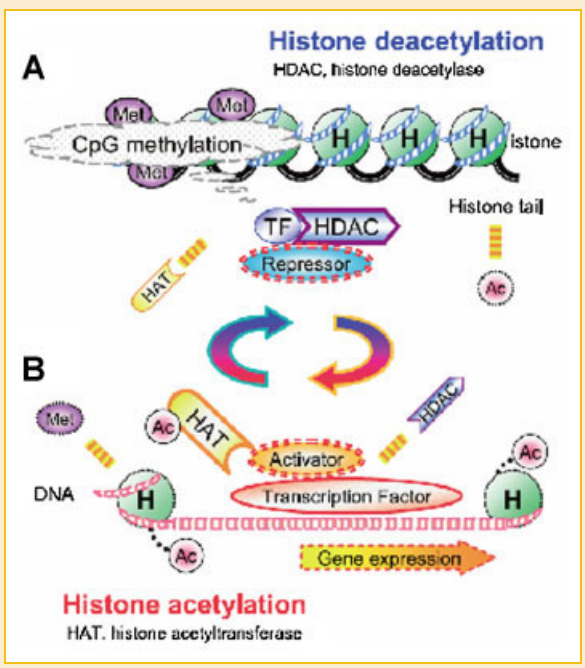

Fig. 3. Epigenetic regulation in the balance between histone acetylation and deacetylation. A: Schematic representation of a condensed heterochromatic structure. CpG islands are methylated in the promoter of the inactive genes on chromatin. In herterochromatic regions, the transcription factor (TF) cannot either recognize or associate with its DNA binding sequence. The repressing molecule and signal favorably associate with TF via the recruitment of corepressor HDAC. B: Schematic model of accessible euchromatic environment. TF, the coactivator HAT and activating molecule (e.g., Sox9, p300, and Smad3/4 trancscriptional complex) co-operatively induce histone acetylation. The chromatin structure changes from an inactive to accessible form by histone acetylation. H, histone; Ac, acetylation; Met, methylation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

primary skeleton. Despite the differences of the formation of chondrocytes from the cells of the mesenchymal origin and the anatomical location of chondrogenesis, it is noted that the formation of the various cartilage proceeds via a fundamentally analogous series of events [Cancedda et al., 2000; Olsen et al., 2000]. In the first stage, undifferentiated prechondrogenic mesenchyme cells migrate to the sites of the prospective skeletal elements, where they initially reside as a dispersed population of progenitor cells. This is followed by the subsequent assembly of the mesenchymal cells to form compact cellular aggregates or condensations—a process that is mediated by a specific combination of precartilage matrix and cell adhesion molecules that inhabit the extracellular space [DeLise et al., 2000]. In the next stage, the intimate juxtaposition of these aggregated mesenchymal cells enables crucial cell–cell surface interactions and signaling events that trigger overt chondrocyte differentiation. Specifically, the chondrogenic progenitor cells exchange their stellate, fibroblastic-like shape for the spherical morphology of hyaline chondrocytes and commence synthesis of cartilage-specific ECM molecules such as collagen types II, IX, and XI [Hoffman et al., 2003] and the highly sulfated proteoglycan aggrecan [Woods et al., 2005]. At this stage, the hyaline chondrocytes may either differentiate into hypertrophic chondrocytes and activate the expression of collagen type X, and contribute to the formation of the growth plate [Beier, 2005], or they may remain undifferentiated so as to maintain the ECM of a persistent hyaline cartilage that is retained throughout the lifespan of the organism.

WHAT MAKES CHONDROCYTES-DERIVED MESENCHYMAL STEM CELLS UNIQUE AND DIFFERENT FROM OTHER MESENCHYMAL STEM CELLS?

Although some similarities occur between chondrocyte-derived MSC and other MSCs, it has been shown [Bernstein et al., 2010] that differences between the two do exist. To confirm the hypothesis it

was imperative to depict gene expression differences between chondrocytes and MSCs during chondrogenic redifferentiation by Pellet Culture (PC). The similarities and differences are listed as follows:

- MSC-derived chondrocytes show inferior mechanical properties and produce less extracellular matrix proteins compared with primary chondrocytes.
- Experimental analysis using immunohistological and quantitative RT-PCR of SOX 9, collagen type I, II, and X using PC-conditioned chondrocytes and PC-conditioned MSC has shown that both chondrocytes and MSC showed a ready progression towards the chondrogenic lineage. Chondrocytes showed a higher SOX 9 expression although there was no significant differences in the collagen I and II mRNA expression.
- MSC's are less efficient at translating, processing, or incorporating collagen into the extracellular matrix.
- Chondrogenic arrest can be missed in both cell types and can proceed toward hypertrophy.
- Chondrocytes expressed significantly higher levels of aggrecan compared to MSC's.
- Increased glycano-anabolic activity is seen in chondrocytes as compared with MSCs due to chondrocytic upregulation of glycosaminyltransferase MGAT4B.
- Chondrocytes showed upregulated fatty acid metabolism that was validated for HMGCS1 and the cytochrome P450 oxidase CYP51A1, thus verifying the cholesterol-biosynthetic activity in chondrocytes.
- MSC's show an upregulation of the gene osteoprotegerin (TNFRSF11B) thus showing that it readily undergoes osseous differentiation as compared to chondrocytes.
- MSC's had a capability to remain in a sustained undifferentiated state and this was linked to the higher expression of the inhibitors of DNA binding ID3 and ID4 in MSC's.
- Downregulation of the eukaryotic initiation factor EIF2AK4 is linked to the increase of protein biosynthesis in MSC's.
- Chondrocytes expressed more of the intracellular chloride channel CLIC4, which is a conserved gene among vertebrates. CLIC4 is a mediator of TGF-b signaling via its translocation to the nucleus. It shows relevance in endothelial tubule formation, anti-apoptotic action, and fibroblast-to-myofibroblast transdifferentiation in cancer cells.

These evidences have shown that MSC's do not reach the same stage of chondrogenic differentiation as chondrocytes do, regardless of TGFb-supplementation and despite harvesting from younger and healthier donors.

ROLE OF CHONDROCYTE DIFFERENTIATION IN TISSUE ENGINEERING

Tissue engineering is an area of regenerative medicine that basically combines areas of cell biology, engineering, material sciences, and surgery to provide new functional tissues using living cells, biomatrices, and signaling molecules [Langer and Vacanti, 1993]. Recently, this discipline has greatly expanded, with numerous research groups focusing on the development of strategies for the repair and regeneration of a variety of tissues [Bonassar and Vacanti, 1998]. Many of these tissue-engineered approaches have targeted the musculoskeletal system in general, with special emphasis on articular cartilage [Temenoff and Mikos, 2000]. The vast majority of approaches that are focused to repair or regenerate articular cartilage are cell-based, thus aiming to provide a population of reparative cells to the injured site. There are various types of cells that are used to develop these strategies. They may be either differentiated chondrocytes isolated from unaffected areas of the joint surface [Risbud and Sittinger, 2002] or progenitor cells capable of differentiating into chondrocytes which can be isolated from a variety of tissues [Amiel et al., 1985]. As harvesting a tissue biopsy from valuable healthy articular cartilage will result in an additional injury, which ultimately cannot repair itself, this cell source might not be an ideal choice. Thus, considerable research efforts are directed to the isolation of progenitor cells and to understand the mechanisms involved in chondrogenic differentiation.

THE WAY FORWARD AND ITS POSSIBLE IMPLICATIONS IN FUTURE THERAPEUTIC INTERVENTIONS

Although in vitro studies and few clinical trials have demonstrated the effectiveness of chondrocyte transplantation in osteoarthritic patients, many challenges still need to be resolved. Scientists need to study in greater detail the mode of delivery of chondrocytes into the tissue, their long-term sustainability in the recipient after transplant, their integration into the damaged tissue and the biological safety these cells promise when administered must be addressed before bringing such treatments to the clinic. To conclude, chondrocyte stem cell therapy offers exciting promises for cartilage repair, but significant hurdles remain before it becomes an acceptable form of therapy in the years to come.

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