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Review

Recent Progress in Stem Cell Chondrogenesis

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ABSTRACT

Significant efforts have been undertaken in the last decade in the development of stem cell-based therapies for cartilage repair. To improve the clinical efficacy of stem cells for articular cartilage repair, understanding the factors and conditions that influence stem cell chondrogenesis in their lineage-specific differentiation and phenotypic stability of the cartilage formation following differentiation would be necessary. In this progress update, we discuss the use of the various sources of stem cells, induction factors including growth factors, oxygen tension and biomaterial scaffolds, and some of the cell-cell/matrix interactions and underlying mechanisms that regulate stem cell chondrogenesis towards stable cartilage formation.

Keywords:
Stem cells; Chondrogenesis; Biomaterials; Tissue regeneration; Tissue engineering.

Introduction

Stem cells, defined by their capacities for self-renewal and for differentiation into a wide variety of cell lineages, hold great promise for applications in tissue engineering and regenerative medicine. In the context of cartilage tissue engineering, the major sources of stem cells are adult mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs) (Heng et al., 2004). Through reprogramming using defined gene and protein factors, recent advances in stem cell biology have enabled the generation of induced pluripotent stem cell (iPSC) sources

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from somatic cell types such as skin fibroblasts that are easily accessible (Takahashi et al., 2007). This potentially allows for personalized patient-specific therapies.

The study of stem cell chondrogenesis has become increasingly relevant not only for the study of cartilage development but also for the development of useful differentiation strategies to obtain sufficient numbers of stem cell-derived chondrocytes needed for transplantation. Of note, the regulation of chondrogenesis, particularly hypertrophy maturation, is becoming increasingly relevant to clinical applications for articular cartilage repair, as well as screening of drugs for treatment of hypertrophic osteoarthritis.

Owing to the vast differentiation capability of stem cells, in particular pluripotent stem cells (ESCs and iPSCs), controlled differentiation of stem cells into the committed functional chondrocytes has been a major challenge. The development of efficient differentiation protocols aided by purification and selection strategies would reduce spontaneous differentiation of stem cells to divergent lineages other than the desired chondrogenic lineage. This is especially important for preventing the teratoma formation in the case of pluripotent stem cells, and improving the purity of functional cells for better therapeutic efficiency in cartilage repair.

Apart from directing the differentiation of stem cells into the chondrogenic lineage, the control of phenotypic stability of the cartilage formation following differentiation is also critically important. Often, differentiation factors and conditions that enhance chondrogenic differentiation of stem cells would also predispose the differentiated chondrocyte-like cells to premature hypertrophy, with terminal progression to apoptosis and replacement by bone formation in a physiological process known as endochondral ossification.

Functional chondrogenic differentiation and phenotype stability of the cartilage formation are critical factors in the clinical application of stem cells for cartilage repair. In this current review, we will discuss the latest progress in our understanding of the stem cell chondrogenesis, with the focus on various stem cell sources, induction factors including growth factors, oxygen tension and biomaterial scaffolds, and the pertinent cell-cell/matrix interactions and underlying mechanisms that regulate stem cell chondrogenesis towards stable cartilage formation.

Stem Cells

Stem cells for cartilage repair can be derived from two major sources - Multipotent adult MSCs isolated from various adult tissues, and pluripotent ESCs derived from inner cell mass of embryos.

Adult MSCs

Adult MSCs are multipotent adult stem cells present in most tissues of mature organisms serve to repair and regenerate tissues. They are capable of differentiating to different lineages including chondrocytes, osteoblasts and adipocytes, and have been identified in bone marrow (Pittenger et al., 1999; Toh et al., 2005) as well as in other tissues including adipose tissue (Zuk et al., 2001), cartilage (Koelling et al., 2009), synovium (Jones and Pei, 2012), and periosteum (Yoshimura et al., 2007). Notably, autologous bone marrow (BM)-derived MSCs are currently being evaluated in human clinical trials for its functional efficacy for cartilage repair (Nejadnik et al., 2010). Clinical evidence now exists suggesting these uses may translate into effective clinical application for cartilage repair (Nejadnik et al., 2010; Wakitani et al., 2011). With potent immunomodulatory properties, these cells also enable a much wider use in clinical applications through potential allogeneic transplantations.

Indeed, several studies have shown the ability of MSCs to potently modulate the immune and inflammatory responses, improve cell migration and angiogenesis, and prevent fibrosis by expression of specific trophic factors (da Silva Meirelles et al., 2009). As such, the role of MSCs in regenerative medicine has extended from merely tissue replacement through cell differentiation to the expression

of bioactive factors that modulate tissue and immune responses, and facilitate overall tissue regeneration. Currently, the paracrine functions of MSCs and their underlying mechanisms in tissue regeneration are under intense investigations. At this point in time, the understanding of secreted factors in the secretome by MSCs during chondrogenesis and their roles during the process is still very limited. Of note, the MSC secretome contains a complex myriad of trophic factors such as growth factors, cytokines, chemokines and microvesicles, which composition changes dynamically in response to the state of the cell and the surrounding microenvironment. Looking forward, the identification of secreted factors by MSCs is likely to provide novel insights to the autocrine/paracrine factors and mechanisms that are mediating chondrogenesis and cartilage regeneration (Toh et al., 2014).

Although adult MSCs have shown great promise for autologous and allogeneic transplantations, these cells have limited differentiation capability and suffer age-related loss of stem cell functions including proliferation and differentiation (Li and Pei, 2012). These led to the investigation of pluripotent stem cells including ESCs and iPSCs as alternative cell sources for cartilage repair.

ESCs / iPSCs

Human ESCs are pluripotent and can differentiate to cell lineages of all three germ layers (mesoderm, ectoderm, endoderm) (Thomson et al., 1998). To date, several diverse cell types have been derived from hESCs, including renal tubular cells (Li et al., 2014), endothelial cells (Rufaihah et al., 2010), chondrocytes (Toh et al., 2009), osteoblasts (Cao et al., 2005), neurons (Vazin et al., 2009), keratinocytes (Kidwai et al., 2013), and hepatocytes (Hay et al., 2008).

Recent advances in stem cell biology have also enabled the generation of iPSCs from somatic cells by defined factors (Takahashi et al., 2007). The technology to generate iPSCs overcomes the ethical concerns related to the use of hESCs, and potentially allows for personalized patient-specific therapies. Furthermore, the iPSC technology also created a new platform for study of diseases and drug screening through disease modeling that was previously not possible (Rosa et al., 2014).

Much of the current research on both ESCs and iPSCs is focused on understanding the developmental pathways and underlying molecular mechanisms in directing stem cell differentiation to a particular lineage in a well-controlled manner for use in regenerative therapies (Toh et al., 2011a). To date, there have been several approaches in directing differentiation of pluripotent stem cells to chondrogenic lineage (Toh et al., 2011a). Among all, the derivation of lineage-restricted MSCs and chondro-progenitors from ESCs and iPSCs represent the most promising approach that is devoid of teratoma formation, and have therapeutic potential for cartilage regeneration (Hwang et al., 2008; Jung et al., 2012; Toh et al., 2010). Study by Toh et al. demonstrated the use of hESC-derived chondrogenic cells embedded in hyaluronic acid hydrogels for repair of osteochondral defects in rats. In that study, the implanted cartilage constructs regenerated the osteochondral defects by undergoing an orderly remodeling process to form a hyaline cartilage layer with underlying subchondral bone by the end of 12 weeks. Notably, no teratoma was observed throughout the course of study (Toh et al., 2010).

In order to better translate hESC and iPSCs into effective cartilage therapies, a standardized differentiation protocol with highly reproducible differentiation efficiency would be required. Also, the safety concerns related to the tumorigenicity of hESCs and iPSCs and immunogenicity of hESCs need to be addressed through more long-term preclinical studies in animal models. With demonstration of safety, functionality and efficacy, MSCs and progenitor cells derived from ESCs and iPSCs may potentially serve as the off-the-shelf readily available source of cells for cartilage repair.

Microenvironmental Control of Stem Cell Chondrogenesis

Conventional system for *in vitro* stem cell chondrogenesis employs the pellet culture, where pellets comprising between 200,000 and 500,000 cells are subjected to chondrogenic induction with a basal medium containing a cocktail of dexamethasone, ascorbic acid, insulin, transferrin and selenous acid. Transforming growth factor (TGF)-β (TGF-β1, 2 and 3) are the well-established inducers of chondrogenesis that lead to cartilage formation with deposition of sulfated glycosaminoglycans (s-GAG) and collagen II. The pellet culture system recapitulates mesenchymal condensation with cell-cell interactions required for chondrogenesis (Toh et al., 2013). However, increasing evidence is suggesting that this conventional culture system with TGF-β may not be sufficient to maintain the phenotypic stability of the cartilage formation, and more complex cues are required to enhance stem cell chondrogenesis towards stable cartilage formation (Toh et al., 2011b).

With advances in material science and bioengineering, it is becoming evident that other factors including cell-matrix interactions and oxygen tension also exert significant influence on stem cell chondrogenesis. Here, we discuss the varying chondrogenic capacity of different stem cell sources, and the distinct roles and interplay of soluble factors, oxygen tension, cell-cell and cell-matrix interactions in the complex microenvironment that regulates stem cell chondrogenesis and phenotypic stability of the cartilage formation.

Source of Stem Cells

Depending on the tissue origin of derivation, stage of development and differentiation capability, the regulation of stem cell chondrogenesis can be quite different among various stem cell sources.

Adult MSCs from different tissue origins also exhibit differential potency in proliferation and differentiation. In several studies, synovium-derived MSCs reportedly demonstrated superiority in proliferation and differentiation to chondrocytes (Jones and Pei, 2012). In view of the intrinsic differences among the different stem cell sources, detailed investigation and comparison of the different stem cell sources in their chondrogenic potential would be required.

However, it seems to be a common problem in adult MSCs that with increase in chondrogenic potential with expression of collagen II and s-GAG, there is also a concomitant increase in hypertrophy with expression of collagen X, metalloproteinase (MMP)-13 and alkaline phosphatase (ALP), in the conventional pellet culture with TGF-β, as the major inducer of chondrogenesis.

Due very much to differences in origin, stage of development and differentiation capability, response of hESCs to chondrogenic factors differs quite significantly from adult MSCs. Of note, it has been shown that early application of transforming growth factor (TGF)-β1 inhibited chondrogenic differentiation of hESCs, and only enhanced chondrogenic differentiation following embryoid body formation (Toh et al., 2007; Yang et al., 2009). Even so, it has been shown that application of BMP-2 on human embryoid bodies in monolayer culture resulted in differentiation towards endodermal cell fate, and only enhanced chondrogenic differentiation in micromass culture (Toh et al., 2007). Conversely, BMPs and TGF-β have been commonly used in early stages of chondrogenic induction of adult MSCs (Hennig et al., 2007; Toh et al., 2005). From these observations, it is obvious that ESC/iPSC chondrogenesis is very different from adult MSC chondrogenesis, due very much to the distinct developmental differences, and possibly differential growth factor response.

In order to improve the efficiency of ESC/iPSC chondrogenesis, several strategies have been employed which include the derivation of MSCs and progenitor cells by either pre-sorting of specific cell population for further expansion and/or direct culture under selective culture conditions (Liu et al., 2009). Notably, Umeda et al. reported the successful isolation of paraxial mesoderm-like cells from human iPSCs under chemically defined medium conditions. These cells express platelet-derived growth factor receptor-α (PDGFR-α) but not vascular endothelial growth factor receptor (KDR). When treated

with sequential addition of PDGF, TGF-β3 and BMP-4, these isolated $KDR^{-}PDGFR\alpha^{+}$ paraxial mesoderm cells demonstrated superior chondrogenic response, without any sign of hypertrophy following 24 days of *in vitro* differentiation (Umeda et al., 2012). Looking forward, hESC/iPSCs may offer opportunities as the ideal cell source for derivation of chondrocytes that are phenotypically stable for cartilage regeneration.

Although there are intrinsic differences among the different stem cell sources in their chondrogenic capacity, recent studies have suggested the roles of biochemical and biophysical factors in the microenvironment in modulation of stem cell chondrogenesis.

Growth Factors

Growth factors played a pivotal role in cellular signaling through interactions with respective growth factor receptors. Early studies have compared the chondrogenic differentiation capacity of adult BM-derived MSCs and adipose tissue (AT)-derived MSCs, and demonstrated superior chondrogenesis of BM-derived MSCs (Afizah et al., 2007; Liu et al., 2007). This could be explained in part by the reduced endogenous expression of BMP-2, 4 and 6 mRNAs and absence in expression of TGF-β-receptor I in adipose-derived MSCs. This reduced chondrogenic potential of AT-derived MSCs was rescued by BMP-6 treatment that induced TGF-β-receptor I expression and reversed by combined application of TGF-β and BMP-6, that induced a gene expression profile similar to that of the differentiated BM-derived MSCs (Hennig et al., 2007). Similarly, Brown et al. compared the chondrogenic capacity of MSCs derived from hESCs and adult bone marrow, and showed that the hESC-derived MSCs have lower chondrogenic potential for cartilage formation, when treated with TGF-β1 alone (Brown et al., 2014). However, hESC-derived MSCs can be effectively induced as BM-derived MSCs for chondrogenesis, when treated with TGF-β1 and BMP-7 in combination. These studies reflect the differences in growth factor requirements for chondrogenic induction of stem cells from different sources which can be attributed to the intrinsic differences in growth factor receptor repertoire expressed by the cells (Handorf and Li, 2014; Hennig et al., 2007). Undoubtedly, growth factor modulation is a potent approach to alter the chondrogenic potential of different stem cell sources.

Often, inducers that are more pro-chondrogenic also inevitably enhances hypertrophy maturation. It has been shown that several growth factors including BMPs and insulin growth factor (IGF) synergize with TGF-β1 to enhance adult MSC chondrogenesis towards hyaline cartilage formation, but there is also increased propensity towards hypertrophy. Accordingly, hypertrophy maturation is regulated by Indian Hedgehog (Ihh)/parathyroid hormone related protein (PTHrP), Wingless (Wnt)-β-catenin, FGF and BMP pathways. Major efforts are underway to dissect the interplay of growth factors that controls the rate and progression of chondrogenesis, and to determine the optimal growth factor combination to promote stem cell chondrogenesis towards stable cartilage formation. Of interest, PTHrP and FGF-2 were shown to be potent inhibitors of hypertrophic progression of TGF-β3 pre-differentiated MSC pellets *in vitro*. However, these pellets were not able to resist subsequent calcification after 4-week transplantation period in the ectopic model (Weiss et al., 2010). Separately, it is recently shown that the temporal regulation of Wnt/β-catenin signaling influences the hypertrophic maturation of TGF-β-induced MSC chondrogenesis (Yang et al., 2011). In that study, continuous co-treatment of pellets with TGF-β3 and the Wnt activator, lithium chloride (LiCl) resulted in inhibition of progression to hypertrophy. By contrast, transient co-treatment with TGF-β3 and LiCl at early stage of chondrogenesis resulted in heightened hypertrophy and subsequent calcification after subcutaneous implantation.

With more sources of stem cells being identified and isolated from different tissues, mapping of their growth factor and receptor repertoire would pave forward optimal chondrogenesis of individual source of stem cells for cartilage repair. Timely application of the growth factors at the optimal concentration, combination and exposure, in relationship to the expression of the

corresponding growth factor receptors, would need to be determined to enhance stem cell chondrogenesis towards stable cartilage formation.

Oxygen Tension

Apart from soluble factors, biophysical stimulation including oxygen (O_2) tension has also been reported to modulate chondrogenesis. It is generally agreed that hypoxia culture condition (ranged 2-5% O_2) during expansion enhances MSC clonogenicity and expression of stemness genes including *Oct-4* and *Rex-1* (Grayson et al., 2006), as well as modulates the secretion of growth factors and cytokines by these cells (Chang et al., 2013). However, at this point in time, the role of hypoxia preconditioning during MSC expansion on subsequent chondrogenesis is less clear and has yielded conflicting results. Notably, it has been shown in separate studies that hypoxia preconditioning may enhance or impair subsequent MSC chondrogenesis (Adesida et al., 2012; Boyette et al., 2014), which necessitates further research to confirm the findings.

On the other hand, application of hypoxia during chondrogenesis seems to yield more consistent outcomes of enhanced chondrogenic gene expression and matrix deposition in various sources of MSCs including BM-derived MSCs and AT-derived MSCs (Munir et al., 2014), as well as in chondrocytes (Foldager et al., 2011). This underscores the role of hypoxia during chondrogenesis, mediated largely by hypoxia-inducible factor (HIF)-1α and -2α. Of note, HIF-1α was shown to enhance MSC chondrogenesis *via* interaction with the Sox9 promoter (Robins et al., 2005). Similarly, hypoxia enhanced chondrogenic differentiation of MSCs with collagen II and s-GAG upregulation *via* AKT and p38 MAPK (mitogen activated protein kinase) pathways (Kanichai et al., 2008). In addition to the chondrogenic effects, hypoxia also exerts anti-hypertrophy and anti-catabolic effects on MSCs and chondrocytes, with downregulated expression of collagen X, ALP and MMP-13 (Gawlitta et al., 2012; Strobel et al., 2010). However, at this time in point, the interplay and underlying mechanisms of HIF-1α and HIF-2α in the catabolic regulation of chondrogenesis leading to hypertrophy are still unclear.

Understanding of the role of hypoxia in stem cell chondrogenesis is still relatively limited. There exist conflicts in results from different investigators and these most likely stem from the differences in donor source, cell type, culture method, the stringency of hypoxia control, as well as how differentiation is assessed. Looking forward, further research would be required to dissect the role of hypoxia at various stages of chondrogenesis from MSC expansion prior to differentiation to chondrogenic differentiation and hypertrophy maturation. Combination of hypoxia with other approaches using growth factors and biomaterials may represent a well-rounded approach to drive stem cell chondrogenesis towards stable cartilage formation.

Co-culture and Cell-Cell Interactions

Co-culture studies of articular chondrocytes and MSCs have provided evidence that chondrocytes enhanced MSC chondrogenesis through cell-cell interactions by means of direct cell contact and/or by secretion of morphogenetic factors in the conditioned medium that are still yet to be fully characterized. As described in a number of studies (Hubka et al., 2014), MSCs and articular chondrocytes in direct contact pellet and scaffold co-cultures displayed improved chondrogenesis with higher gene expression and synthesis of cartilage matrix proteins than either cell type in monoculture. Further studies also provided evidence that co-culture of MSCs with chondrocytes improved chondrogenesis in both pellets (Acharya et al., 2012) and hydrogels (Bian et al., 2010) with inhibition of further progression to hypertrophy, by action of PTHrP secreted by the chondrocytes (Fischer et al., 2010).

Apart from chondrocytes enhancing MSC chondrogenesis, recent studies have also suggested trophic effects of MSCs on chondrocyte matrix synthesis and functions. However, the results obtained from these studies at this point in time are still conflicting and

warrants further investigation. For instance, it was found that BM-MSCs exert trophic effects in chondrogenesis by promoting proliferation and ECM deposition of chondrocytes (Wu et al., 2011), and MSCs from different tissue sources including synovial membrane, bone marrow and adipose tissue seem to exert these trophic effects, irrespective of the tissue origins (Wu et al., 2012). On the other hand, there are also studies describing the inhibitory effects of MSC trophic factors on chondrocytes, where MSCs were observed to downregulate chondrocyte differentiation and matrix deposition (Lee et al., 2012; Xu et al., 2013).

Moving ahead, delineating the cell-cell interactions between chondrocytes and MSCs are likely to shed light on the autocrine/paracrine factors and mechanisms that are mediating chondrogenesis and cartilage regeneration.

Biomaterials and Cell-Matrix Interactions

Biomaterials serve an important role as the synthetic extracellular matrix (scaffold) to enhance stem cell chondrogenesis through cell-matrix interactions. To date, a wide range of natural and synthetic polymers have been investigated as scaffolds for cartilage tissue engineering (Chung and Burdick, 2008). Natural polymers include collagen (Lee et al., 2003), gelatin (Wang et al., 2014), hyaluronic acid (HA) (Toh et al., 2012), and many more. Natural polymers mimic the natural ECM found within the body and can often interact with cells through surface receptors to influence cell fate and functions. For example, HA has been shown to modulate stem cell chondrogenesis through CD44 interaction, and may also influence cell aggregation to foster cell-cell interactions (Huang et al., 2011; Wu et al., 2013). Synthetic polymers commonly used for cartilage tissue engineering include polyethylene glycol (PEG) (Williams et al., 2003), poly (propylene) fumarates (Fisher et al., 2004; Lim et al., 2013), and polyurethanes (Werkmeister et al., 2010). Synthetic polymers are more controllable and predictable than the natural polymers, but require additional modification to enhance their biocompatibility and bioactivity. In this instance, specific ligands may be incorporated into these synthetic matrices to confer properties of cell adhesion and degradation, therefore allowing cellular infiltration and matrix remodeling for tissue formation.

Advances in material science and engineering have enabled design of biomaterial scaffolds with incorporation of select cues in the microenvironment to influence stem cell chondrogenesis (Toh et al., 2011b). These signaling cues may range from biophysical cues (*i.e.* scaffold architecture, geometric and mechanical cues) to biochemical cues (*i.e.* adhesive motifs and soluble cues). Readers are referred to several recent reviews in this area (Chung and Burdick, 2008; Toh and Loh, 2014). Notably, it is recently shown that the crosslinking degree and the stiffness of the scaffold could modulate the differentiation of MSCs into formation of different types of cartilage by controlling the extent of mesenchymal condensation during chondrogenesis. In that study (Toh et al., 2012), lower cross-linked HA matrix enhanced chondrogenesis with increases in the percentage of cells with chondrocytic morphology, biosynthetic rates of s-GAG and collagen II, and hyaline cartilage tissue formation. With increasing cross-linking degree and matrix stiffness, a shift in MSC differentiation towards fibrous phenotypes with the formation of fibrocartilage and fibrous tissues was observed. Separately, it is also recently demonstrated that conjugation of N-cadherin mimetic peptides onto HA hydrogels promoted early MSC chondrogenesis by facilitating mesenchymal condensation through cell-cell interactions, although the effects on subsequent hypertrophy are less clear (Bian et al., 2013). Notably, chondrogenesis and subsequent hypertrophy may also be related to the cell degradability of the material system. It was found that hMSCs seeded in MMP-sensitive hydrogels exhibited increased cell spreading and better chondrogenesis with lesser hypertrophy, compared to those seeded in MMP-insensitive hydrogels (Feng et al., 2014).

Looking forward, biomaterials science is likely to provide innovative platforms for better control of stem cell chondrogenesis towards stable cartilage formation for cartilage tissue engineering and regeneration.

Conclusions and Future Perspectives

Proper control of stem cell chondrogenesis and hypertrophy maturation is necessary for stable cartilage formation. In this progress update, we have discussed the use of the various sources of stem cells, induction factors including growth factors, oxygen tension and biomaterial scaffolds, and some of the pertinent cell-cell/matrix interactions that regulate stem cell chondrogenesis towards a stable cartilage formation.

At present, there exist significant challenges in combining the above-described biochemical and biophysical factors in a well-concerted and timely-coordinated fashion to control stem cell chondrogenesis. The elucidation of the complex network of molecular factors and signaling pathways would likely require more sophisticated biomaterial and microfluidics systems.

Moving ahead, with the emerging use of advanced biomaterial systems and microtechnologies, a deeper understanding of the cellular microenvironment and the pertinent cell-cell/matrix interactions that regulate stem cell chondrogenesis will likely advance our understanding and tools for specific induction of chondrogenesis towards a stable cartilage formation for long-lasting cartilage regeneration.

Abbreviations

MSCs: Mesenchymal stem cells; ESCs: Embryonic stem cells; iPSCs: induced pluripotent stem cells; BM: Bone marrow; AT: Adipose tissue; TGF: transforming growth factor; BMP: bone morphogenetic protein; IGF: insulin growth factor; FGF: fibroblast growth factor; Ihh: Indian hedgehog; PTHrP: parathyroid hormone related protein; LiCl: lithium chloride; s-GAG: sulfated glycosaminoglycans; MMP: metalloproteinase; ALP: alkaline phosphatase; PDGFR: platelet-derived growth factor receptor; HIF: hypoxia-inducible factor; HA: Hyaluronic acid; PEG: polyethylene glycol.

Competing interests

The author declares that he has no competing interests.

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