Curr Mol Bio Rep (2017) 3:276–287
https://doi.org/10.1007/s40610-017-0082-6

Advances in Adult Stem Cell Differentiation and Cellular Reprogramming to Enhance Chondrogenesis

Saliya Gurusinghe[1] · **Nadeeka Bandara**[2] · **Padraig Strappe**[3]

Published online: 28 October 2017

Abstract
Purpose of Review Cell-based therapies to treat articular cartilage and osteochondral defects as a result of osteoarthritis or traumatic injury are a promising approach. Traditional sources of cells have been autologous chondrocytes which are culture expanded and implanted; however, dedifferentiation of these cells results in a type of fibrocartilage which has reduced therapeutic benefit. Advances in cellular reprogramming technology are either through generation of induced pluripotent stem cells (iPSCs) and subsequent chondrogenic or through direct reprogramming of adult cells to chondrocytes. These approaches have the potential to provide an unlimited source of cartilage for therapeutic applications; however, challenges remain in terms of efficient cellular differentiation and ability to integrate and repair tissues.
Recent Findings Growth factor-based strategies previously used in chondrogenic differentiation of adult stem cells and embryonic stem cells have been successfully applied to induced pluripotent stem cells, enhancing the ability of iPSCs to produce both patient-specific chondrocytes and to produce large quantities of these cells. A combination of novel biomaterials and additive bioprinting have also opened new approaches to recapitulate zonal cartilage structure and repair of osteochondral defects.
Summary The development of innovative protocols to generate chondrocytes from a variety of primary cells continues to proceed rapidly, allowing fine tuning of differentiation processes to produce an articular cartilage phenotype with improved mechanical and tissue integration capabilities.

Keyword Induced pluripotent stem cells · Osteoarthritis · Fibrocartilage · Adult stem cells · Chondrogenesis

Introduction

Bone, cartilage, tendons and ligaments make the basic component of the skeletal system that enables humans and other animals to retain structural form and function. Of these, cartilage acts as a template in skeletogenesis during embryonic and neonatal development [1]. In the adult, articular cartilage tissue lines the ends of long bones reducing frictional forces while maximising shock absorption. It is a highly specialized tissue consisting of chondrocytes and the extracellular matrix (ECM) secreted by the chondrocytes, which is a network of mainly type II collagen and proteoglycans giving cartilage its strength, unique shape and resistance to torsion [2].

Cartilage lesions lead to joint degeneration and diseases such as osteoarthritis (OA) [3]. The self-healing capacity of articular cartilage is reduced when damaged, due to the lack of a vasculature and poor wound-healing response from localized progenitors [4]. OA is the leading cause of pain and disability among the elderly population, and those affected amount to three million in Australia, approximately 15% of the population, adding an approximated 40,000 cases of osteoarthritic sufferers annually [5]. The high prevalence of the illness in society impacts the economy significantly with age,

This article is part of the Topical Collection on *Molecular Biology of Skeletal Tissue Engineering*

✉ Padraig Strappe
p.strappe@cqu.edu.au

[1] Graham Centre for Agricultural Innovation, Charles Sturt University, Boorooma St, Wagga Wagga, NSW 2678, Australia

[2] OBI Department, St Vincent's Institute of Medical Research, Melbourne, VIC 3065, Australia

[3] School of Health, Medical and Applied Sciences, Central Queensland University, Rockhampton, QLD 4701, Australia

genetic predisposition and injury considered as the major risk factors associated with the epidemiology of the disease. Current treatment strategies involve encouragement of bone marrow stimulation, in which subchondral bone is accessed by micro-fracture to encourage bone marrow-derived cells and cytokines to migrate to the site of injury through multiple perforations or abrasion [6]. These techniques usually result in the formation of fibrocartilage that is biochemically and biomechanically inferior to articular cartilage and increase the risk of tissue degradation [7, 8].

Autologous chondrocyte implantation (ACI) was the first cell therapy applied to cartilage repair where an implantation of an autologous periosteal flap was carried out, with the insertion of culture-expanded articular chondrocytes [9]. Two years post-implantation, the majority of the implantations were successful, yielding pain-free and operational knee joints. However, in a few patients, the development of fibrous cartilage required trimming and a second operation. The main disadvantages observed in ACI were donor site morbidity and the need for in vitro expansion of chondrocytes limiting their therapeutic potential. Poor proliferation and reduced ECM production by culture-expanded chondrocytes reduced successful integration of the transplanted cells [10].

Dedifferentiation of Chondrocytes

Articular cartilage homeostasis is maintained by the interaction of three main components: chondrocytes, ECM and growth factors [11]. Mechanical stimuli such as stretch and directional load also effects articular cartilage structure [12–14]. Chondrocytes secrete components of the ECM and maintain higher ratios of the different types of collagen such as collagen type II:type I and collagen type II:type X [15]. However, during the progression of osteoarthritis (OA) or during monolayer expansion of chondrocytes for ACI, this interaction is lost and the chondrocyte phenotype drastically changes [16]. This is referred to as chondrocyte dedifferentiation.

Previous work has shown that monolayer expansion of chondrocytes in two-dimensional culture results in dedifferentiation within a few passages [17]. Two-dimensional culture disrupts the extracellular three-dimensional ECM-laden natural scaffolds that chondrocytes reside in and lose cell-ECM signals via intermembraneous receptors known as integrins [11]. Cell-ECM commnication is maintained via many integrins, of which some are known to be associated with collagen type I, collagen type II and fibronectin [18, 19]. Specifically, the $\alpha 5\beta 1$ fibrinectin receptor expression has been shown to suppress chondrocyte differentiation when chondrocytes were cultured as a monolayers by promoting cell proliferation while inhibiting chondrocyte specific gene expression [20, 21].

Cell-cell and cell-ECM communication in chondrocytes is also carried out by growth factors. In particular, it has been shown that members of the transforming growth factor β (TGFβ) superfamily of growth factors are affected during chondrocyte differentiation. mRNA expression of TGFβ-2 has been shown to decrease significantly during monolayer expansion of human chondrocytes [22], while a significant reduction has been reported in expression of insulin-like growth factor 1 (IGF-1) during monolayer expansion of human chondrocytes [23]. The expression of the major chondrocyte-specific transcription factor Sox9, and concurrently, the expression of collagen II also decreases significantly during chondrocyte dedifferentiation [24].

Another aspect of chondrocyte dedifferentiation is the effect of catabolic gene expression encoding inflammatory cytokines and proteases. In particular, interleukin 1 β (IL-1β) expression increases in dedifferentiated chondrocytes and contributes to the dedifferentiated phenotype [25]. Its expression was shown to reduce *Col2a1* expression and also enhance the expression of *Col1a1* and *Col3a1* mRNA transripts. It has been shown that IL-1β elicits this result through the activation of canonical Wnt signalling and activation of β-catenin [26]. It has also been shown to degrade articular cartilage through the stimulation of nitric oxide (NO) which enhances extracellular signal-regulated kinase (ERK-1/2) [27]. Furthermore, IL-6 has also been shown to inhibit aggrecan expression through activation of the Notch signalling pathway [28].

Proteolytic enzymes such as matrix metalloprotease (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are both involved in cartilage degradation [29]. The expression of MMP-1, 3, 7, 8, 9 and 14 mRNA has been shown to increase during chondrocyte dedifferentiation [23, 30]. Also, MMP-13 has been shown to cleave collagen type II during osteoarthritis and express strongly in dedifferentiated chondrocytes [31, 32]. ADAMTS-4 and 5 have been shown to specifically cleave aggrecan and do not cleave other ECM components such as type II or type I collagen [33–35].

Strategies for Chondrocyte Redifferentiation

As chondrocyte senescence and dedifferentiation are major roadblocks in the application of chondrocytes for the treatement of cartilage defects, many studies have focused on enhancing the proliferation and maintainence of the chondrogenic phenotype. A number of studies have shown the effects of growth factors to enhance the chondrocyte phenotype of cultured chondrocytes. Jakob [36] and others demonstrated that epidermal growth factor (EGF), platelet-derived growth factor (PDGFbb), fibroblast growth factor (FGF)-2 and TGFβ-1 treatment in monolayer-expanded chondrocytes enhanced proliferation while also increasing dedifferentiation

characteristics. However, three-dimensional pellet culture of the cells with growth factor treatment regained the chondrocyte phenotype with strong expression of GAG and specifically collagen type II. Olney and co-workers [37] also showed that growth plate chondrocyte proliferation was increased following the addition of IGF-1, TGFβ1-3 and FGF-2 in monolayer culture while BMP 2, 4 and 6 showed an inhibitory effect on chondrocyte proliferation.

Recent studies involving growth factor-mediated redifferentiation of chondrocytes heavily utilize three-dimensional scaffolds to promote the chondrocyte phenotype by increasing cell-cell and cell-ECM interactions. Human chondrocytes from osteoarthritic cartilage have been shown to both proliferate and redifferentiate with a growth factor combination of TGFβ-1, FGF-2 and PDGFbb when cultured with horse serum and stimulated by three-dimensional culture in alginate beads [38]. Dahlin and others [39] demonstrated that the use of a hypoxic culture system with 5% O_2 and a porous, electro spun poly (ε-caprolactone; PCL) scaffold enhanced chondrogenic redifferentiation of bovine articular chondrocytes while also increasing cell proliferation. They also utilized a flow perfusion bioreactor to enable uniform flow of culture media, which further contributed to chondrocyte redifferentiation through constant exposure to the growth factors and nutrients.

Stem cells have been suggested as an alternative source of cells for the treatment of degenerative diseases with unique characteristics in comparison to differentiated cells. They are unspecialized cells capable of self-renewal through indefinite cell division, while maintaining their differentiation potential [40]. They are also capable of differentiation into cells of particular lineages under certain physiological and chemical conditions. Three different types of stem cells have been described in the literature: embryonic stem cells, umbilical cord blood or placenta-derived stem cells, and adult or somatic stem cells [41].

Embryonic Stem Cells

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst and can proliferate indefinitely, in an undifferentiated state while being able to differentiate into any cell type of the developing body or into extra-embryonic tissues, in vivo, referred to as totipotency [42]. In particular, they have been shown to differentiate to end-stage cell types of the mesoderm including chondrocytes [43, 44], osteoblasts [45–47] and adipocytes [48, 49]. This versatility of ES cells to produce many cell types makes them an ideal source of cells for use in tissue engineering applications. However, the sourcing and use of ES cells has many ethical considerations and is strictly regulated [50]. Furthermore, they have also been shown to result in teratoma formation following in vivo transplantation of undifferentiated ES cells in mouse knee joints where site-specific differentiation of ES cells to osteoblast was expected [51]. Furthermore, human ES cells have displayed genomic instability over multiple population doublings resulting in amplification of some genes while having no effect on others making tissue-specific differentiation of stem cells difficult [52].

Induced Pluripotent Stem Cells

Takahashi and Yamanaka demonstrated for the first time that ectopic expression of the transcription factors Oct3/4, Sox2, c-Myc and Klf4 (OSKM) reprogrammed mouse embryonic fibroblasts and mouse adult fibroblasts, to exhibit ES cell morphology and growth characteristics [50]. The reprogrammed cells were named induced pluripotent stem (iPS) cells. Following the success with mouse iPS cell generation, it was extended to adult human fibroblasts where the iPS cells derived were described as having similar growth and differentiation characteristics to human ES cells [53]. Importantly, it was shown that the transgene expression was silenced following full reprogramming of the donor cells to iPS cells. An important consideration in these early experiments was the possibility of reactivation of the oncogene c-Myc which may lead to tumour formation. However, Nakagawa et al. showed that iPS cells could be derived by the expression of only Oct3/4, Sox2 and Klf4 (OSK) [54]. Omitting c-Myc resulted in a significantly lower number of reprogrammed colonies, but stopped the tumour incidences in chimeric mice relative to the chimeric mice derived with the four original factors.

Since their discovery, iPS cells have been derived from monkey [55], dog [56], horse [57, 58], swine [59, 60] etc. Alternative methods for delivering transgenes for iPS generation have been studied with the central aim of either controlling integrated transgene expression or using non-integrating vectors. To this end, iPS cells have been derived using a doxycycline-driven lentiviral Tet-On system, transient plasmid transfection [61, 62], adenovirus [63, 64], mRNA [65] and protein delivery of the four pluripotency factors [66].

Adult Stem Cells

As an alternative to ES cells, adult mesenchymal stem cells (MSCs) have become popular in recent years for derivation of tissue-specific cells of the mesoderm [67]. The relative ease of isolation, efficient in vitro culture expansion and multipotency signal their importance as an alternative source of cells [68, 69]. They have been shown to successfully differentiate into chondrocytes, adipocytes and osteoblasts while the differentiation potential is also a requirement for the characterisation of the cell type as a mesenchymal stem cell [70]. Functional

niches of adult stem cells fulfil the role of regenerating damaged or diseased tissue throughout the lifetime of an individual [71]. However, this self-healing capacity reduces with age. While the differentiation potential of MSCs is limited when compared to ES cells, as an allogenic cell source, it shows great potential for immunosuppression [72, 73]. However, the maintainence of the immunosuppressive qualities in vivo is debatable with a recent study showing host immune reactivity following the chondrogenic differentiation of transplanted allogeneic MSCs in a rat model [74].

Sources of Mesnchymal Stem Cells and Effect of Ageing on the Differentiation Potential

MSCs were first isolated from the bone marrow by Friedenstein et al. and referred to as colony-forming unit-fibroblasts (CFU-Fs) [75]. Other tissues have also been identified to contain niches of MSCs. MSCs from 'adipose, periosteum, synovial membrane, synovial fluid, muscle, dermis, deciduous teeth, pericytes, trabecular bone, infra-patella fat pad, articular cartilage and umbilical cord' have been successfully isolated from all of these tissues [76]. However, the source of MSC has been shown to play an important role in their differentiation potential under identical culture conditions. As such, a comparison between the differentiation potential in bone marrow mesenchymal stem cells, umbilical cord blood-derived stem cells and adipose-derived stem cells revealed that umbilical cord blood-derived stem cells could not differentiate towards adipocytes whereas the stem cells from the bone marrow and adipose demonstrated differentiation potential to all three lineages [77]. Importantly, it was noted that the stem cell population was heterogeneous and that some colony-forming units were only able to differentiate to one or two of the lineages. Apart from the source of the stem cells, the age of the donor and the passage used for differentiation have a strong influence on the differentiation potential. Kretlow et al. demonstrated that cell attachment and proliferation decrease with the increasing age in mouse bone marrow-derived stem cells [78]. They also showed that the differentiation potential of the isolated cells to adipocytes, osteoblasts and chondrocytes decreases dramatically with age and passage. This observation has been supported in recent experiments using in vitro differentiating human bone marrow-derived MSC [79] and human adipose-derived stem cells [80].

Immunophenotype and Characterisation of MSC

In terms of standardising MSCs across the many tissue sources, the international society of cell therapy (ISCT) requires the fulfilment of the following criteria for the characterisation of multipotent mesenchymal stromal cells: '(a) adherence to plastic (b) specific surface antigen (Ag) expression (c) multipotent differentiation potential' [81]. Furthermore, it is required that the expression of cell surface markers, CD105, CD73 and CD90, is required in 95% or more of the cell population. The standard also states that the cell surface markers CD45, CD34, CD14 or CD11b, CD79α or CD 19 and HLA class II should not be expressed in over 2% of cells. Thirdly, the mesenchymal cells need to be capable of differentiation to osteoblasts, adipocytes and chondrocytes under standard in vitro differentiation conditions. Interestingly, greater understanding of the immunophenotype of MSC allows phenotypical separation of chondrocytes from MSC, although they show striking similarity in morphology and growth characteristics during monolayer culture. Diaz-Romero and others showed that the immunophenotype of chondrocytes and particularly dedifferentiated chondrocytes varies considerably in comparison to MSC [82]. Their findings also demonstrate that dedifferentiated chondrocytes have significantly different cell surface antigen presentation to those of MSC and primary chondrocytes. The study suggests CD14 and CD90 as indicators of distinct chondrocyte phenotype and state of dedifferentiation, respectively, as CD90 expression is significantly upregulated following monolayer expansion of chondrocytes whereas CD14 expression is not evident in MSC whereas it is strongly expressed in primary chondrocytes.

Chondrogenic Differentiation of MSC

Chondrogenesis of MSCs in vivo involves the chondrogenic lineage commitment of cells, aggregation of committed cells and differentiation to mature chondrocytes, with further maturation leading to chondrocyte hypertrophy and matrix mineralisation [83]. Figure 1 shows the stages involved in the expression of site-specific ECM proteins during chondrogenesis. The in vitro differentiation of MSCs to chondrocytes involves the proliferation of sufficient numbers of cells that can be used in a three-dimensional, high-density cell culture system increasing cell-cell interaction, in the presence of growth factors [85].

Previous studies have shown efficient chondrogenesis of mesenchymal stem cells with growth factor stimulation. As such, TGFβ-2 and TGFβ-3 [86], and TGFβ-3 and IGF-1 [87, 88] have been shown to possess chondroinductive properties. Similarly, accelarated chondrogenic differentiation of adipose-derived stem cells has been shown with the use of the mitogenic factor fibroblast growth factor (FGF)-2 [89, 90], BMP6 [91], TGFβ-2 and IGF-1 [92] and BMP2 and IGF-1 [93]. Recent studies have investigated the combination of growth factor supplementation with culture in three-dimensional scaffolds, both natural and synthetic. The main advantages noted were accelarated cell proliferation and

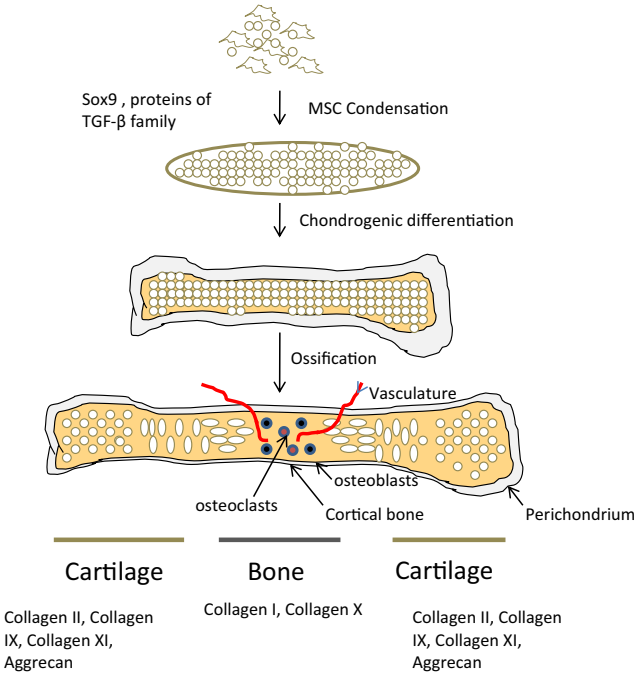

Fig. 1 Chondrogenic differentiation of MSCs and endochondral ossification. MSC condensation initiates chondrogenic differentiation followed by chondrocyte maturation resulting in ECM synthesis, predominantly with collagen types II, VI, IX and XI and aggrecan. Further maturation of chondrocytes leads to chondrocyte hypertrophy, laying the template for endochondral ossification. The predominant collagens secreted change to types I and X upon maturation. Adapted from Woods et al. 2007 [84]

chondrogenic differentiation potential due to targeted increase in collagen type II expression while suppressing collagen type I expression [94–96]. These studies have shown great potential for the use of adult stem cells in cartilage tissue regeneration. However, some previous reports comparing growth factor-mediated chondrogenic differentiation have highlighted that cartilage tissue derived from MSCs was inferior with regard to articular cartilage-specific collagen II production when compared to that from redifferentiated chondrocytes [97, 98].

Co-culture of chondrocytes and MSCs has been shown to increase the differentiation potential of MSCs while suppressing chondrocyte differentiation and hypertrophy. A previous study showed that co-culture of MSCs with chondrocytes in a methacrylated hyaluronic acid hydrogel enhanced the chondrogenic potential of the MSCs while suppressing chondrocyte hypertrophy [99]. Utilizing a PCL scaffold for three-dimensional culture, Meretoja and others showed a similar outcome in co-cultures of MSCs and chondrocytes from bovine and rabbit species with TGFβ-3 inclusion [100].

Cellular Reprogramming

In OA, the loss of cartilage integrity and the phenotypical changes in resident chondrocyte populations occur due to the reduction in growth factor production, low response to growth factors by chondrocytes and chondrocyte senescence. An earlier study showed that chondrocytes in cartilage regions closer to a lesion undergo mitotic division during OA, resulting in telomere erosion in the chondrocytes and also resulted in an increase in the expression of cartilage-degrading matrix metalloprotease (MMP) enzymes MMP8 and MMP13 throughout the joint [101]. This result demonstrates that cartilage degradation due to OA is not isolated to a distinct region, but widespread throughout the joint surface. Chondrocytes from human OA joint cartilage and culture-expanded chondrocytes have similar gene expression profiles for collagen types I and II [102]. The chondrocyte phenotype lost through dedifferentiation has been shown to recover with anabolic growth factor treatment such as transforming growth factor β (TGF β) superfamily of proteins, including bone morphogenetic protein (BMP) and TGF β, in effect regaining cartilage homeostasis. Of these, BMP2 has been shown to induce chondrogenesis through its binding to the TGFβ receptors which then activate the Smad signalling pathway [103]. The downstream effect of this cascade is the transcriptional activation of Sox9 leading to the enhancement of collagen type II expression. However, Sox9 expression also results in the enhancement of osteogenic factors such as Runx2 and the hypertrophic marker collagen type X [104]. Similarly, fibroblast growth factors (FGFs) have been used for enhancing proliferation in culture-expanded chondrocytes and for maintaining the chondrogenic phenotype. FGF-2 in particular not only is required for long-term monolayer expansion of chondrocytes but also drives hypertrophic differentiation earlier than untreated cultures [90, 105]. TGFβ-1 and 3 have been used for chondrogenic enhancement of differentiating mesenchymal stem cells [106]. We have also shown previously that TGFβ-3 inclusion during chondrocyte redifferentiation in Sox9-overexpressing canine chondrocytes had a synergistic effect with a significant increase in the chondrogenic phenotype [107].

Reprogrammed somatic cells which can recapitulate the articular chondrocyte phenotype have been suggested as an alternative to growth factor supplementation for regaining the chondrocyte characteristics. Previous work has shown that a select group of pluripotency factors Klf4, c-Myc and chondrogenic factor Sox9 differentiates mouse and human fibroblasts directly to articular chondrocytes [108, 109]. Similarly, human placental cells have also been reprogrammed directly to chondrocytes using the transcription factors BRACHYURY, c-Myc, MITF and BAF60C [110]. Such reprogrammed cells have the distinct advantage of tissue-specific gene expression leading to chondrocytes that actively suppress dedifferentiation and chondrocyte hypertrophy. In a previous study, we demonstrated that this approach is also applicable to reprogramming extensively culture-expanded canine chondrocytes to an articular chondrocyte

phenotype, with suppression of dedifferentiation and significantly lower hypertrophic differentiation when compared to chondrocytes differentiated with Sox9 overexpression alone [107].

CRISPR-Cas9 as a Tool to Investigate Chondrogenic Pathways

Gene editing is revolutionising molecular and cellular biology at a rapid pace, and applications of this technology to further understanding chondrogenic pathways and translational applications to enhancing differentiation of adult stem cells are promising.

CrispR-Cas9 has been used to generate chondrocyte cell lines with specific gene knockout (KO) results in novel phenotypic characteristics; Yang et al. established an aggrecan-specific KO through modification of the commonly used rat chondrosarcoma cell line (RCS) to stably express the Cas9 nuclease allowing subsequent editing specific guide RNAs [111]. An aggrecan KO displayed interesting in vitro characteristics, including string attachment to tissue culture plastic, and surprisingly, the loss of Ag reduced the ability of the Ag Ko cells to form a chondrosarcoma in athymic mice and the upregulation of host-infiltrating T cells suggested that lack of the aggreccan promoted immune surveillance. Comparing the gene expression profile of the Ag KO cells with WT also revealed fold changes in genes associated with cell extracellular interactions. Hyaluron (HA) plays an important role in cartilage integrity through maintaining aggrecan via interaction with the cell plasma membrane. Huang et al. generated HA-deficient chondrosarcoma cell lines through Cas9-CrispR-mediated ablation of the HA-Synthase-2 (HAS-2) enzyme [112•]. The HAS-2 KO cells showed a deficit in their ability to produce a functioning ECM, and exogenous addition of purified aggrecan failed to produce substantial matrix. Particle exclusion from matrix was also impacted upon loss of aggrecan, over expression of Has-w via an adenoviral vector restored aggrecan incorporation and yielded a similar ECM profile to wild-type cells. CrispR-Cas9 provides a rapid means of specific gene knockout which can aid in the elucidation of signalling pathways involved in chondrogenesis and highlighting the importance of key cartilage matrix proteins in KO cell lines. Clinical translation of Crispr-Cas9 in cartilage repair may be through the ability to produce engineered cells that enhance ECM production or are protected from immune-mediated destruction. The latter rational is demonstrated in a study by Brunger et al. who applied Crispr-Cas9-mediated knockout of interleukin 1 (IL-1) receptor type I in murine induced pluripotent stem cells (iPSCs) and subsequently differentiated these cells into chondrocytes as an approach to generate inflammation-resistant cells which may have enhanced survival in vivo [113••]. This study highlighted the need to achieve complete homozygous removal of the IL-R1 gene, as only these cells were completely resistant to IL-1alpha cytokine-mediated damage compared to heterozygous mutants and wild-type cells. In a similar study, the same group targeted the inflammatory cytokine receptors, TNFR1 and IL1R, in human adipose-derived stem cells (ADSCs) with CrispR/Cas9 and showed in vitro resistance to cytokine mediated damage in chondrogenic-differentiated cells which also maintained their immunomodulatory properties and ability to produce cartilage-specific ECM [114].

Induced Pluripotent Stem Cells and Cartilage Regeneration

The landmark work of Yamanaka in the generation of iPSCs has continued to provide the field of chondrogenesis and cartilage repair with powerful cell-based reagents. Efficient differentiation of iPS cells towards a chondrogenic lineage has been demonstrated in numerous studies using a variety of chemical/small molecule or gene modification techniques. Early studies revealed the potential of murine iPSC differentiation towards chondrogenic lineage using BMP-4 and dexamethasone in a 3D micromass culture [115]. Initial passage 1 cells showed significant chondrogenic gene expression in cells purified on the basis of a Col2a1-driven GFP promoter. However, subsequent monolayer expansion revealed reduced expression of chondrogenic-associated Col2a1 and Acan and increased hypertrophic Col10a1. Human iPS cells as a potential source of chondroprogenitors were investigated by Guzzo et al. [116], and using a differentiation approach combining BMP-2 and micromass culture showed the development of a heterogeneous cell population with articular and fibrocartilage characteristics. The challenges of efficient chondrogenic differentiation of iPS cells have been highlighted [117] and include formation of mesenchymal progenitors through stepwise formation of embryoid bodies and subsequent outgrowth of monolayer cells. A landmark paper by Yamashita et al. successfully differentiated human iPSCs over a period of 42 days using a cocktail of ascorbic acid, BMP-2, TGFbeta1 and GDF-5 and suspension culture [118••]. Transplantation of differentiated cells into subcutaneous spaces in SCID mice showed formation of hyaline cartilage without tumour formation together with a degree of repair of an articular cartilage defect in SCID rats and in mini-pigs. The use of cartilage-specific promoters driving expression of a reporter gene is commonly used to select differentiated iPS cells, such as Col2a1-eGFP [119] or Col11a2-eGFP [118••], and the use of potential *Col10a1* promoters may allow monitoring of hypertrophic markers. Generation of iPSCs using an integrating viral vector may leave a genetic footprint which may interfere with reprogramming; in an approach to reduce this risk, Borestrom et al. used synthetic mRNA expressing the Yamanaka factors to generate iPSCs

from donor human chondrocytes [120]. Subsequent differentiation of iPS cells to chondrocytes in a monolayer culture, led to redifferentiated chondrocytes displaying enhanced cartilage matrix characteristics similar to primary chondrocytes and superior to cells derived from fibroblasts. Articular chondrocytes are readily available from patients undergoing arthroplasty, and in a similar study, Guzzo et al. demonstrated that redifferentiation of chondrocyte-derived iPS cells resulted in an improved cartilage gene expression and proteoglycan profile compared to differentiation of fibroblast-derived iPS cells to chondrocytes [121].

Methods to differentiate embryonic stem cells (ESCs) to chondrocytes have been successfully applied to iPS cells, and Cheng et al. developed a stepwise approach to differentiate ESCs and iPS cells to mesendoderm with Activin A, Wnt3A and BMP4 followed by differentiation towards mesoderm and then chondrogenic differentiation using a cocktail of GDF5, FGF-2 and NT-4, over a total period of over 3 weeks with a substrate consisting of fibronectin and gelatin [122]. Chondrogenic differentiation of iPS cells yielded a high proportion of Sox9-positive cells together with increased Col2a1 expression. Applying a similar differentiation protocol, Lee et al. first directed human iPS cells towards mesoderm and used follistatin to suppress endodermal differentiation with subsequent chondrogenic differentiation resulted in cells with a high expression of Sox9 and Col2a1 and together with reduced expression of hypertrophic and fibrocartilage markers [123]. With loss of pluripotency gene expression, in vivo transplantation of iPS-derived chondrocytes in a PEG-chondroitin sulfate hydrogel implanted subcutaneous showed similar levels of GAG production when compared to primary chondrocytes. Teratoma detection in transplanted iPS cell-derived chondrocytes is a safety concern; Saito et al. showed the development of an immature teratoma in one of 36 NOD/SCID mice transplanted with iPS cell-derived chondrocytes following the above differentiation protocol [119]. Improvements in the safety profile of iPS cell-derived chondrocytes may also include approaches to generation of iPS cells without the use of virus-mediated vectors have included transposon-mediated delivery of the iPS cell factors by 'Piggy Bac' [124] and subsequent in vitro chondrogenic differentiation of the reprogrammed rat embryonic fibroblasts.

For successful in vivo repair, transplanted iPS cell-derived chondrocytes should not cause an immune response that would lead to targeted cell removal and an exacerbated inflammatory reaction. While an autologous source of cells would limit this possibility, allogeneic sources of chondrocytes could allow large-scale expansion of well-characterised cell banks. In a promising in vitro study by Kimura et al., iPS-derived cartilage was found to be no more antigenic than primary human cartilage showing suppression of T cell proliferation limited expression of MHC classes I and II [125].

Traditional methods to induce chondrogenesis includes 3D pellet formation which can have a profound effect on cell proliferation and ultimately gag production which impact on efficacy of transplanted cells in repairing cartilage defects. In an approach to maintain cell proliferation in differentiated murine iPS cells, the cell cycle inhibitor p12 was targeted by short hairpin RNA (shRNA) which had a profound effect on proliferation while maintaining GAG production [126].

Employing a novel viral gene delivery system for human iPS cell generation based on minicircle plasmid DNA [127] and subsequent stepwise differentiation to MSCs and chondrocytes and avoiding embryoid body formation resulted in increased GAG and Col2a1 expression and transplantation of iPS derive chondrocytes seeded within a Polyethylene-Methacrolate hydrogel into osteochondral defects of arthritic rats produced in vivo matrix production compared to undifferentiated iPS cells. Non-invasive assessment of therapeutic benefit of transplanted cells allows measurements over a time course, and in this study, a novel application of MRI was used to determine T2 relaxation times as an indicator of decreasing water content and increasing matrix formation.

Disease Modelling of Musculoskeletal Disease

Probing patient- and disease-specific changes in iPSC-derived cartilage has yielded interesting findings in a number of recent studies. Phillips et al. highlighted how results from in vitro differentiation assays may not be recapitulated in vivo, and vice versa, especially human iPS cells derived from either skin or bone marrow stromal cells which showed potent osteogenic in vitro differentiation but yielded low levels of bone formation in vivo [128]. Interestingly, patient-derived iPS cells and subsequent chondrogenic differentiation allow modelling and elucidation of cellular signalling pathways in a specific disease which may lead to improved drug screening and design. In a proof of principle study, Lee et al. demonstrated the successful generation of patient-specific iPS cells from fibroblast like synoviocytes harvested from patients suffering from rheumatoid arthritis or osteoarthritis [123]. Lentiviral vector delivery of factors resulted in iPS cells showing characteristic pluripotency markers and the ability to form tissues corresponding to developmental lineages when transplanted in vivo. Recapitulation of a disease phenotype has been shown by Yamasaki et al. who generated iPS cells from cleidocranial dysplasia (CCD)-specific patient dental pulp cells reprogrammed using a novel integration-free sendai virus vector [129•]. CCD is characterised by a missense mutation in exon 3 in Runx2 which affects chondrocyte maturation, teratoma formation of the subcutaneous transplanted cells resulted in cartilage tissue containing swollen cytoplasm and a lack of normal hypertrophic chondrocytes. Xu et al. took a similar approach in analysing cartilage tissue associated with

teratoma from subcutaneously transplanted retroviral vector generated-iPS cells from patients with familial osteochondritis disecans (FOCD) [130]. FOCD is characterised by a heterozygous mutation in the aggrecan gene, and phenotypic recapitulation of this disease was seen in the iPS-derived cartilage showing aggrecan primarily within chondrocytes and depletion within the ECM. A sparse ECM and ER stress are also characteristics of a type II collagenopathy and showed irregularities in teratoma/cartilage tissue with accumulated collagen derived from patient-specific iPS cells [131].

Enhancing iPSC-Chondrocyte Differentiation and Articular Repair with Biomaterials

A number of studies have now refined chondrocyte differentiation protocols from iPS cells which will contribute to consistency and scale up, necessary for clinical translation. Biomaterials acting as novel cellular substrates or scaffolds may contribute to differentiation and in vivo therapeutic potential. In a study by Liu et al., murine iPS cells were seeded onto an electrospun polycaprolactone/gelatin scaffold and differentiated in chondrogenic media [132]. Scaffolds were used to repair an articular cartilage defect in rabbits and showed increased matrix production and cartilage gene expression profiles compared to treatment with scaffold alone. However, an analysis of hypertrophic or fibrocartilage markers was not performed or how these cell-laden scaffolds might perform in a weight-bearing environment. Indeed, load bearing may be a synergistic approach to in vitro iPS cell differentiation together with specific growth factors. Using ESCs, McKee et al. demonstrated that cells seeded on a polydimethylsiloxane scaffold and subject to compression underwent chondrogenic differentiation with RhoA playing a pivotal role in mechanical stimulation [133].

Summary

Strategies to enhance chondrogenesis of adult stem cells have been a major focus of regenerative medicine; however, limitations have been associated with differentiation efficiency and long periods of growth factor exposure. Successful culture expansion of cells is often challenged by donor age, and increasing incidence of co-morbidities such as diabetes which can contribute to rapid cell senescence. Chondrogenic differentiation of adult stem cells in vitro has remained largely dependent on a similar set of growth factors, with increasing use of biomaterial-based scaffolds to recapitulate zonal changes in articular cartilage and the osteochondral interface while maintaining cells in a 3D growth environment. Recent advances in bioprinting [134•] and the development of cartilage-specific bioinks will contribute to enhanced in situ chondrogenic differentiation and tissue integration. Reprogramming cells to pluripotency through generation of iPS cells and then subsequent differentiation to chondrocytes shows potential in providing large quantities for therapeutic applications based on an allogeneic cell source. However, efficient in vitro differentiation is dependent on a relatively complex schedule of growth factors and stepwise progress through developmental lineages. Generation of iPS cells through reprogramming of readily accessible blood cells [135, 136] reduces the need to access tissue though invasive methods may also provide a source of chondrogenic differentiated cells for cartilage repair.

Compliance with Ethical Standards

Conflict of Interest Saliya Gurusinghe, Nadeeka Bandara, and Padraig Strappe each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent This article contains no studies with human or animal subjects performed by any of the authors.

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