Directed differentiation of induced pluripotent stem cells into chondrogenic lineages for articular cartilage treatment

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Abstract

In recent years, increases in the number of articular cartilage injuries caused by environmental factors or pathological conditions have led to a notable rise in the incidence of premature osteoarthritis. Osteoarthritis, considered a disease of civilization, is the leading cause of disability. At present, standard methods for treating damaged articular cartilage, including autologous chondrocyte implantation or microfracture, are short-term solutions with important side effects. Emerging treatments include the use of induced pluripotent stem cells, a technique that could provide a new tool for treatment of joint damage. However, research in this area is still early, and no optimal protocol for transforming induced pluripotent stem cells into chondrocytes has yet been established. Developments in our understanding of cartilage developmental biology, together with the use of modern technologies in the field of tissue engineering, provide an opportunity to create a complete functional model of articular cartilage.

Keywords

Cartilage, stem cells, regenerative medicine, osteoarthritis

Introduction

Articular cartilage (AC) is a highly specialized and organized tissue that enables painless motion in diarthrotic joints. Although cartilage cells (chondrocytes) make up only 1% of the tissue volume, their high metabolic activity provides the unique composition of extracellular matrix (ECM). Major components of the ECM are collagen fibres (approximately 15%) and proteoglycans (about 12%). The principal collagen in AC is type II collagen, comprising 80% of all fibres. AC also contains small amounts of collagens types IX, XI, III, VI and XII.[1] Proteoglycans that form large molecules (aggrecans) consist of glycosaminoglycan (GAG) subunits containing negatively charged chondroitin sulphate and keratan sulphate chains that bind water, up to 50 times their weight. This mechanism endows AC with high resistance to mechanical stress and load distribution.[2] The unique structure of cartilage allows it to resist mechanical forces ranging from 1 to 4 MPa.[3] The presence of collagen fibres and proteoglycans also enables

minimum friction at the articular surface.[4] Non-collagenous protein fibres such as cartilage oligomeric protein (COMP), tenascin and fibronectin account for less than 5% of the wet weight of AC.

Variations in cell morphology and specific ECM composition and organization allow us to distinguish four cartilage zones: superficial, transitional, middle (radial) and deep (calcified). The superficial zone, which has high water content

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and collagen concentration, is the major barrier responsible for resisting shear forces. Deeper zones with higher concentrations of proteoglycans allow for the equal distribution of mechanical stress, thus protecting the subchondral bone from increased loading.[5] Excessive loads that damage collagen and proteoglycan fibre networks, thereby causing water loss, result in numerous morphological and biochemical changes in cartilage structure. AC is devoid of blood and lymphatic vessels and nerves; for this reason, it has a decreased ability to self-renew after injury. Cartilage injuries are thought to be very common, but their true incidence is unknown. Curl et al. (1997) reported chondral lesions present in 63% of patients who undergo knee arthroscopy.[6]

Many attempts have been made to restore injured AC to recover joint function, but cartilage resurfacing remains a formidable challenge. Treatment options for symptomatic cartilage lesions range from conservative treatments (non-steroidal anti-inflammatory drugs (NSAIDS), analgesics and physical therapy) to the most advanced cell-based tissue engineering methods. The aim of conservative treatment is to reduce symptoms; however, evidence of the efficacy of such treatments in improving joint structure is controversial.[7] A more aggressive intervention involves various surgical approaches, all of which seek to fully restore or regenerate the cartilage, with its unique properties. Advances in imaging methods have led to much better recognition of the frequency and types of injuries, thus resulting in more accurate planning of the treatment algorithm.[8,9] Both computed tomography (CT) and high-resolution magnetic resonance imaging (MRI; 1.5 tesla or greater) are useful for evaluating joint structures. Moreover, MRI allows physicians to assess the cartilage and monitor the results of cartilage repair procedures.[10–12] In case of full-thickness, symptomatic cartilage lesions in young patients, surgical intervention is recommended since untreated isolated injuries may progress and lead to joint degeneration and premature osteoarthritic changes.[13] Osteoarthritis (OA) is now considered a civilization disease. It is estimated that 80% of the population have radiographic evidence of OA by the age of 65 years, although only 50% of these present clinical symptoms.[14,15] OA changes in the elderly are exacerbated due to decreased capacity of chondrocytes to synthesize ECM components and age-related limitations in maintaining tissue homeostasis.[16–18] The clinical symptoms of OA (pain, stiffness, crepitus, effusions and restricted range of motion) make OA the leading cause of disability and impaired quality of life in the world.

Currently available surgical procedures are classified as palliative (debridement, lavage), reparative (marrow stimulation techniques) or restorative (osteochondral grafting, autologous chondrocyte implantation (ACI)).[19] The choice of the strategy is based on lesion location, patients' physical demands and pre-operative status.[20] Each factor should be carefully assessed since each surgical method has its own specific limitations. Lavage of the joint and debridement are

considered first-line treatment options in smaller lesions in order to wash out and remove debris, loose cartilage fragments and inflammatory mediators.[21] Marrow-stimulating techniques, such as microfracture or subchondral bone drilling, attempt to restore the cartilage surface by creating a blood clot from subchondral bone blood vessels. These techniques are recommended for small lesions in patients <40 years of age, given that the success rate of this intervention is highly age-dependent.[22] Moreover, microfracture enables further treatment with ACI due to its high failure rate (26%) compared with 8% failure rate in patients treated previously with debridement alone.[23] At present, the most advanced methods are based on injecting cells with chondrogenic potential into the lesion site. However, the cell types most appropriate for harvesting and seeding have yet to be determined. First experiences with cartilage resurfacing using autologous cells date back to the early 1980s.[24] ACI is a two-stage procedure. The first step includes debridement of the lesion site and harvesting of cartilage slices from non-weight-bearing areas of the joint. Then, cells are cultured to multiply the number of cells to provide sufficient number of cells to fill the defect.[25] During the second stage of treatment, chondrocytes are implanted into the lesion and covered with periosteal flap (first-generation ACI) or resorbable membranes (second-generation ACI). The application of proper biomaterial cover may enhance cell proliferation and differentiation.[26] Despite improvements in surgical techniques, some challenges remain, mostly related to the limited number of chondrocytes available for cell culture, preservation of the cells' chondrogenic potential and re-differentiation of cells during tissue formation after implantation. The use of recently investigated mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) could help to overcome these limitations.

The exploration of source of pluripotency

Stem cell–based therapy is a promising tool for degenerative diseases associated with age and/or environmental factors. The term 'stem cell' was first used by Ernst Haeckel at the end of 19th century.[27] This scientific term describes an ancestor cell giving rise to a multicellular organism or fertilized egg and eventually developing into a new being.[27] Nowadays, the term 'stem cell' describes an undifferentiated, self-renewing population of pluripotent cells able to form tissues derived from primary germ layers. This ability is one of the most desired in regenerative medicine.

Pluripotency is the main characteristic of embryonic stem cells. The use of stem cells in medicine has been the subject of much controversy, given the ethical debate surrounding the use of human embryos.[28] In 2006, Takahashi and Yamanaka[29] successfully developed a technique to retrieve iPSCs from adult mouse fibroblasts by changing cell fate. The following year, they obtained human

pluripotent stem cells by cell transduction using selected transcription factors SOX2 (SRY (sex determining region Y)-box 2), c-MYC (v-myc avian myelocytomatosis viral oncogene homolog), KLF4 (Kruppel-like factor 4) or OCT3/4 (octamer-binding transcription factor 3 and 4).[30] That discovery was a breakthrough in stem cell research and gave hope of obtaining a tool for gene therapy and tissue engineering. Moreover, this technique resolved the ethical concerns associated with the application of human embryos in regenerative medicine.[28,31–33]

However, obtaining iPSCs is not a simple process and presents many obstacles and dangers. Some transcriptional factors used during the cell reprogramming process (c-MYC, OCT3/4) have an oncogenic potential due to the possibility of non-specific integration of lentiviral vectors with genomic DNA. Currently, scientists are working on developing non-viral, safe protocols to derive reprogrammed cells, thus decreasing risks of spontaneous cancerogenesis and teratoma formation after treating patients with iPSC cell–based therapies.[34–36]

The promising potential of iPSCs in many pathological and degenerative conditions has been widely described.[35] An increased incidence of OA among the ageing societies of the developed countries has been observed.[37–39] It is well established that OA substantially decreases the quality of life and gradually leads to disability. Although standard methods of treatment bring temporary relief of symptoms and provide a short-term solution, in most cases, the end result is joint replacement.[33,40,41] The primary goal of improving quality of life has been partly achieved. However, these treatment procedures are not without problems, and important side effects – primarily decreased mobility can negatively affect the quality of life.[42]

The aim of this review is to present a methodology for generating chondrocytes from iPSCs based on current knowledge of cartilage development. This new approach to AC and OA treatment based on cell therapy has a great potential to support or replace current standard procedures.

Chondrogenesis

The formation of cartilage from mesenchymal cells during embryogenesis is a multistep process regulated by several different groups of proteins. Most of these proteins belong to the transforming growth factor (TGF-β) family of proteins, including TGF-β1, TGF-β2 and TGF-β3, bone morphogenic proteins (BMPs) and wingless- and int-related proteins (WNTs), among many others.[43–47] The origin of the process can be found in skeletal blastemas' mesenchymal precursor cells, which are able to differentiate into muscles, cartilage, tendons or perichondrium.[44,46] The cartilage lineage formation includes four steps: (1) cell migration, (2) aggregations of mesenchymal–epithelial cell interactions, (3) condensation and (4) chondrocyte differentiation.

After appearance of three germ layers of embryos, precursor mesenchymal cells migrate to the determined field

of primeval limb formation called the lateral mesenchymal plate (LMP). The cartilage anlage is created by dense, elongated LMP cells. Formed nodules are able to differentiate into chondroblasts or chondrocyte cells. The further development and differentiation of cartilage tissue yield the beginning of primary bones and joints.[48,49] Chondrogenesis is a complex, multicellular process involving many biomolecules, both in vitro (Table 1) and in vivo. Some of these proteins (i.e. Noggin, BMP) have antagonist properties or can work as silencers or enhancers depending on their concentration or expression level (e.g. retinoic acid and WNT).[50]

Milestones at cartilage construction

Many protocols using different concentrations of various growth factors have been established to induce chondrogenesis in vitro. One approach is to create a universal procedure to obtain cells, which will allow the patients to fully recover their activities of daily living with less invasive procedures.[83] During the cell culture process, functionality, molecular profile and methods of cell expansion must be properly assessed due to the limited proliferation in primary cell cultures.[84] The main problems to be solved when working with models of AC are as follows: (1) appropriate cell population should be chosen for further differentiation protocols, (2) a small quantity and low concentrations of prochondrogenic growth factors should be used to decrease costs of the procedure, (3) cell culture expansion to scale up the bioprocess and (4) neutral biomaterial for cell and organism microenvironment should be employed.[50,85]

To date, only a few protocols have been developed to obtain appropriate amounts of cells for cartilage autologous grafts.[86] These procedures include biomaterial scaffolds or free-scaffold three-dimensional (3D) cell culture (micromass, spheroid and pellet culture).[86,87]

The appropriate cell population

MSCs – including bone marrow stem cells (BMSCs), adipose stem cells (ASCs) and synovial-derived stem cells (SDSCs) – have been identified as the most suitable cell populations for cartilage regeneration.[88–91] The main limitations of utilizing MSCs in clinical research are their age-related decreased ability to proliferate and the limited number of the desired cell populations obtained from patients' tissue; as a result of these limitations, the costs of using such cells are high due to the need for intensive cell culturing and advanced surgical techniques. A related issue is the need for optimization of cell culture protocols in order to obtain viable cells.[83]

The iPSCs seem to have the features appropriate for clinical applications. Newly developed protocols make it easier to obtain safe and stable cell lines that possess the same characteristics as human embryonic stem cells

Table 1. Growth factors involved in in vitro chondrogenesis process.[50]

Enhancers of chondrogenesis	Inhibitors of chondrogenesis
Activin[51]	Actin[70]
Bone morphogenic proteins (BMPs)[45]	Activator protein (AP-1)[71]
C-1-1 transcription factor[52]	Epidermal growth factor (EGF)[72]
Dexomethasone[53]	Fibroblast growth factors (FGF2, FGF-4, FGF-8)[73]
Fibroblast growth factors (FGF2, FGF2-4, FGF2-8)[54]	Homeodomain (HOX) transcription factors (MSX2)[74]
Growth and differentiation factor 5 (GDF5)[55]	Indian hedgehog (IHH)[75]
Histone deacetylases (HDAC1–4)[56]	Leukaemia/lymphoma-related factor[76]
Homeodomain (HOX) transcription factors (BARX2, NKX3-2, MSX1, PAX1, PAX9)[57]	Matrix metalloproteinases (MMP-2, MMP-13)[77,78]
Insulin-like growth factor-1 (IGF1)[58]	Noggin[79]
Lymphocyte enhancer–binding factor-1 (LEF1) transcription factor[59]	Retinoic acid[80]
	Rho GTPases family[81]
Matrix metalloproteinases (MMP-1)[60]	RUNX2[82]
NCAM[61]	WNT signalling molecules (WNT1, WNT-3a, WNT-5a, WNT-7)[68,69]
PGE2[62]	
Protein kinase C (PKC) family[63]	
Retinoic acid[64]	
SMAD transcription factors[65]	
SRY-type high mobility group box (HMG-box) DNA-binding domain SOX) transcription factors[66]	
Sonic hedgehog signalling protein (SHH)[67]	
TGF-β family[48]	
Wingless- and int-related protein (WNT) signalling molecules (WNT1, WNT-3a, WNT-5a, WNT-7)[68,69]	

NCAM: neural cell adhesion molecule; PGE2: prostaglandin E2; TGF-β: transforming growth factor-β;   RUNX2 : Runt-related transcription factor 2.

(hESCs). Described protocols involve (1) Cre/lox-mediated excision from the host genome; (2) delivery of non-integrating with genome episomal vectors, containing plasmids that harbour reprogramming factors; (3) adenoviral transduction; (4) reprogramming proteins linked to the cell-penetrating peptide (CPP) and (5) transposase-mediated excision.[34,92] Application of these findings should enable us to obtain safe iPSCs.

Induction chondrogenesis of iPSCs

Oldershaw's group (Oldershaw et al.[93]) established a three-stage protocol for differentiation of hESCs into chondrocytes using various different matrices and growth factors (WNT-3a, activin, follistatin, BMP4, fibroblast growth factor 2 (FGF2), growth and differentiation factor 5 (GDF5) and neurotrophin 4 (NT4)). Yang et al.[40] simplified Oldershaw et al.'s[93] protocol by using only six growth factors (excluding NT4) and only one type of matrix in a two-stage process. Both of these protocols have been shown to be capable of producing – in only 2 weeks – chondrocyte-like cells with higher COL2A1 (Collagen type II, alpha 1) and SRY (sex determining region Y)-box 9 (SOX9) expression and decreased pluripotent marker expression compared to control cell lines.[40,93]

Obtaining a chondrocyte-like cell population through embryonic body (EB) formation or micromass pellet culture has also been described.[94] During this process, stem cells start to spontaneously differentiate into three germ layers.[95] Stimulation of mesenchymal cells found in formed EB, with chondrogenic factors such as TGF-β, BMP2 or BMP4, resulted in the expression of type II collagen and aggrecan. The main disadvantage of this procedure is the time-consuming intermediate step, that is, the EB culture.[96] Another disadvantage of these methods is the non-homogeneous differentiated cell population. However, this problem can be solved by cell selection based on the increased expression of green fluorescent protein (GFP), which causes concomitant transfection of the cells with GFP-col2 plasmid.[97]

The modification of in vitro culture systems and medium environment can also result in improved chondrocyte stability or may induce increased chondrogenic markers in stimulated cells during propagation. For example, culturing dermis isolated adult stem cells (DIAS) under hypoxic conditions increases GAGs and type II collagen expression compared to normoxia.[98] Additionally, one study found that differentiating cord blood MSCs in a membrane system under hypoxia demonstrated increased wet mass and more homogeneous histological architecture compared to pellet cell culture and standard air condition.[99] Another important factor involves the ingredients used in the cell culture medium. Expansion of fresh isolated chondrocytes in monolayer at lower glucose concentrations (1 mM) improves the stability of propagated cells. However, 3D cell culture in the presence of higher glucose concentrations (10 mM) increases the amount of ECM proteins.[84]

Many studies have demonstrated the influence of physical factors in the chondrogenesis process. One such study was carried out with human ASCs under in vitro stimulation of low-intensity ultrasound (LIUS) (continuous 1 MHz wave for 10 min/day). Compared to the control group, the treated cells displayed increased expression of cartilage differentiation markers such as Col2A (mostly), Col2B, aggrecan and Col10.[43] The chondro- and osteoinductive properties of appropriate frequencies (1 Hz, 100 Hz) have also been confirmed in human and swine umbilical mesenchymal cells.[100] However, in vivo studies on damaged rabbit joint model did not confirm enrichment of collagen type II by BMSC stimulated by low-intensity pulsed ultrasound.[101]

Due to the physiology of the joints and the water-rich ECM of cartilage, chondrocytes are subject to high pressure, a condition that permits the exchange of nutrition and metabolism products by diffusion due to the avascular nature of AC.[102] Based on this observation, some researchers have proposed using hydrostatic pressure to create a specific microenvironment similar to cartilage tissue. The results are ambiguous. Some studies have demonstrated that using only hydrostatic pressure for differentiation of cells can cause lack of influence on aggrecan, SOX9 and type II collagen expression.[102,103] The differentiation process is complicated and depends on many factors, which is why adding 1 ng/mL TGF-β_3 to cell culture medium and applying hydrostatic pressure caused up-regulation of Sox9 expression and increased production of ECM proteins in porcine tissue–derived stem cells.[103]

Biomaterials and scaffolds

A tissue-specific 3D microenvironment is created by the aforementioned ECM, a secreted cellular biopolymer. The ECM of AC consists mostly of various types of collagens (II, X, XI) and proteoglycans (GAGs). In the ACI procedure, several billion chondrocytes are used to fill in the substantial cartilage loss. However, that technique had a number of defects from the very beginning. The disadvantages included cell leakage from the flap site and graft hypertrophy. Another problem was the propagation of the monolayer of chondrocytes, whose morphology changed from round-shaped cells into fibroblast-like cells with a dramatic decrease in cell proliferation. However, this state was reversible when the cells were seeded onto 3D scaffolds. This confirmed the crucial role of spatial methods of cell expansion for future AC models.[42,104,105]

In general, the main features of biomaterials used as cell scaffolds in tissue engineering are (1) biocompatibility and biodegradability, yielding non-toxic degradation end-products; (2) mechanical and physical properties similar to healthy tissue and (3) an appropriate microarchitecture created for cells to easily exchange oxygen, nutrition ingredients and ions through scaffold pores.[106]

Biomaterials can be classified by origin into (1) natural and (2) synthetic materials. Natural biomaterial include protein-based (collagen, fibrin) materials and polysaccharides (alginate, chitosan, hyaluronic acid, cellulose). Natural biomaterials provide a more favourable environment for the cells. However, their application may be limited by the risk of disease transfer, ability to trigger immunological response, lack of mechanical strength or a too-heterogeneous architecture. Synthetic biomaterials include polylactic-co-glycolic acid (PLGA), polylactic acid (PLA) and polyethylene glycol (PEG). Synthetic biomaterials are not as bioactive as protein-based ones, although they have higher mechanical strength. Moreover, they present a decreased risk of contamination by pathogens. The biggest advantage of synthetic materials is that their architecture can be adapted to obtain desirable properties during the fabrication process.[105,106]

For therapeutic targets, those biomaterials could form a few types of scaffolds such as hydrogels (whose viscoelastic properties are closest to those of cartilage), meshes and sponges (highly porous and mechanically durable) and composites created for better integration with the native tissue by cross-linking various polymers, for example, collagen + chondroitin 6-sulphate gels or PLA sponge + alginate gels.[107] New opportunities, such as 3D printers combined with high-resolution diagnostic equipment (MRI or CT), could be another step towards personalized medicine regeneration of injured tissues by printing individually designed prostheses or applying ECM-based scaffolds suitable for cell seeding onto transplanted organs.[108]

ECM as a natural scaffold fulfils many crucial functions in cartilage development due to their complex composition. Chondrocytes are immersed in a dense protein and proteoglycans mixture, which leads to decreased cell functionality and repair ability. The various histological layers in cartilage structure have different amounts of GAGs, and this determines the specific shape of chondrocytes for each zone and also determines their phenotype. To obtain a suitable scaffold for cartilage regeneration, a few crucial biophysical and biological aspects must be observed. First, the scaffold stiffness has a significant influence on ECM organization, deposition of proteoglycans, chondrocyte proliferation and differentiation of MSC into chondrocytes.[109,110] Another aspect is appropriate pore size. Large pores enable easy exchange of gas and nutrition, but decrease cell adhesion and attachment to scaffold surface.[111] Studies of collagen type II–based scaffolds (150–250 μm size pores) have shown increased Col2a1 and Acan expression and an increased amount of GAGs in bovine AC.[112]

Conclusion

The iPSCs generated through innovative and tumour formation–free methods are a promising approach to the treatment of OA or AC injuries. Optimization of procedures and

knowledge of developmental biology allow for the establishment of cell differentiation protocols that yield fully functional chondrocyte-like cell populations. When the injury has been carefully and comprehensively assessed, the use of modern techniques such as 3D printing will enable physicians to apply less invasive procedures that offer faster recovery and, most importantly, a greatly improved quality of life.

Declaration of conflicting interests

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