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Pluripotent Stem Cells: Differentiation Potential and Therapeutic Efficacy for Cartilage Repair

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Abstract

Articular cartilage injuries, often caused by trauma, have a limited potential to heal, which over time, may lead to osteoarthritis, an inflammatory and degenerative joint disease frequently associated with activity-related pain, swelling and impaired mobility. Many treatment modalities have been introduced but with limited success due to the formation of inferior fibrocartilage at the damaged area/injured site during the repair process. Pluripotent stem cells (human embryonic stem cells and induced pluripotent stem cells) provide a valuable cell source for critical understanding of pluripotency and lineage-specific differentiation, as well as for derivation of therapeutic mesenchymal cells and chondrocytes for articular cartilage repair. Here, we discuss the characteristics of pluripotent stem cells, their differentiation pathways to mesenchymal progenitors and chondrocytes, and their emerging roles in cartilage regeneration.

Keywords: pluripotent stem cells, mesenchymal stem cells, chondrocytes, cartilage, chondrogenesis

1. Introduction

Articular cartilage is a unique hypocellular, aneural, an avascular load-bearing tissue, supported by the underlying subchondral bone [1]. The extracellular matrix (ECM) is composed of a hydrated network of type II collagen fibrils, which are specifically arranged architecturally, and enforced with water-retaining aggrecan molecules linked to hyaluronic acid. The type II collagen network is further stabilized by other collagen types, IX and XI, in the territorial/inter-territorial matrix and biglycan, decorin, matrilins, perlecan, type IV collagen, laminins, type VI and

XVIII/endostatin collagens in the pericellular matrix surrounding the chondrocytes [2–5]. This combination of molecules gives the articular cartilage its unique ability to withstand the repetitive compressive loading in daily activities [1, 5, 6].

Articular cartilage injuries, commonly caused by excessive sports activities, have high incidence. When left untreated, cartilage lesions can lead to osteoarthritis, a degenerative joint disease characterized by the progressive degradation of the articular cartilage, subchondral bone, meniscus and ligaments, and the formation of painful osteophytes [7]. Osteoarthritis is the most common form of arthritis that affects 27 million people in United States, resulting in over 50% of total joint replacements, and the number of cases is increasing due to aging and obesity [6].

Current treatment modalities for articular cartilage injuries include microfracture, abrasion, drilling, osteochondral grafting, and more recently autologous chondrocyte implantation (ACI) [8]. Many of these methods help to repair the tissue and reduce pain to some degree. However, there are pertinent issues of inferior fibrocartilage repair, donor site morbidity and loss of chondrocytic phenotype upon expansion that necessitates the need for an alternative approach.

In recent years, stem cells have emerged as a promising cell source for treatment of cartilage lesions and osteoarthritis [9, 10]. Major advantages of applying stem cells for cartilage repair stem from their availability, proliferative capacity and multi-lineage differentiation potency [9, 10]. Major sources of stem cells include the adult and embryonic stem cells. Adult mesenchymal stem cells (MSCs), commonly isolated from bone marrow and adipose tissue, are currently evaluated in clinical trials for treatment of cartilage defects and osteoarthritis and have yielded promising results [11, 12]. However, one major limiting factor in use of adult MSCs relates to the impaired cellular proliferation and differentiation with increasing donor age [13, 14]. Furthermore, it has been established that cartilage differentiated from adult bone marrow MSCs is not phenotypically stable and has the propensity for hypertrophy development [15–17].

Pluripotent stem cells (PSCs) have the capacity for unlimited self-renewal and ability to differentiate into all cell types present in the body, thus representing an immortal cell source that could potentially provide unlimited supply of cells for cell-based therapies [18, 19]. However, one of the greatest challenges in pluripotent stem cell research is to understand, control, and develop an efficient and stable culture milieu for directing differentiation to a particular lineage, in this instance, chondrocytes [9]. In order to advance pluripotent stem cell therapies to the clinic, several biological challenges need to be overcome that include as follows: (1) directing lineage-specific differentiation, (2) defining the functional phenotype of derived chondrocytes, (3) eliminating the risk of teratoma formation, (4) designing adjuvant and tissue-engineering strategies to enhance the efficacy of cartilage repair and (5) deciphering the underlying mechanisms of cartilage repair. This chapter aims to discuss the latest advances in use of human PSCs in cartilage tissue engineering and regeneration.

2. Pluripotent stem cells

Pluripotent stem cells (PSCs) possess unlimited self-renewal and differentiation capacity, and include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are isolated from the inner cell mass of embryos [18], while iPSCs are created from somatic cells through reprogramming with defined gene and protein factors [19]. Human PSCs are well-poised as an alternative cell source to adult stem cells for transplantation. These cells proliferate in culture without loss of differentiation potential, thus enabling the generation of specific desired cell types in large numbers [9]. Furthermore, these pluripotent cells are capable of differentiating into the various cellular intermediates and committed cell types of a specific lineage by recapitulating the developmental embryogenesis. Specifically, in cartilage therapy, pluripotent stem cells enable the generation of early-staged chondroprogenitors and chondrocytes for cartilage-specific transplantation (i.e. hyaline, fibrocartilage and elastic cartilage). For repair of articular cartilage of the knee joint, hyaline cartilage repair is desired, so as to provide the long-term durability and ability to withstand repetitive mechanical loading in daily activities [1].

Major hurdles to clinical use of human PSCs include the risk of teratoma formation for human ESCs and iPSCs, and the risk of immune rejection in the case of human ESCs. Although nuclear reprogramming technology has been established that enables the generation of patient-specific PSCs, there is still safety issue of chromosomal variation as a result of genomic alteration [20]. These genetic alterations may in a long term lead to tumours, as observed in cases of gene therapy [21, 22]. On this note, several groups are exploring non-integrative methods such as transient viral transfections and small molecules to generate iPSCs [23, 24]. Such methods may enable generation of iPSCs that are safe for transplantation in near future.

3. Differentiation of PSCs into chondrogenic lineage

Development of an efficient and reproducible culture protocol for directing the differentiation of human PSCs into a defined specific chondrogenic lineage prior to transplantation is critical, as any remnants of PSCs could result in teratoma formation [9, 25]. On this note, as few as 245 human ESCs were reported to induce teratoma formation in 10 weeks' time [26]. Establishing the functional phenotype and competence of PSC-derived chondrogenic cells for transplantation is also a challenge. Hence, understanding the factors and underlying signaling pathways involved in lineage-specific differentiation of human PSCs to chondroprogenitors and chondrocytes, and establishing the functionality of these derived cells are necessary. We will discuss the various strategies utilizing approaches of growth factors, co-culture, conditioned medium and morphogenetic factors, gene modification, as well as derivation of mesenchymal progenitor cells. Additionally, tissue-engineering approaches incorporating three-dimensional (3-D) scaffolds and sustained release of soluble factors to influence stem cell chondrogenesis will be discussed.

3.1. Growth factors, chemicals and inductive medium

Early studies in PSC chondrogenesis involved differentiation *via* embryoid body (EB) formation, an *in vitro* culture system designed to mimic developmental embryogenesis with the formation of three germ layers, namely mesoderm, ectoderm and endoderm, and their tissue derivatives [27, 28]. In these studies, EBs were formed in suspension culture, followed by direct plating or dissociation into single cells and further differentiation into chondrocytic cells [27, 29]. Specific growth factors and chemicals may be added in certain fashion and combinations to induce specific cellular signalling and differentiation. Accordingly, Kramer et al. [27] investigated the differentiation of mouse ESCs *via* 5 days of EB formation followed by culture of EB outgrowth for 30 days. In that study, addition of bone morphogenetic protein (BMP)-2 (2 ng/ml) or BMP-4 (10 ng/ml), but not TGF- β 1 (2 ng/ml), increased the number of cartilaginous nodules when applied for the entire culture period. It was further observed that application of BMP-2 from day 2 to 5 of EB formation had the optimal stimulatory effect on chondrogenesis. It was further demonstrated that co-stimulation with BMP-2 (10 ng/ml) and TGF- β 1 (2 ng/ml) during day 3–5 of EB formation, followed by BMP-2 stimulation in EB outgrowth had a synergistic enhancement on chondrogenesis [29].

These findings somewhat agree with the study performed on human ESCs, where early and continuous application of TGF- β 1 (10 ng/ml) had an inhibitory effect on chondrogenesis [30, 31], and only enhanced chondrogenesis when applied at a later stage, following 5 days of EB formation [30]. The early inhibitory effect of TGF- β 1 on human ESC chondrogenesis relates to the role of TGF- β /activin/nodal signalling in the maintenance of undifferentiated state of human ESCs [30, 32]. Similarly, Toh et al. [33] showed that BMP-2 (100 ng/ml) was capable of inducing chondrogenic differentiation of dissociated human EB cells seeded as high-density micromass over a 21-day culture period. Notably, dissociation of the human EBs and replating them as high-density micromass enhanced the kinetics of chondrogenesis with early upregulation of type II collagen and glycosaminoglycan (GAG). This finding is in agreement with the high-density culture of adult MSCs in pellet and hydrogel cultures that cellular condensation is required for chondrogenesis [5, 34].

These studies subsequently prompted the investigation of growth factors and molecules involved in early germ layer induction of PSCs prior to chondrogenic differentiation. Kawaguchi et al. [35] showed that retinoic acid (10^{-7} M) added during EB formation enhanced early induction of pre-somitic mesoderm and neural crest markers including Mox1 and FoxD3. Subsequent stimulation with TGF- β 3 (10 ng/ml) in EB outgrowth resulted in heightened type II collagen expression. Recently, Yamashita et al. [36] demonstrated a direct differentiation approach without the need of cell sorting and expansion. In that study, mesendodermal differentiation of human iPSCs was induced by Wnt3a (10 ng/ml) and Activin A (10 ng/ml), followed by chondrogenic differentiation under co-stimulation of BMP-2 (10 ng/ml), TGF- β 1 (10 ng/ml) and GDF-5 (10 ng/ml) over a period of 42 days was able to produce homogenous hyaline cartilaginous particles at high efficiency. Further subcutaneous transplantation of the human iPSC-derived cartilaginous particles generated hyaline cartilage that expressed type II collagen, but not type I collagen, in immunodeficiency mice. Collectively, the TGF- β and BMP signalling have distinct roles during embryonic chondrogenesis, and the

interplay of these factors, in addition to the co-factors and culture systems, have a significant impact on PSC chondrogenesis.

3.2. Co-culture, conditioned medium and morphogenetic factors

The co-culture approach represents one of the earliest strategies used in chondrogenic differentiation of PSCs [37–39]. In this approach, the PSCs are co-cultured with chondrocytes in cell–cell contact or *via* a transwell system that allows only exchange of the morphogenetic factors and diffusible signals.

Bigdeli et al. [39] performed co-culture of human ESCs with irradiated neonatal or adult articular chondrocytes in pellet cultures in the presence of TGF- β 1 for 14 days, prior to dissociation. In that study, isolated mesenchymal cells were expandable and demonstrated chondrogenic potential with deposition of GAG upon differentiation in pellet culture in the presence of TGF- β 3. In another study, Vats et al. [38] showed that co-culture with chondrocytes over transwell inserts induced chondrogenic differentiation of human ESCs with expression of type II collagen and GAG by the end of 28 days, without the need of exogenous growth factors. When transplanted subcutaneously into the backs of severe combined immunodeficient (SCID) mice, these co-cultured human ESCs formed cartilaginous tissue that stained positive for type II collagen and GAG. Further studies showed that co-culture with bovine chondrocytes induced chondrogenic commitment of human ESCs and iPSCs, generating expandable chondrogenic cells that expressed type II collagen at early passage, and maintained the chondrogenic potential for cartilage formation [40, 41].

In those studies, the morphogenetic factors secreted by the chondrocytes were sufficient to induce PSC chondrogenesis, without the need of cell–cell contact [40, 41]. However, the morphogenetic factors are poorly defined, and the exact factor(s) responsible for differentiation of PSCs remains a question.

3.3. Gene modification

Gene transfer strategies to induce chondrogenesis of dedifferentiated chondrocytes and MSCs have been widely studied [42, 43]. To date, gene transfection of the Sox Trio (Sox5, Sox6 and Sox9) seems to be the most promising approach in generating stable hyaline cartilage, with the suppression of hypertrophic maturation and osteogenesis [42]. However, this strategy has yet to be explored in chondrogenic induction of human PSCs.

To date, the use of gene transfer strategy in induction of PSC chondrogenesis is limited. Wei et al. [44] showed that iPSCs can be generated from human osteoarthritic chondrocytes and subsequently induced to differentiate into chondrocytes. In that study, lentiviral transfection of TGF- β 1 was applied singly or in combination with primary chondrocytes in co-culture to induce differentiation of human iPSCs in alginate matrix. Notably, overexpression of TGF- β 1 and chondrocyte co-culture demonstrated synergistic enhancement of iPSC chondrogenesis *in vitro* and ectopic cartilage formation *in vivo*. Recently, Diekman et al. (2015) demonstrated that inhibition of cell cycle inhibitor p21 in mouse iPSCs enhanced monolayer proliferation

and subsequent chondrogenic differentiation with increased synthesis of GAG in pellet cultures over the control cells [45].

3.4. Derivation of mesenchymal progenitor cells from PSCs

Adult mesenchymal stem cells (MSCs) are present in several adult tissues, with the ability for lineage differentiation at least to bone, cartilage and adipose tissue [46–48]. Developmentally, MSCs that are involved in cartilage formation are derived mainly from mesoderm for limb joint and rib chondrocytes, and cranial neural crest for craniofacial chondrocytes. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, the minimal criteria to define human MSCs include adherence to plastic surface, specific antigen expression (CD73⁺ CD90⁺ CD105⁺ CD34⁻ CD11b⁻ CD14⁻ CD19⁻ CD79a⁻ HLA-DR⁻) and multi-lineage differentiation potential under standard *in vitro* differentiation conditions [49]. Due to the expression of major histocompatibility complex (MHC) class I, but not MHC II, adult MSCs are considered non-immunogenic, making them a valuable cell source for allogeneic transplantation without the need of immunosuppression [50].

Several strategies have been reported for the derivation of mesenchymal stem/progenitor cells from PSCs [51–53]. Earlier studies have employed co-culture with stroma cells or growth factor induction with or without sorting for an enriched cell population [51, 52]. Barberi et al. [51] was the first to report the derivation of MSCs from human ESCs. In that study, mesenchymal differentiation of human ESCs was induced by co-culture with OP9 stromal cells followed by sorting of CD73⁺ mesenchymal precursor cells. These mesenchymal precursor cells expressed MSC markers including CD44, CD73, CD105, CD166, VCAM, ICAM1 and STRO-1 and demonstrated multi-lineage differentiation to cartilage, bone, fat and skeletal muscle cells under inductive conditions. Lian et al. [52] subsequently reported a clinical compliant protocol that involved direct culture of human ESCs in culture medium supplemented with 10% serum replacement medium, 5 ng/ml of fibroblast growth factor (FGF)-2 and 5 ng/ml platelet-derived growth factor (PDGF)-AB. This was followed by sorting of CD105⁺ CD24⁻ mesenchymal precursor cells that displayed a MSC surface antigen profile (CD29⁺, CD44⁺, CD49a⁺, CD49e⁺, CD105⁺, CD166⁺, CD34⁻ and CD45⁻) and tri-lineage (osteogenic, chondrogenic, adipogenic) differentiation potential. Others have employed simple expansion of PSCs or EB outgrowth cells in culture medium conditions, typically used for culture of MSCs [53–55]. In those studies, the initial cultures consisted of mixed cell populations. After a few passages over 3–5 weeks, a homogenous population of spindle-shaped MSC-like cells was obtained. These cells expressed key MSC surface markers and displayed the capacity to differentiate into osteoblasts, adipocytes and chondrocytes. When injected into thigh muscles of SCID mouse, these human PSC-derived mesenchymal cells did not form teratoma [52].

The chondrogenic response of mesenchymal cells depends on the developmental origin. Studies have shown that MSCs generated by different methods from various sources of iPSCs could display variability in their differentiation capacity [53, 56, 57]. In all the above-mentioned protocols of deriving MSCs from human PSCs, the developmental origin of derived MSCs is a question. On this note, Umeda et al. [58] reported the isolation of paraxial mesoderm-like cells from human iPSCs under chemically defined medium conditions. These cells

expressed platelet-derived growth factor receptor (PDGFR)- α , but not vascular endothelial growth factor receptor (KDR). When cultured in chondrogenic conditions sequentially supplemented with PDGF-BB, TGF- β 3 and BMP-4, these mesodermal mesenchymal cells were able to form hyaline-like cartilage that resembles the hyaline cartilage of the knee joint articular cartilage.

To advance the clinical use of human PSC-derived MSCs, the immunogenicity and immunological properties of these cells are important considerations in order to rule out potential adverse immune rejection upon cell transplantation. To date, few studies have studied the immunological characteristics of human PSC-derived MSCs [59–61]. Similar to human bone marrow MSCs, human ESC-derived MSCs express cell surface HLA class I (HLA-ABC), but not HLA class II (HLA-DR) molecules, and suppress T lymphocyte proliferation induced by allogenic cells or mitogenic stimuli [59–61]. Importantly, hESC-derived MSCs demonstrated therapeutic efficacy with marked increase in survival of lupus-prone mice and a reduction of symptoms in autoimmune model of uveitis [60]. These findings support the use of human PSC-derived MSCs as a potential therapeutic candidate for further clinical development and application.

4. Cartilage tissue engineering

Utilizing the combination of cells, scaffolds and/or biomolecules, tissue engineering has emerged as a potential alternative to tissue/organ transplantation in tissue/organ replacement, and a strategy to enhance the overall therapeutic efficacy in treatment of injuries and diseases. Due to its limited ability to self-repair, cartilage is an ideal candidate for tissue engineering [62]. The principles of tissue engineering not only apply to cell seeding in scaffolds for implantation, but also extend to modulation of various cellular processes and cell–cell–matrix interactions that are critical in tissue repair and regeneration [63]. Here, we provide an overview of the scaffolds (matrices) and stimulating factors that have been used to influence PSC chondrogenesis.

4.1. Scaffolds

Different biomaterials have been investigated in their ability to support PSC chondrogenesis in 2-D and 3-D. This ranges from natural polymers such as type I collagen [64], type II collagen [65], agarose [66, 67]), alginate [44, 68]), fibrin [69], fibronectin [69], hyaluronic acid [70] and gelatin [69, 71] to synthetic polymers including polycaprolactone (PCL) [71, 72], polylactic-co-glycolic acid (PLGA) [73, 74], poly(L-lactic acid) (PLLA) [73] and polyethylene glycol diacrylate (PEGDA) [40, 75, 76], and may be fabricated in the forms of sponges, meshes and hydrogels. Several parameters need to be considered in scaffold design for cartilage tissue engineering. These include the scaffold architecture (e.g. shape, porosity and pore size); mechanical properties (e.g. elasticity); biochemical properties (e.g. ligands for cell adhesion); biocompatibility and biodegradability (to enable cell survival, matrix deposition and tissue remodelling). Scaffold design parameters for cartilage tissue engineering have been exten-

sively reviewed [63]. Biomaterials that have been used in PSC-based cartilage tissue engineering are summarized in **Table 1**.

Biomaterial	References
<i>Natural polymers</i>	
Agarose	[66, 67]
Alginate	[44, 68]
Gelatin	[69, 71]
Fibrin	[69]
Fibronectin	[69, 73]
Hyaluronic acid	[70]
Matrigel™	[73]
Type I collagen	[64]
Type II collagen	[65]
<i>Synthetic polymers</i>	
Polycaprolactone (PCL)	[71, 72]
Poly(lactic-co-glycolic acid) (PLGA)	[73, 74]
Poly(L-lactic acid) (PLLA)	[73]
Polyethylene glycol (PEG)	[40, 75, 76, 98]

Table 1. Types of biomaterials used in cartilage tissue engineering.

4.1.1. *Natural biopolymers*

Natural polymers resemble the natural extracellular matrix (ECM) found in the body and can interact with cells through surface receptors to influence cell fate and functions [77]. To recapitulate the native cartilage ECM environment, several earlier studies have investigated the use of type I and II collagens, and GAG as the major ECM components in fabrication of 2-D substrates and 3-D scaffolds for chondrocytes [78] and stem cell culture and chondrogenesis [79]. When cultured on type II collagen substrates, isolated human EB-derived mesenchymal cells retained the chondrogenic potential and displayed enhanced chondrogenesis with increased matrix deposition of type II collagen and GAG in subsequent pellet culture in the presence of TGF- β 1 [65]. Other ECM proteins such as fibronectin and basement membrane molecules (e.g. type IV collagen, laminin, nidogens) have also been reported to regulate progressive stages of chondrogenesis [80–82]. Notably, substrate combination of fibronectin and gelatin was reported to enhance chondrogenic differentiation of human iPSCs, compared to gelatin alone [69].

However, the use of natural polymers has disadvantages of batch variability and difficulty in control of material properties including degradation, mechanics and bioactivity [77]. To overcome these limitations, blended combination of natural and synthetic polymers, chemi-

cal and physical modifications, or use of pure synthetic polymers such as PEG may be considered [83].

Levenberg et al. [73] was the first to study the role of 3-D PLGA/PLLA scaffolds coated with either Matrigel™ or fibronectin for directed differentiation of human EB cells in the presence of various factors including retinoic acid, activin-A, TGF- β 1 or insulin growth factor (IGF)-I. It was concluded that TGF- β 1 was needed for human EB chondrogenesis in 3-D PLGA/PLLA scaffolds, despite the presence of other lineage structures. Bai et al. [74] further demonstrated differentiation of human EB-derived cells in monolayer chondrogenic conditions supplemented with TGF- β 3 and BMP-2, before dissociating the cells for suspension in alginate and seeding in PLGA scaffolds. Following 8-week subcutaneous implantation, cell-alginate-PLGA complexes formed cartilaginous tissues with lacunar cell morphology and stained positive for Sox9, type II collagen and GAG, amid at a low efficiency and the presence of fibrous tissue-like structures. On this note, the synthetic PLGA scaffold provides the mechanical framework [74], while alginate hydrogel enables the encapsulation of cells and maintains them in spherical cellular morphology that is supportive of chondrogenesis [68, 74]. Liu et al. [71] further showed that electrospun PCL/gelatin nanofibrous scaffolds enhanced chondrogenic differentiation of mouse iPSC-derived EB cells over plate controls. When implanted into the full-thickness defects created in the rabbit model, cell-seeded PCL/gelatin scaffolds restored the defects with hyaline-like repair tissue that stained positive for GAG and type II collagen.

4.1.2. *Modifications of natural polymers*

Modification of natural polymers aims to improve the overall composition, physical and mechanical properties of the biomaterial scaffold. Modifications of hyaluronic acid [84, 85], gelatin [86, 87] and chondroitin sulphate [88, 89] to include chemical functionalities (e.g. thiols, methacrylates, tyramines) have been frequently performed to improve the properties of these natural polymers for various biomedical applications. Some of these semi-synthetic matrices have been used as scaffolds for PSCs and PSC-derived cells in cartilage tissue engineering. Of note, HA has been reported to influence chondrogenesis through interactions with surface receptor CD44, making this natural biopolymer an ideal candidate for chemical modifications for application in cartilage tissue engineering [85, 90]. Prestwich's group first reported thiol-modification of hyaluronic acid and gelatin that allow crosslinking by addition of PEGDA to form hydrogels for biomedical applications including cartilage tissue engineering [84, 91]. When used to encapsulate human EB-derived mesenchymal cells and culture in chondrogenic medium supplemented with TGF- β 1 and BMP-7, these cell-seeded constructs displayed hyaline cartilaginous tissue characteristics including chondrocytic lacunar morphology, and deposition of high amounts of type II collagen and GAG, and minimal amount of type I collagen (**Figure 1**) [70]. When further implanted into rat osteochondral defects, these cell-seeded constructs regenerated the defects by undergoing an orderly remodelling process to form a hyaline cartilage layer and underlying subchondral bone by the end of 12 weeks. Similarly, thiol-modified dextran/PEG hybrid hydrogels have been reported for encapsulation of chondrocytes and mouse ESCs, and both cell types produced cartilaginous tissue [92].

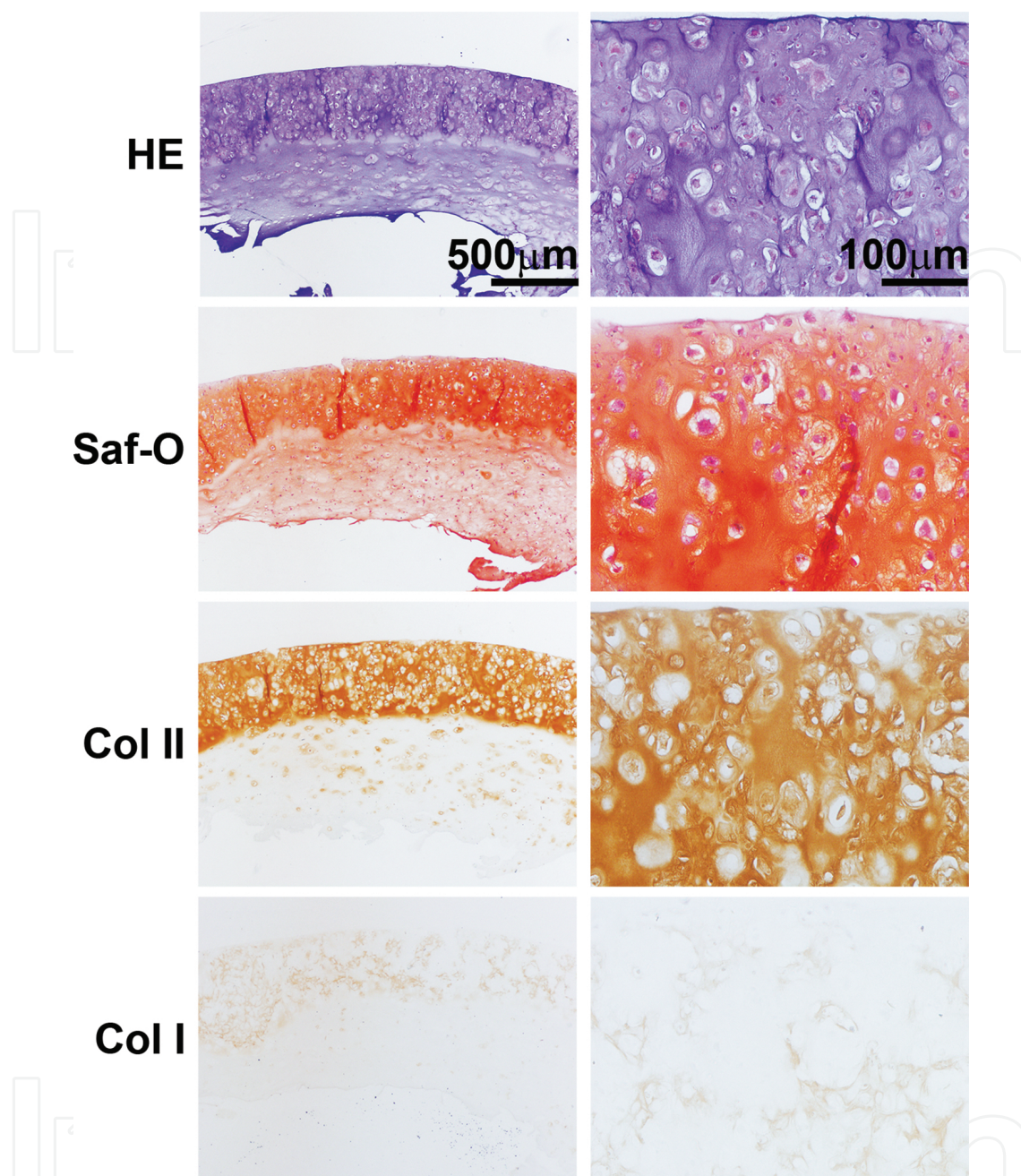


Figure 1. Tissue-engineered cartilage construct based on human ESC-derived mesenchymal cells. Histological staining by haematoxylin and eosin (HE) and safranin O/fast green (Saf-O) staining, as well as immunohistochemical staining for type II collagen (Col II) and type I collagen (Col I), was performed. Analysis revealed hyaline cartilaginous tissue characteristics of the tissue-engineered cartilage construct including chondrocytic lacunar morphology, abundant deposition of GAG and Col II, and minimal deposition of Col I.

4.1.3. Synthetic polymers

Synthetic polymer-based scaffolds with the defined composition and chemistry have evolved to become increasingly attractive as cell culture platforms to gain insights on effects of specific biophysical and/or biochemical cues on cell behaviour and function [93]. However, these

synthetic polymers usually require modifications with peptides or blending with natural ECM proteins to confer bio-functionality to support various cellular functions including cell adhesion, proliferation and differentiation [40, 73, 75]. Among these synthetic polymers, PEG is widely used due in part to its high hydrophilicity, bio-inert property and versatility. The hydroxyl groups of PEG are easily modified with other chemical functionalities (e.g. acrylates, methacrylates, thiols, azides) that can react to form a 3D network [94, 95]. Furthermore, peptide sequences such as Arg-Gly-Asp (RGD) can be tethered to PEG to facilitate cell adhesion and spreading [95]. On this note, Hwang et al. [75] showed that mesenchymal cells derived from human EB outgrowth displayed heightened cartilage-specific gene expression and matrix production, when encapsulated in RGD-modified PEG hydrogels compared to PEG hydrogel alone. Similarly, expanded mesenchymal chondrogenic cells derived from co-culture of human ESCs and bovine chondrocytes, encapsulated in RGD-modified PEG hydrogels formed homogenous cartilaginous tissue *in vitro* and *in vivo* [40].

4.2. Stimulating factors

Apart from growth factors and cytokines that have been described above to influence chondrogenesis, other stimulating factors have also been commonly employed to induce, accelerate and/or enhance cartilage formation, as part of tissue-engineering triad strategy. These additional stimulating factors include the use of small molecules and application of mechanical stimulation (e.g. hydrostatic pressure, dynamic compression, bioreactors).

Small molecules including kartogenin, TD-198946, prostaglandin E2, glucosamine, dexamethasone and cytochalasin D have been reported to promote stem cell chondrogenesis [96]. Notably, cytochalasin D treatment has been shown to enhance chondrogenesis of mouse EB-derived cells through actin filament reorganization [97]. Glucosamine, an amino monosaccharide present in GAG, was also reported to increase the levels of cartilage-specific gene expression and matrix accumulation when applied to hydrogel cultures of mouse EBs in chondrogenic conditions supplemented with TGF- β 1 [98].

The influence of biomechanical signals on stem cell chondrogenesis is widely reported [62, 99, 100]. Specifically, Terraciano et al. [101] showed that mesenchymal cells derived from human EBs responded differently to mechanical stimulation, depending on the extent of chondrogenic differentiation. In the absence of TGF- β 1, EB-derived mesenchymal cells exhibited a downregulation of chondrogenic genes including Sox9, aggrecan and type II collagen. However, after TGF- β 1 pre-conditioning, mechanical compression enhanced chondrogenic differentiation of EB-derived mesenchymal cells with increased chondrogenic gene expression and matrix deposition.

5. Cartilage regeneration using PSCs and cell derivatives

To date, several studies have applied PSCs and PSC-derived cells in cartilage repair in ectopic and orthotopic models *in vivo* [40, 69, 70, 76]. The effective application of PSCs and the associated outcomes in cartilage repair depends on several factors including 1) differentia-

tion efficiency and homogeneity of cartilage formation; 2) delivery strategy; 3) site of transplantation, as previously reviewed [9]. Here, we discuss the latest developments of PSCs, and underlying mechanisms in cartilage regeneration from animal studies.

Although a number of studies have demonstrated the functionality of PSCs in cartilage repair in orthotopic models, few have investigated the survival of the implanted PSCs over the course of cartilage tissue repair [69, 70, 76]. In a study by Toh et al. [70], tissue engineered constructs based on human EB-derived mesenchymal cells were implanted into full-thickness cartilage defects in an immunosuppressed rat model. In that study [70], it was observed that human chimerism of human EB-derived mesenchymal cells declined drastically over a period of 12 weeks. The percentage human cell chimerism and survival declined from ~70% at 2 weeks to ~30% at 6 weeks and <5% at 12 weeks. Despite the rapid decline in cell survival, there was full cartilage regeneration composed of mainly the host rat chondrocytes populating the defect site at the end of 12 weeks, implicating the role of paracrine functions of human EB-derived mesenchymal cells in cartilage repair. It was further reported in another study [69] that significant number of implanted human ESC-derived mesenchymal cells could still be detected at the repair site by the end of 12 weeks, although the exact percentage human cell chimerism was not determined. It therefore remains unclear if extended period of cell survival may promote a better and long-lasting cartilage repair. There are, however, good reasons to hypothesize that human PSC-derived cells mediate tissue repair and regeneration *via* multiple mechanisms that require thorough investigations.

Based on the current understanding, it is well-accepted that MSCs mediate tissue repair and regeneration by mechanisms of direct differentiation into target cells to replace the damaged tissue, and secretion of trophic factors to orchestrate endogenous cell response to mediate tissue regeneration [10]. Proteomic analysis of MSC conditioned medium (secretome) revealed a broad range of bioactive molecules secreted by MSCs, including growth factors, cytokines, chemokines, extracellular matrix molecules and vesicles [102], some of which reported to play important roles in chondrogenesis immunomodulation, and tissue repair [103, 104]. Similarly, human PSC-derived MSCs have been reported to produce a broad range of these trophic factors that largely constitute the biological significance of these cells in tissue repair [105]. Among these bioactive molecules, extracellular vesicles (EVs) produced by human ESC-derived MSCs have been demonstrated in their efficacy to reduce the infarct size in a mouse model of myocardial ischemia/reperfusion injury [106], promote hepatic regeneration in a mouse model of drug-induced liver injury [107], and more recently to promote cartilage regeneration in a rat model of osteochondral defect [108].

6. Conclusions and perspectives

The discovery of human PSCs, including human ESCs and iPSCs, has opened new avenues and possibilities for treatment of cartilage injuries and diseases such as osteoarthritis. The field is progressing rapidly to develop protocols for differentiation of human PSCs into MSCs and chondrogenic cells for cartilage tissue engineering and repair.

Looking ahead, the therapeutic potential of these cells will depend largely on identifying and fine-tuning the strategies, conditions, and factors required to induce PSC chondrogenesis in a defined, reproducible, and clinically compliant manner. Interdisciplinary efforts of culture technologies, biomaterials and tissue engineering are converging to develop effective PSC therapies, including intra-articular cell injection and implantation of tissue engineered cartilage graft, for cartilage repair.

The application of PSCs in cartilage regeneration not only restricts to cellular therapies, but extends to secretome components including growth factors, cytokines, extracellular matrix molecules and vesicles that likely underpin the mechanistic roles of PSC-derived cells in tissue repair and regeneration. Deciphering the composition and the components of the secretome is going to shed light on the paracrine functions of PSC-derived cells in cartilage repair, as well as development of novel therapeutics and strategies for cartilage regeneration.

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