European Cells and Materials Vol. 36 2018 (pages 96-109) DOI: 10.22203/eCM.v036a08 ISSN 1473-2262

eCELLS & MATERIALS

OPEN ACCESS

INDUCED PLURIPOTENT STEM CELLS FOR CARTILAGE REPAIR: CURRENT STATUS AND FUTURE PERSPECTIVES

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Abstract

The establishment of cartilage regenerative medicine is an important clinical issue, but the search for cell sources able to restore cartilage integrity proves to be challenging. Human mesenchymal stromal cells (MSCs) are prone to form epiphyseal or hypertrophic cartilage and have an age-related limited proliferation. On the other hand, it is difficult to obtain functional chondrocytes from human embryonic stem cells (ESCs). Moreover, the ethical issues associated with human ESCs are an additional disadvantage of using such cells.

Since their discovery in 2006, induced pluripotent stems cells (iPSCs) have opened many gateways to regenerative medicine research, especially in cartilage tissue engineering therapies. iPSCs have the capacity to overcome limitations associated with current cell sources since large numbers of autologous cells can be derived from small starting populations. Moreover, problems associated with epiphyseal or hypertrophic-cartilage formation can be overcome using iPSCs.

iPSCs emerge as a promising cell source for treating cartilage defects and have the potential to be used in the clinical field. For this purpose, robust protocols to induce chondrogenesis, both *in vitro* an *in vivo*, are required. This review summarises the recent progress in iPSC technology and its applications for cartilage repair.

Keywords: Induced pluripotent stem cells, pluripotency, cartilage, osteoarthritis, cell therapy, tissue engineering, regenerative medicine, chondrogenesis.

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Introduction

The development of a multicellular organism begins from a single-cell, the zygote, which divides in a quick and regulated manner during gestation. The cells from the first two divisions, namely the totipotent cells, are able to generate both embryonic and extra embryonic tissues (placenta). As these cells divide, their potency to generate extra embryonic tissues is lost and they become pluripotent cells, which are able to differentiate into cells that arise from the three germ layers: endoderm, mesoderm and ectoderm (Evans and Kaufman, 1981).

Although embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Thomson *et al.*, 1998), and the cells in the germinal ridge of the embryo (Nichols and Smith, 2007) were thought to be the only type of pluripotent cells known, Takahasi and Yamanaka (2006) show that it is possible to activate a class of genes within

adult cells that cause a reversion to a pluripotent state. Using retroviruses to deliver and force the expression of four transcriptional factors [octamer-binding transcription factor 4 (*Oct4*), sex determining region Y-box 2 (*Sox2*), *c-Myc* and Kruppel-like factor 4 (*Klf4*)], mouse tail-tip fibroblasts are reprogrammed into cells similar to ESCs, called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). The same protocol can successfully reprogram human fibroblasts (Takahashi *et al.*, 2007). The generation of iPSCs is now achieved from a variety of species (Harding and Mirochnitchenko, 2014) and a wide range of somatic cell types (Web ref. 1; Web ref. 2). Although the reprogramming efficiency varies among somatic cell types, accessible and abundant differentiated cell types, such as fibroblasts, keratinocytes or blood cells, can be directed into a pluripotent cell state (Brouwer *et al.*, 2016; Driessen *et al.*, 2017; Higgins *et al.*, 2012).

iPSCs and ESCs share the properties of unlimited self-renewal and pluripotency (Narsinh *et al.*, 2011). Also, they are similar in terms of surface marker expression, morphology, proliferation, gene expression profiles, *in vivo* teratoma formation capacity and telomerase activity (Hirschi *et al.*, 2014; Narsinh *et al.*, 2011; Zhao *et al.*, 2013a). However, human iPSCs have several advantages over the use of human ESCs. iPSCs represent an easily-accessible source of patient-specific pluripotent cells and, despite the possibility of an immunological response and rejection of iPSCs by a recipient patient cannot be completely precluded, they are expected to reduce the immune response when transplanted (Zhao *et al.*, 2013a). Also somatic cell nuclear transfer (SCNT) can be used to create patient-specific ESCs (Tachibana *et al.*, 2013). However, the widespread application of SCNT to human cells is challenging due to the need for mature, developmentally competent human oocytes, which leads to issues regarding the use of human oocytes for research. In this sense, iPSCs can bypass the ethical and political issues related to the use of human ESCs because the destruction of human embryos is not needed (Hirschi *et al.*, 2014).

iPSCs are considered to be a promising tool for disease modelling, drug discovery and regenerative medicine applications (Hirschi *et al.*, 2014; Lian *et al.*, 2010; Okano and Yamanaka, 2014; Rony *et al.*, 2015; Sayed *et al.*, 2016; Singh *et al.*, 2015; Zhao *et al.*, 2013a). In fact, the iPSC technology is already implemented to investigate tissue dysfunction at the cell and molecular level and to treat both genetic and non-genetic diseases (Zhao *et al.*, 2013a).

Functional cartilage is spontaneously produced from human iPSCs in teratomas (Kumazaki *et al.*, 2014; Takahashi *et al.*, 2007b; Yu *et al.*, 2007), making these cells possible candidates for cartilage regenerative medicine applications and a tool for studying disease mechanisms and new treatments.

The aim of this review was to discuss the role of iPSCs over recent years in drug discovery, disease modelling and regenerative medicine applications. Specifically, the focus was on differentiation protocols, treatment strategies and results obtained in the field of cartilage therapy.

Chondrogenic differentiation of iPSCs

To employ the iPSC technology for cartilage damage treatment, drug discovery and disease modelling applications, a robust *in vitro* chondrogenesis is required (Augustyniak *et al.*, 2015). To evaluate the different methodologies suggested, it is important to understand the normal developmental pathways that lead to the production of chondrocytes.

Chondrocyte differentiation is regulated by multiple signal transduction pathways, which regulate a complex series of events, including condensation of mesenchymal progenitor cells and nodule formation followed by chondrogenic differentiation (Li and Dong, 2016). Several critical signalling molecules regulate this process, including soluble factors, such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), transforming growth factor beta (TGFb), Wnt and cell adhesion molecules (N-CAM, N-cadherin, b-catenin) (Matta *et al.*, 2014). These factors activate essential targets for initiation and maintenance of the chondrocyte phenotype. Cartilage-specific transcription factors, such as SRY-related high-mobility group-box gene 9 (*Sox9*), are also required for the initial condensation of mesenchymal progenitor cells and for the maintenance of the chondroprogenitor phenotype; therefore, *Sox9* is highly expressed during chondrogenesis (Li and Dong, 2016). Apart from these factors, macromolecules of the cartilage extracellular matrix, such as type II collagen, hyaluronan, aggrecan or fibronectin, can also act as signalling molecules (Matta *et al.*, 2014).

To assess the effectiveness of chondrogenic differentiation, it is important to know to what extent the differentiated cells share a similar phenotype and genotype with their native counterparts. Since no chondrogenic cell surface markers are known, chondrogenic differentiation is usually evaluated by measuring histological and molecular markers, rather than flow cytometry markers (Driessen *et al.*, 2017). The chondrogenic phenotype is characterised by the expression of type II collagen, Sox9, aggrecan and high levels of proteoglycans (Dehene *et al.*, 2009; Nejadnik *et al.*, 2015). Furthermore, both epiphyseal and fibroblastic markers, such as the fibrocartilage marker type I collagen and the hypertrophic cartilage type X collagen, should be assessed to robustly study cells functionality (Dehene *et al.*, 2009; Nejadnik *et al.*, 2015). Run-related transcription factor 2 (RUNX2), matrix metalloproteinase-13 (MMP13) and vascular endothelial growth factor (VEGF) are also used as markers of epiphyseal phenotype, due to their role in the formation of hypertrophic chondrocytes during skeletal development (Augustyniak *et al.*, 2015).

Table 1. Human iPSC chondrogenic differentiation protocols and quality of the chondrogenesis obtained. Quality of chondrogenesis is classified according to: expression of positive markers and absence of hypertrophic/fibrocartilage markers (good); expression of both positive and negative markers (intermediate); lower expression of positive markers than in control (low); if expression of negative markers is not studied, the quality of differentiation obtained is not evaluated in the table. Abbreviations: EBs (embryo bodies), MSC (mesenchymal stromal cell), COLII (type II collagen), ACAN (aggrecan), COLI (type I collagen), COLX (type X collagen), GAGs (glycosaminoglycans), OCN (osteocalcin), NE (non-evaluable).

Reference	Chondrogenic differentiation protocol	Assessment of the differentiation							Quality of differentiation
		Positive markers				Negative markers			
		COLII	Sox9	ACAN	Others	COLI	COLX	Others	
Wei *et al.*, 2012	Co-culture	+		+	GAGs (+)			VEGF (−)	Good
Qu *et al.*, 2013	Co-culture	+	+			+			Intermediate
Medvedev *et al.*, 2011	EBs formation	+	+	+	GAGs (+)	+			Intermediate
Umeda *et al.*, 2012	EBs formation	+			GAGs (+)	−			Good
Craft *et al.*, 2015	EBs formation	+	+	+		−	−	RUNX2 (−)	Good
Lee *et al.*, 2015	EBs formation	+	+	+	Sox5 (+) Sox6 (+)	−	−		Good
Liu *et al.*, 2012	MSC-like population	+	+	+	GAGs (+)				NE
Villa–Diaz *et al.*, 2012	MSC-like population	+		+	GAGs (+)				NE
Koyama *et al.*, 2013	MSC-like population	+/−	+	+/−	GAGs (+)		−		Intermediate
Zou *et al.*, 2013	MSC-like population				GAGs (+)				NE
Nejadnik *et al.*, 2015	MSC-like population	+	+	+		+	+		Low
Chijimatsu *et al.*, 2017	MSC-like population	+	+	+	GAGs (+)		+		Intermediate
Borestrom *et al.*, 2014	Growth factors	+	+	+	GAGs (+)		−		Good
Cheng *et al.*, 2014	Growth factors	+	+		GAGs (+)				NE
Saito *et al.*, 2015	Growth factors	+	+	+	Sox6 (+) GAGs (+)				NE
Yamashita *et al.*, 2015	Growth factors	+			GAGs (+)	−	−	OCN (−)	Good

Strategies for chondrogenic differentiation of iPSCs *in vitro*

Knowledge of iPSC chondrogenesis is still in its infancy and, therefore, standardisation is lacking, leading to many different methodologies showing variable results. Very few iPSC studies reach the standards of a high quality chondrogenesis, with rounded cells embedded individually in extracellular matrix rich in type II collagen and proteoglycans (Archer and Francis-West, 2003). To offer a wide vision of the field, this section summarises the different methodologies developed over recent years and classifies the protocols according to the quality of the chondrogenesis obtained (summarised in Table 1).

Protocols to differentiate iPSCs chondrogenically are grouped in three or four categories (Tsumaki *et al.*, 2015): (1) co-culture with primary chondrocytes, (2) through embryoid body (EB) formation and (3) using combinations of growth factors. All these methods are believed to transition through an mesenchymal stromal cell (MSC)-like state, but some authors perform a more specific procedure to obtain an intermediate population of MSC-like cells, thus establishing a fourth category: (4) induction of MSC-like cells. Furthermore, new approaches are reported recently, such as the use of conditioned medium obtained from human cartilage chondrocytes (Suchorska *et al.*, 2017).

Co-culture with primary chondrocytes

The primary advantage of co-culture is that the paracrine factors secreted from the chondrocytes may stimulate the differentiation of iPSCs into

chondrocytes by mimicking the *in vivo* environment found in the tissue (Tsumaki *et al.*, 2015; Wei *et al.*, 2012). By contrast, the co-culture conditions may increase the risk of contamination of differentiated cells with other undesired cells (Lietman, 2016). This strategy is followed by Wei *et al.* (2012): to promote chondrogenic differentiation, lentiviruses are used to transduce human iPSCs with TGFb type 1 (TGFb1); next, the iPSCs are co-cultured *in vitro* with human healthy chondrocytes. After differentiation, proteoglycans presence is detected inside the matrix, the expression levels of type II collagen and aggrecan are similar to that of human chondrocytes and the presence of the VEGF is not detected (Wei *et al.*, 2012). Qu *et al.* (2013) co-culture iPSCs in a trans-well system along with bovine articular chondrocytes. Then, differentiated cells are expanded and pellet culture is performed in chondrogenic medium supplemented with TGFb3. Using this protocol, an incomplete chondrogenesis is obtained. The iPSC-derived chondrocytes show similar expression levels of type II collagen and Sox9 to the bovine chondrocytes, but they also present higher levels of type I collagen (Qu *et al.*, 2013). Adding soluble molecules such as BMP or other members of the TGFb family to the culture medium could improve the quality of the differentiation.

Embryoid bodies formation

In vitro chondrogenic differentiation of iPSCs through EB formation is the most common approach for obtaining human iPSC-derived chondrocytes (Suchorska *et al.*, 2017). Their three-dimensional (3D) structure is considered to be similar to that in the early post-implantation embryo and, therefore, the cells in the EB should be able to differentiate into cells from the three germ layers (Cheng *et al.*, 2014).

Several groups employ this strategy with certain success. Craft *et al.* (2015) reach a high-quality chondrogenesis by culturing human iPSC-derived EBs in the presence of mesoderm-specific transcription factors. After this first differentiation step, the EB is dissociated, the cells are sorted according to primitive streak/early mesoderm markers and the resultant cells are cultured at high density/micromasses in the presence of TGFb3. Resultant cells express high levels of type II collagen, Sox9 and aggrecan in comparison with both healthy chondrocytes and hypertrophic chondrocytes; no presence of type I collagen, type X collagen or RUNX2 is detected (Craft *et al.*, 2015).

Umeda *et al.* (2012) differentiate iPSCs to paraxial mesoderm using EB culture, using specific time and dose-combination of growth factors and cell sorting techniques, both with EB cells and EB-outgrowth cells. To stimulate chondrogenic differentiation, platelet-derived growth factor (PDGF), TGFb3 and BMP4 are used. Although most of the differentiated cells are ESCs, also iPSC-derived cells showing robust chondrogenesis and generating hyaline-like cartilaginous particles are obtained (Umeda *et al.*, 2012).

Lee *et al.* (2015) report a factor-based protocol for differentiating human iPSCs into chondrocytes, also through EB formation. Sprouted cells obtained after two weeks of differentiation are seeded as a 3D pellet and cultured in chondrogenic medium supplemented with growth factors, as previously described (Oldershaw *et al.*, 2010). As a novelty, p160-Rho-associated coil kinase (ROCK) inhibitor Y27632 is added, which promotes pluripotent stem cells survival and enhances *Sox9* expression. Immunohistochemical stainings and molecular analysis reveal good-quality chondrogenesis in the 3D pellets (Lee *et al.*, 2015).

Medvedev and colleagues (2011) culture EBs in chondrogenic medium supplemented with TGFb3 and BMP2. After EB dissociation, chondrogenic cells can self-assemble dense cartilage-like aggregates *in vitro*, with glycosaminoglycans, Sox9 and aggrecan detected (Medvedev *et al.*, 2011). By using this protocol, good cell functionality is obtained, but the evaluation of fibrocartilage and hypertrophic markers within the aggregates would have been desirable.

Intermediate MSC cellular differentiation

Since MSCs are able to differentiate into the chondrogenic, osteogenic and adipogenic lineages (Dominici *et al.*, 2006; Sanjurjo-Rodriguez *et al.*, 2016) and mesenchymal condensation is a prerequisite for chondrogenesis induction during embryonic cartilage formation (Lietman, 2016), another differentiation strategy is to first stimulate iPSC differentiation into a MSC-like population, followed by their differentiation towards chondrocytes. To establish MSC-like populations from iPSCs, several strategies are investigated, including the use of coatings (Liu *et al.*, 2012), growth factors (Nejadnik *et al.*, 2015) and EB formation (Koyama *et al.*, 2013; Villa-Diaz *et al.*, 2012).

Differentiation of iPSCs into MSCs by forming EBs is performed by Koyama *et al.* (2013) and Villa-Diaz *et al.* (2012). These protocols involve EB formation, monolayer culture of sprouted cells from EBs into MSC medium and 3D pellet culture in chondrogenic medium supplemented with i) dexamethasone and TGFb3 or ii) ascorbic acid, dexamethasone and TGFb3. Comparison with human native chondrocytes is only performed in Koyama's protocol, revealing that human iPSCs differentiate into pre-chondrogenic-like cells but are not equivalent to fully mature chondrocytes.

Zou *et al.* (2013) establish a method for deriving MSCs from human iPSCs avoiding the EB formation, culturing the iPSCs directly in MSC differentiation medium followed by serial trypsinisation-based passaging. For chondrogenic differentiation, pellets are formed, which are cultured in chondrogenic medium supplemented with TGFb3. Similarly, Nejadnik and colleagues (2015) report another approach involving direct induction of human MSCs under specific cell culture conditions, followed by chondrogenic differentiation with TGFb3. Cells at day 14 of chondrogenic differentiation present

significantly increased gene expression of the hyaline chondrogenic markers, compared to human iPSCs-MSCs before differentiation. However, an increased expression of type I and type X collagen is also shown (Nejadnik *et al.*, 2015). Chijimatsu *et al.* (2017) generate MSC-like cells from iPSCs by using specific culture media, which are subjected to 3D pellet culture system using TGFb3 and BMP2. Molecular and histological evaluation of chondrogenic differentiation show that pellets undergo chondrogenesis. However, cells exhibit features of hypertrophic chondrogenesis with type X collagen upregulation, suggesting that incomplete chondrogenesis is obtained (Chijimatsu *et al.*, 2017).

Another strategy for generating MSCs from iPSCs is based on the use of coatings during cell culture (Liu *et al.*, 2012). Besides being an osteogenic and chondrogenic differentiation promoter of MSCs, type I collagen stimulates the epithelial-to-mesenchymal transition (Medici and Nawshad, 2010) and, thus, it is used for the generation of MSCs from pluripotent stem cells. For example, Liu *et al.* (2012) report a one-step method to derive MSC-like cells from human iPSCs using fibrillar type-I-collagen-coated plates. This method consists in the generation of a thin layer of collagen fibrils onto the plates, which successfully stimulates the derivation of MSC-like cells, as shown by the expression of MSC surface markers. For chondrogenic differentiation, the standard protocol originally described by Johnstone *et al.* (1998) is used.

Growth factors

Several studies test the efficacy of administrating growth factors, such as BMPs, TGFs, insulin-like growth factors and FGFs. Since this approach is based on reproducing the events that during the embryo development "guide" the differentiation process, it is also known as "directed differentiation" (Augustyniak *et al.*, 2015; Suchorska *et al.*, 2017). Cheng *et al.* (2014) successfully apply in iPSCs the protocol already developed for the direct differentiation of human ESCs towards chondrocytes. This protocol is based on a sequence of pathways activated during development and involves the use of different growth factors, such as activin-A, Wnt3a, FGF2, BMP4, neurotrophin-4 and the growth differentiation factor 5 (GDF5). Chondrogenic cell aggregates, expressing Sox9 and positive for safranin O staining, are observed at the end of the protocol. Also, gene expression of chondrogenic markers is higher than in iPSCs before differentiation but no evaluation of hypertrophic or fibrocartilaginous markers is performed (Cheng *et al.*, 2014). With similar results, this protocol is also applied by Saito *et al.* (2015) to differentiate human iPSCs.

The Yamashita *et al.* (2015) protocol consists in initially differentiating human iPSCs into mesendodermal cells, as previously reported (Oldershaw *et al.*, 2010; Umeda *et al.*, 2012), and, then, culturing them in chondrogenic medium supplemented with ascorbic acid, BMP2, TGFb1 and GDF5. Next, chondrogenically-committed cells are sorted according to collagen type II expression and cultured in 3D. Following this protocol, cartilaginous particles with rounded cells embedded in extracellular matrix rich in collagen type II, proteoglycans and with no presence of collagen type X are obtained.

A good-quality chondrogenesis is achieved by Borestrom *et al.* (2014). Their protocol involves a 3D pellet pre-differentiation stage, followed by monolayer expansion of chondrogenic progenitors. These progenitors are cultured in a second chondrogenic 3D pellet and differentiated into chondrocytes using chondrogenic medium supplemented with growth factors. Gene expression levels of *Sox9*, type II collagen, aggrecan and type X collagen are similar to that of human articular chondrocytes. Furthermore, intense blue colour is observed inside the 3D pellets following alcian blue staining and immunostaining for type II collagen results positive.

Achievements and current challenges in iPSC chondrogenesis

The protocol by Oldershaw *et al.* (2010) has an enormous impact in the field of stem cells and cartilage research. However, its application to iPSCs is unsuccessful mainly due to the low cell viability. The optimisation of this directed differentiation method performed by Umeda *et al.* (2012) focusses on the modulation of Wnt and TGFb signalling and it succeeds in overcoming the cell viability problems. It also allows the generation of high quality hyaline cartilage-like tissue and the identification of key molecules to trigger iPSC chondrogenesis, becoming one of the highest impact protocols in the field. Additionally, achieving homogeneous chondrogenic cell populations is another important challenge, especially when protocols involving EB formation are developed (Lietman, 2016; Liu *et al.*, 2012; Nejadnik *et al.*, 2015). The establishment of purification steps along the differentiation protocol is a big advance, as confirmed by the results achieved by Craft *et al.* (2015) and Yamashita *et al.* (2015).

Apart from these achievements, there are still many challenges and questions that remain open. First, chondrogenic differentiation of iPSCs is cost- and time-consuming. Second, the quality of the derived chondrocytes is not always evaluated in depth and sometimes they show hypertrophic or fibrocartilaginous characteristics. Third, it is not yet clarified which is the best method for deriving chondrocytes from iPSCs. Regarding this last point, it is not easy neither to establish a comparison between all the methods available nor to determine the quality of the chondrogenesis objectively, mainly due to the different methodologies presented. Moreover, each protocol uses different iPSC lines, derived from different somatic cell types by using different reprogramming methodologies, which

could interfere in the research outcome. Few research studies comparing different methodologies in a structured way are conducted and even less comparing different cell sources or different cell lines. Suchorska *et al.* (2017) try to elucidate some of these questions by comparing four methods to stimulate chondrogenesis of iPSCs: 1) monolayer culture with the addition of growth factors (direct protocol), 2) EBs in chondrogenic medium with TGFb3, 3) EBs in chondrogenic medium with conditioned medium from human chondrocytes and 4) EBs in chondrogenic medium with conditioned medium from human chondrocytes and TGFb3. These protocols differentiate iPSCs into cells having similar expression levels of chondrogenic markers to human chondrocytes. Specifically, the direct protocol or the conditioned-medium protocol are the most cost-effective methods (Suchorska *et al.*, 2017). Similarly, Augustyniak and collaborators (2015) compare the conditioned protocol and the EB formation protocol with TGFb3. Cells differentiated in the conditioned medium present characteristic features of mature chondrocytes. In contrast, cells cultured in the presence of TGFb3 present characteristics of hypertrophic chondrocytes (Augustyniak *et al.*, 2015).

All the methods for obtaining chondrocytes from iPSCs have paved the way to develop cartilage regenerative medicine therapies. However, it remains unclear whether chondrogenically-induced iPSCs can be considered to be real human chondrocytes, namely cells with the same phenotype and cellular activity as the native chondrocytes. Also, it would be important to confirm if iPSC chondrogenesis truly matches or even improves chondrogenesis of MSCs or healthy articular chondrocytes. Disease modelling, drug discovery and regenerative medicine applications require robust *in vitro* chondrogenesis and, therefore, more research in this field is needed to achieve uniform differentiation of the desired cell type and to better understand the process of chondrogenesis.

Therapeutic applications of iPSCs

Robust and reproducible protocols to induce iPSC chondrogenesis are not yet available; perhaps this is the reason why there are so few published studies about therapeutic applications of iPSCs in cartilage diseases (Lietman, 2016), in comparison with other diseases, such as neurodegenerative disorders or cardiomyopathies, among others (Lian *et al.*, 2010). Nevertheless, with further optimisation of the protocols to induce chondroprogenitors and promote their chondrogenic differentiation, iPSCs are destined to be an attractive cell source for applications in drug discovery, disease modelling and regenerative therapies, to treat and repair chondral defects.

Disease modelling

Disease modelling consists in the recapitulation of the disease of interest in a Petri dish. The idea behind this is to derive iPSCs from a patient's somatic cells and differentiate them *in vitro* into the cell type desired (Stadtfeld and Hochedlinger, 2010). Two properties of iPSCs are indispensable for this application: unlimited self-renewal and differentiation potential (Lian *et al.*, 2010; Singh *et al.*, 2015). Since iPSCs provide an unlimited source for any desired specialised cells, their potential is irrefutable. Hence, many studies use patient-specific iPSCs (Das and Pal, 2010; Menon *et al.*, 2016; Zhao *et al.*, 2013a). Regarding cartilage diseases, most of the studies focus on monogenic cartilage-diseases. For example, Xu *et al.* (2016), using an *in vitro* model of familiar osteochondritis dissecans based on the employment of iPSCs, reveal that several characteristics of the differentiated chondrocytes can help in explaining the disease phenotype and susceptibility to cartilage injury. Studying chondrocytes differentiated from patients' MSCs and iPSCs, large deregulation and aberration in assembled extracellular matrix and deregulated cell fate are found, which are caused by an abnormal accumulation of mutated aggrecan protein. Moreover, the composition of the extracellular matrix in patients' iPSC-derived chondrocytes reflects the changes seen in advanced osteoarthritis (OA), presenting further evidence of the association between familial osteochondritis dissecans and early-onset OA (Xu *et al.*, 2016). Patient-derived skeletal dysplasia iPSCs are generated by Saitta *et al.* (2014) to study early stages of aberrant cartilage formation *in vitro*. Briefly, the mutation causing the dysplasia is found to disrupt the normal sequence of differentiation that occurs during endochondral bone formation, showing inappropriate expression of cartilage markers and abnormal TGFb1 and BMP2 signalling (Saitta *et al.*, 2014). Yokoyama *et al.* (2015) study an auto-inflammatory disease, called neonatal-onset multisystem inflammatory disease (NOMID), which is caused by NACHT, LRR and PYD domains-containing protein 3 (NALP3) mutations, through the iPSC technology. They establish wild-type and mutant iPSCs, stimulate them to trigger chondrogenic differentiation, both *in vivo* and *in vitro*, and compare the phenotypes of the chondrocytes and the chondrogenic tissue generated. Finally, Sox9 is found overexpressed *via* the cAMP/PKA/CREB signalling pathway in chondrocytes with disease-causing mutations in NLRP3, causing extracellular matrix overproduction (Yokoyama *et al.*, 2015).

Besides for examining genetic diseases, patient-specific iPSCs are also useful for studying complex diseases affected by several factors, such as genetic background and environmental modifications (Lee *et al.*, 2014; Liu *et al.*, 2016). Thus, iPSC modelling may be promising for studying OA and other polygenic cartilage disorders, as they present a complex pathophysiology. In this sense, iPSCs are also generated from patients with rheumatoid arthritis (Lee *et al.*, 2014) and OA (Kim *et al.*, 2011; Lee *et al.*, 2014). On the other hand, multiple single nucleotide polymorphisms (SNPs) play different

roles in the pathogenesis of OA and its subtypes (Wang *et al.*, 2016) and, therefore, it would be of great interest to develop a model of OA based on the use of these SNPs, for which many susceptibility loci are known(Wang *et al*., 2016). The establishment of human iPSC-based models of OA could help researchers to identify important molecules and events that play a key role in the development and progression of OA directly in the susceptible cell type. At this point, it is important to find a phenotype directly linked to the disease, which would enable to model it *in vitro*. Another important issue is the generation of stable chondrocytes/cartilage and hypertrophic chondrocytes/cartilage to perform comparisons between disease forms. Although these concerns are still to be solved, the iPSC potential in this field is exciting and all the studies developed to date represent a first step towards this goal. Findings from modelling diseases may help to better understand the disease and ultimately develop a treatment (Singh *et al.*, 2015) that could be tested in these disease models.

Drug discovery

Before performing human clinical trials, the toxicity of any novel drug should be tested in animal models, to obtain reliable data about the drug effects and side effects (Singh *et al.*, 2015). However, these models are limited because animals and humans possess significant differences (genetic background, anatomy and pathophysiology) and animal models are incapable of exactly replicating human physiological conditions (Lian *et al.*, 2010; Okano and Yamanaka, 2014). Also, some compounds may have species-specific toxicity in animals (Singh *et al.*, 2015). Focussing specifically on OA, the disease progress is slower in humans than in animals and the pathological changes of the OA animal model may not be entirely consistent with those of the human disease (Liu *et al.*, 2016). Thus, additional drug screening model systems are needed to better mimic human conditions and to evaluate whether the results could be extrapolated to humans.

iPSC culture would represent an alternative to developing animal models of the disease. The use of iPSCs offers a better alternative to conventional pharmacological and toxicological tests, allowing the generation of human disease-specific cell types to enable better prediction of therapeutic response and toxicology (Qi *et al.*, 2014). Additionally, iPSCs are a valuable tool for seeking compounds or growth factors that can improve current differentiation protocols. In this sense, Yang *et al.* (2012) show a screening platform using human iPSCs in a multi-well plate format to identify compounds that can promote chondrogenesis. They generate iPSCs from human keratinocytes and establish reporter lines that can represent endogenous collagen II expression levels based on luciferase activity. Two chimeric ligands of activin/BMP2 are tested at two different doses and their effects are compared to cells treated with GDF5. Higher concentrations of each of these two compounds can improve chondrogenic differentiation as compared to GDF5 (Yang *et al.*, 2012). Similarly, Yamashita *et al.* (2015) generate a chondrocyte-specific reporter human iPSC line that expresses GFP when differentiated into chondrocytes. This line is used to search for the culture conditions that drive the differentiation of iPSCs towards chondrocytes. For this purpose, the cells are firstly differentiated into mesendodermal cells, then, the medium is changed to basal medium with three different types of supplementation. BMP2, TGFb1 and GDF5 result to be critical for GFP expression and, therefore, for the chondrogenic differentiation of iPSCs (Yamashita *et al.*, 2015).

Some drugs are already tested on iPSC models of different diseases (Zhao *et al.*, 2013a). With regard to cartilage disorders, Willard *et al.* (2014) show a screening of candidate OA drugs in murine iPSC and find that the NF-kB inhibitor SC-514 effectively reduces cartilage loss in response to inflammatory conditions.

These studies illustrate that the use of iPSCs as a screening platform could pave the way for a more precise and personalised medicine, which would enable the study of the effect of various novel drugs at an individual level (Novak *et al.*, 2014). Combining these screening platforms with a library of iPSCs from different patients with the same disease may provide insight into the genetic and potentially epigenetic variation of a population (Lian *et al.*, 2010).

Regenerative medicine and cartilage tissue engineering using IPSCs

Articular cartilage has a poor intrinsic capacity for repair due to its avascular nature and the poor stem or progenitor cells access. Therefore, cartilage defects due to traumatic or pathological conditions slowly grow over time, eventually leading to more complex disorders, such as OA (Diekman *et al.*, 2012; Oldershaw *et al.*, 2010; Yamashita *et al.*, 2015). Currently, there is no cure for OA. Treatments are mainly focussed on pain management, slowing degradation and reducing inflammation, with total joint replacement usually needed in the end phase of the disease (Fuentes-Boquete *et al.*, 2007; Willard *et al.*, 2014; Zhang *et al.*, 2016). New treatments based on regenerative medicine and cartilage tissue engineering could offer new solutions to preserve, repair and restore the integrity of articular cartilage (Fig. 1). By treating small or focal defects in cartilage, it could be possible to avoid or reduce damage progression and generalised degradation, as seen in OA (Fuentes-Boquete *et al.*, 2007.

iPSCs are widely used in cartilage tissue engineering because they allow for the use of abundant, accessible and autologous cells for cartilage formation, bypassing ethical concerns. In contrast, other cell sources as MSCs or chondrocytes are difficult to obtain and show changes in their phenotype and differentiation potential after several

passages (Diekman *et al.*, 2012). For this purpose, the application of an appropriate scaffold plays a decisive role (Cavallo *et al.*, 2010) and, therefore, many studies using scaffolds or gel carriers to enhance the chondrogenesis of iPSCs have been performed. However, most of the investigations already published are carried out using mouse-derived iPSCs (Table 2).

Scaffolds employed in cartilage tissue engineering using iPSCs are commonly composed of different natural biopolymers or synthetic polymers, ranging from agarose hydrogels to polycaprolactone scaffolds. Diekman *et al.* (2012) report the creation of tissue-engineered cartilage constructs from murine iPSCs by using a starting population of prechondrogenic cells embedded in an agarose gel. Their potential to be used for repairing cartilage is examined using an *in vitro* defect model system. Cells show cartilaginous matrix production and integration with the surrounding explant cartilage, as determined by histology. In addition, the engineered cartilage has similar mechanical properties to the native cartilage (Diekman *et al.*, 2012).

Uto *et al.* (2013) embed iPSCs within a collagen hydrogel and introduce it inside a defect prepared at the mice patellar groove. Joints filled with iPSCs embedded within the collagen hydrogel reproduce the smooth contour of the joint and, most importantly, iPSCs remain in the transplanted site after 8 weeks, showing that these cells can engraft and persist inside the damaged area (Uto *et al.*, 2013). On the other hand, tumour formation at the surgical site is observed in some of the mice, which may be avoided by *in vitro* differentiation before implantation.

Gel scaffolds are highly permeable and allow for the homogeneous distribution of the cells (Zhao *et al.*, 2013b). Also, they are highly biocompatible and present low cytotoxicity (Apelgren *et al.*, 2017). Despite being relatively easy to implant, their viscous properties may complicate the construct handling (Apelgren *et al.*, 2017). Therefore, another strategy is to mix gel scaffolds with more rigid ones. Liu *et al.* (2014) fabricate polycaprolactone/gelatine scaffolds to enhance chondrogenic differentiation of mouse iPSCs. Subsequently, they implant the constructs into cartilage defects performed in the rabbit knee and assess the efficacy of cartilage restoration. They observe by scanning electron microscopy that cells can attach to the surface of the scaffold and, also, some can infiltrate between the fibres. The formed tissue shows a large number of chondrocytes and upregulated expression of type II collagen and aggrecan. Moreover, after three months, the defects are almost entirely filled (Liu *et al.*, 2014).

Studies using human iPSCs show that human iPSC-derived chondrocytes can be grown in 3D cultures or scaffolds to create cartilage-like tissue *in vitro* and *in vivo* (Tsumaki *et al.*, 2015), although with different results in terms of the quality of the cartilage-like tissue obtained. Kim *et al.* (2011) reprogram human synovial cells from OA patients and culture them in polycaprolactone polymer scaffolds, finding high levels of chondrogenic markers, such as type II collagen and Sox9, but also the presence of type I and type X collagen. Similarly, Wei *et al.* (2012) improve the quality of the human iPSC chondrogenesis by using an alginate matrix. Ko *et al.* (2014) also use an alginate hydrogel to

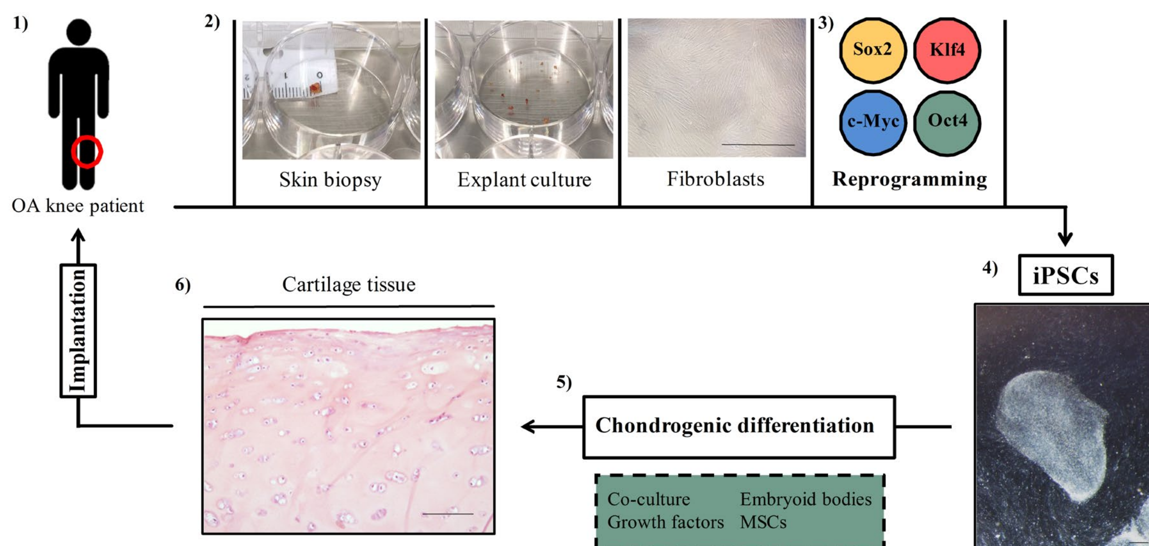

Fig. 1. Scheme illustrating the proposed methodology to perform regenerative medicine for articular cartilage by generation of iPSCs. 1) OA patient selection. 2) Harvest of skin biopsy and fibroblast isolation by the explant culture technique (scale bar: 200 µm). 3) Reprogramming with *Oct4*, *Sox2*, *Klf4* and *c-Myc*. 4) Culture of iPSC colonies (scale bar: 300 µm). 5) Chondrogenic differentiation of iPSCs to obtain cartilage-like tissue. 6) Image showing a slice of healthy human articular cartilage stained with haematoxylin-eosin (scale bar: 100 µm).

Table 2. Summary of *in vitro* and *in vivo* protocols for cartilage regenerative medicine and tissue engineering by using iPSCs. Abbreviations: EB (embryo body), TGFb (transforming growth factor beta), BMP (bone morphogenetic protein), OA (osteoarthritis), RA (rheumatoid arthritis), ACI (autologous chondrocyte implantation), MEF (mouse embryonic fibroblasts). A (ascorbic acid), ABT (ascorbic acid + BMP type 2 + TGFb type 1), ABTG (ascorbic acid + BMP type 2 + TGFb type 1 + GDF5), BMCs (bone marrow cells).

Reference	Somatic cell type	Chondrogenesis	*In vitro*/ *In vivo*	Animal model	Animal
Kim *et al.*, 2011	Human OA synoviocytes	EB formation. Pellet culture or agarose culture and 3D polycaprolactone scaffold.	*In vitro*	–	–
Diekman *et al.*, 2012	Dermal mouse fibroblasts	Micromasses formation, digestion, cell sorting and pellet formation. Factors added: BMP4 and TGFb3.	*In vitro*	–	–
Wei *et al.*, 2012	OA chondrocytes	EB formation. Lentiviral transduction of iPSCs with TGFb1 and co-culture with chondrocytes in alginate matrix.	Both	Subcutaneous implant	Mouse
Uto *et al.*, 2013	Mice BMCs and MEFs (ASP0001 iPS-MEF-Ng-20D-17)	Micromasses embedded in collagen hydrogel. Factors added: TGFb3.	Both	Model of joint defect in patellar groove	Mouse
Cheng *et al.*, 2014	Human dermal fibroblasts and ESCs	Three-steps protocol based on the addition of growth factors. Factors added: activin A, Wnt3a, FGF2, BMP4, GDF5, follistatin and neurotrophin 4.	*In vivo*	Osteochondral defect in trochlear groove of the femur	Rat
Ko *et al.*, 2014	hiPSC line SC802A-1 (human fibroblasts)	EB formation, disaggregation and culture in pellets and alginate hydrogel. Factors added: TGFb3.	*In vivo*	Osteochondral defect on the patellar groove of the femur	Rat
Liu *et al.*, 2014	Mouse iPSCs	EB formation, trypsinisation and cells seeded onto polycaprolactone scaffolds. Factors added: TGFb1.	*In vivo*	Knee joint defect	Rabbit
Lee *et al.*, 2015	Human fibroblasts	Three-steps protocol based on the addition of growth factors + EB formation. Factors added: Activin A, Wnt3a, FGF2, BMP4, GDF5, follistatin and neurotrophin 4.	Both	Subcutaneous implant	Mouse
Saito *et al.*, 2015	Human neonatal dermal fibroblasts	Three-steps protocol based on the addition of growth factors. Factors added: Activin A, WNT3A, FGF2, BMP4, GDF5, follistatin and neurotrophin 4.	*In vivo*	Full thickness cartilage defect in the medial femoral condyles.	Mouse
Yamashita *et al.*, 2015	hiPSC line 409B2 and mouse cell line 604B1	Three-steps protocol based on the addition of growth factors. Three types of supplementation were tested: A, ABT o ABTG.	Both	Subcutaneous implant. Knee joint surface defects.	Mouse Rat Pig
Nguyen *et al.*, 2017	A2B iPSC line	Micromass formation, digestion, four passages in monolayer and second micromass formation. Factors added: TGFb1 and TGFb3. 3D printing with irradiated chondrocytes in nanocellulose and alginate bioink.	*In vitro*		

differentiate human iPSCs chondrogenically. Then, they assess the *in vitro* capacity of these cells for cartilage regeneration using an osteochondral rat defect model. The new tissue generated reveals a good restoration of the articular surface. Nonetheless, despite the persistence of implanted iPSCs *in situ*, reduced amount of proteoglycans as compared with adjacent normal cartilage is observed (Ko *et al.*, 2014).

A pioneering approach in cartilage tissue engineering is to 3D print combinations of scaffolds and cells to generate a cartilage-like tissue. 3D printing consists in laying down successive layers of material in different shapes, each one printed directly on top of the previous one according to a computer program (Dodziuk, 2016). The 3D shape of the bio-printed constructs can be very precise, which is important for the reconstruction of specific structures (Apelgren *et al.*, 2017). Thus, this technique allows the distribution of different cells and supporting biomaterials in different ways to resemble the microarchitecture of the tissues. Nguyen *et al.* (2017) use this approach, combining nanofibrillated cellulose composite bioink with human iPSCs. They print the mixture of cells and ink as six-layer grids into 24-well plates and culture

them in chondrogenic differentiation medium. iPSCs can maintain a pluripotent phenotype after 3D bioprinting and cartilage-like tissue expressing type II collagen is observed after five weeks of culture in chondrogenic medium (Nguyen *et al.*, 2017). Although this approach is still in its infancy, this research suggests that 3D bioprinting with iPSCs may be the future treatment to repair damaged cartilage in joints.

Discussion

The remarkable properties of pluripotent stem cells, as ESCs and iPSCs, make them promising tools with great potential for cell therapy and regenerative medicine applications. However, the use of human ESCs is linked to ethical barriers, as well as problems related to the immune response. Since Takahashi and Yamanaka made the landmark discovery of reprogramming differentiated cells into a pluripotent state in 2006, allowing for the procurement of pluripotent cells in a non-invasive manner and eluding ethical problems, researchers can work with pluripotent cells bypassing the concerns of using human ESCs.

Reproducing different diseases in a Petri dish, testing new drugs and compounds in the human cells of interest and generating successful tissues for implantation and repair is getting more feasible thanks to the use of iPSCs. In this sense, numerous studies show the potential of iPSCs for treating cartilage lesions. Nevertheless, to be able to apply iPSC for cartilage repair, efficient protocols to differentiate the iPSCs chondrogenically are required. Many protocols of chondrogenic differentiation are described but all of them using different growth factors, varying culture times, intermediate steps and different culture systems (monolayer and pellet). There is still no general agreement concerning the best approach to obtain chondrocytes; for this reason, studies systematically comparing the different approaches would be helpful. Likewise, it is too soon to firmly state that iPSCs are better than MSCs for cartilage therapies, but there is no doubt that iPSCs can improve several issues, such as accessibility, cell number, fibrocartilage formation or phenotype loss with passages.

Monogenic cartilage diseases are more likely to be modelled by using iPSCs and recently, the use of these cells to model disorders, such as skeletal dysplasia, has produced positive results (Liu *et al.*, 2016). In the field of iPSCs and OA, opportunities and challenges coexist. iPSC models can be a valuable tool for understanding disease mechanisms and perhaps to test potential drugs for clinical use, although it is still necessary to find a specific phenotype *in vitro*. According to Liu *et al.* (2016), the establishment of the human iPSC-based models of the OA can accelerate the exploration of the pathogenesis of the disease and be a significant step towards potential treatments.

The uses of iPSCs for tissue engineering applications are more focussed in the fields of cardiology or neurology than rheumatology. Studies developed to date using iPSCs with and without scaffolds have obtained cartilage-like tissue, which is, however, still different from hyaline articular cartilage. The important part is that iPSCs can engraft and persist inside the lesions and produce extracellular matrix when chondrogenic differentiation is stimulated. Finally, although the 3D printing with iPSCs approach is still in its infancy, it has an enormous potential and may be the future treatment to repair damaged cartilage in joints.

Conclusion

To continue progressing in this field, it is extremely important to reach a consensus about what are the minimum requirements for considering that chondrogenically differentiated iPSCs fulfil the threshold of mature chondrocytes. These criteria would likely be (1) obtaining rounded or polygonal cells (2) embedded individually in extracellular matrix (3) rich in type II collagen and proteoglycans and in (4) the absence of epiphyseal markers, such as alkaline phosphatase and type X collagen.

Acknowledgements

CIBER-BBN; Rede Galega de Terapia Celular and Grupos con Potencial de Crecemento, Xunta de Galicia (R2016/036, R2014/050 and GPC2014/048); Fondo de Investigación Sanitaria; Instituto de Salud Carlos III (PI17/02197). Clara Sanjurjo-Rodríguez is beneficiary of a postdoctoral fellowship from the Xunta de Galicia; Rocío Castro-Viñuelas and María Piñeiro-Ramil are granted by a predoctoral fellowship from the Xunta de Galicia and the European Union (European Social Fund - ESF); Fundación Española de Reumatología (2014 grant); Universidade da Coruña; Fundación Profesor Novoa Santos.

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Discussion with Reviewer

Solvig Diederichs: Is *in vitro* chondrogenesis of iPSCs already sufficiently efficient to consider iPS cells an attractive alternative to primary chondrocytes and MSCs?

Authors: Multiple cell-based approaches attempt to restore hyaline cartilage and prevent degeneration (Fuentes-Boquete *et al.*, 2007) but formation of high-quality cartilage is not yet achieved. The use of chondrocytes and MSCs is linked to several drawbacks, such as limited availability, loss of phenotype in culture, invasive harvesting procedures and fibrocartilage or hypertrophic cartilage formation. iPSCs represent a promising alternative, mainly

due to two properties: unlimited self-renew and chondrogenic differentiation potential. However, few studies show high-quality cartilage from iPSCs(Chiramatsu *et al.*, 2017; Craft *et al.*, 2015; Lee *et al.*, 2015; Umeda *et al.*, 2012; Wei *et al.*, 2012). Indeed, the availability of many studies applying different protocols introduce significant variability and create confusion. iPSCs are destined to be a very useful source for cartilage therapies and small molecule and drug screening applications, since they allow the use of abundant, accessible and autologous cells bypassing ethical concerns; however, iPSC chondrogenesis is not yet efficient enough to completely substitute chondrocyte- or MSC-based therapies. More studies critically comparing chondrogenic differentiation between different cell types, as MSCs and iPSCs, are needed to elucidate this open question.

Solvig Diederichs: Would young cells, such as perinatal tissues cells, including cord blood cells, be preferable over adult cells that accrue many genetic variances during their life time?

Authors: Since the first generation of iPSCs in 2006, a multitude of somatic cell types have been reprogrammed to pluripotency. However, the conversion is still highly inefficient (< 1 %) (Maherali *et al.*, 2008, additional reference). It is proposed that successful generation of iPSCs may be easier to achieve from actively dividing cells than from slow/non-dividing cells (Streckfuss-Boemeke *et al.*, 2013; Utikal *et al.*, 2009, additional references), which may be explained by a higher capacity of the cells to incorporate the reprogramming factors into the host genome or to activate the reprogramming signalling pathway. Fibroblasts are one of the cell types most commonly used for reprogramming because of their high proliferation capacity at early passages (Streckfuss-Boemeke *et al.*, 2013, additional reference), easy cultivation, propagation, cryopreservation properties and viability in culture. However, adult cells are more likely to accumulate nuclear and mitochondrial mutations, which cannot be erased during the reprogramming processes and, therefore, can interfere in the function and tumour risk of iPSCs (Wang *et al.*, 2013, additional reference). In contrast, young cells, such as the ones derived from umbilical cord blood or placenta, are expected to incorporate few somatic mutations when compared to adult donor cells (Cai *et al.*, 2010, additional reference). Thus, the umbilical cord and placenta emerge as promising extra-embryonic tissues for cell therapy purposes. Additionally, some studies suggest that cells expressing certain degree of multipotency can provide a better source for reprogramming (Kato *et al.*, 2000, additional reference). In this sense, umbilical cord cells express genes found in ESCs, such as *Oct4*, *Nanog* or *Rex-1*. Since these cells already express several genes required for pluripotency, are readily available and are not limited by ethics, they seem to be a great cell source to use in reprogramming. The advantages and disadvantages of reprogramming human cells from different tissues are currently unclear but will need careful comparison to find the optimal cell source for reprogramming.

The use of "young cells" is not exempt from problems. For example, probably because the formation of extra-embryonic tissues occurs very early after implantation, extra-embryonic tissue cells retain an immature phenotype (Red-Horse *et al.* 2004, additional reference). Thus, an important parallel question that emerges is whether modelling of late-onset diseases, such as OA could be possible when using this kind of "immature cells" for reprogramming. This is an important issue that needs to be solved for disease modelling, drug screening and, eventually, translational applications of iPSCs.

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Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.