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Review

Chasing Chimeras – The elusive stable chondrogenic phenotype

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ABSTRACT

The choice of the best-suited cell population for the regeneration of damaged or diseased cartilage depends on the effectiveness of culture conditions (e.g. media supplements, three-dimensional scaffolds, mechanical stimulation, oxygen tension, co-culture systems) to induce stable chondrogenic phenotype. Herein, advances and shortfalls in *in vitro*, preclinical and clinical setting of various *in vitro* microenvironment modulators on maintaining chondrocyte phenotype or directing stem cells towards chondrogenic lineage are critically discussed. Chondrocytes possess low isolation efficiency, limited proliferative potential and rapid phenotypic drift in culture. Mesenchymal stem cells are relatively readily available, possess high proliferation potential, exhibit great chondrogenic differentiation capacity, but they tend to acquire a hypertrophic phenotype when exposed to chondrogenic stimuli. Embryonic and induced pluripotent stem cells, despite their promising *in vitro* and preclinical data, are still under-investigated. Although a stable chondrogenic phenotype remains elusive, recent advances in *in vitro* microenvironment modulators are likely to develop clinically- and commercially-relevant therapies in the years to come.

1. Introduction

Articular cartilage defects have been reported in about 60–66% of routine knee arthroscopies [1] and full-thickness defects are found in 4.2–6.2% of arthroscopies of patients younger than 40 years [2]. They are caused by joint diseases, genetic or metabolic conditions or as a result of trauma. The most common symptoms are pain, swelling and reduced mobility [3]. Articular cartilage defects increase the risk of osteoarthritis (OA) [4–8], are frequently found in association with OA and correlate with symptoms and disease progression [6,9,10]. Although the aetiology is not well known, probably in chronic cartilage injury, breakdown molecules induce inflammation in the joint [11–13] that in turn induces alteration of cell phenotype and increases the synthesis of cytokines and matrix-degrading enzymes by chondrocytes [11,12,14–17]. In U.S., the estimated direct and indirect costs of OA and related diseases are about US$ 128 billion per year [18].

Numerous cell types are under intense investigation for cartilage repair and regeneration (Table 1). Autologous chondrocyte implantation techniques (ACI) rely on the isolation of chondrocytes from a non-load bearing region of articular cartilage and their *in vitro* expansion and implantation in the defect site [19–21]. Despite ACI treatments showed initially promising results, recent clinical trials have suggested that they are ineffective as a long-term treatment [22] and that they are not superior to other surgical treatments [23,24]. This is mainly due the fact that during *in vitro* expansion, chondrocytes gradually lose their phenotype, in a process known as dedifferentiation. Their synthesis of collagen type II decreases, parallel with an increase in collagen type I [25]. *In vitro* expansion usually results in production of fibrocartilaginous substitutes with inferior mechanical properties as compared to physiological hyaline cartilage [1]. A wide branch of scientific research is aimed at optimising cell culture systems to longer maintain chondrogenic phenotype during *in vitro* expansion. In parallel, researchers are searching for cells sources alternative to chondrocytes that would minimise donor-site morbidity, reducing the number and costs of interventions. Different cell populations have been investigated and, particularly, mesenchymal stem cells (MSCs) have gained considerable attention, due to their ease of isolation, and great expansion and chondrogenic differentiation capacity. ACI techniques have been used for the treatment of chondral defects even in cases with concomitant inflammatory and degenerative pathologies [26–34] and, in this context, an advantage of MSCs over adult cells would be their anti-inflammatory property. However, MSCs possess a high tendency to

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Table 1
Rationale of various cell populations and numbers of studies available (Source: PubMed; Date:11-05-2018).

Cell type	Advantages	Concerns	Terms searched	No. studies
Nasal and articular chondrocytes	Natural population in cartilage	Low proliferation capacity and tendency to dedifferentiate Scarce availability. Additional knee surgery needed	Cartilage tissue engineering chondrocytes	4257
BMSCs	High chondrogenic potential	Surgical procedure needed. Tendency to hypertrophy	Cartilage tissue engineering bone marrow mesenchymal cells	876
ADSCs	High proliferation rate. Ease of isolation. High yield of isolation	Low chondrogenic potential	Cartilage tissue engineering adipose derived stem cells	356
SDSCs	High chondrogenic potential over extended passages. Their matrix is similar to hyaline cartilage in collagen type I and collagen type II	Scarce availability. Additional knee surgery needed	Cartilage tissue engineering synovial derived stem cells	97
Periosteum MSCs	High expansion potential	Invasive surgical procedure needed. Scarce availability	Cartilage tissue engineering periosteum MSCs	31
Peripheral blood MSCs	Ease of isolation, high yield	Not well studied	Cartilage tissue engineering peripheral blood MSCs	19
Skeletal muscle MSCs	Chondrogenic potential	Invasive surgical procedure needed. Scarce availability	Cartilage tissue engineering skeletal muscle MSCs	36
Umbilical cord MSCs	Abundant supply, painless collection, no donor site morbidity, fast and long self-renewal *in vitro*	Low isolation efficiency, tendency to produce fibrocartilage rather than hyaline cartilage	Cartilage tissue engineering umbilical cord MSCs	76
ESCs	High chondrogenic potential, low tendency to display hypertrophic phenotype	Safety and ethical concerns	Cartilage tissue engineering embryonic stem cells	189
iPSCs	High chondrogenic potential, low tendency to display hypertrophic phenotype	Safety concerns	Cartilage tissue engineering induced pluripotent stem cells	78
SF MSCs	Not well studied	Scarce availability. Additional knee surgery needed	Cartilage tissue engineering synovial fluid MSCs	20

acquire a hypertrophic phenotype when exposed to chondrogenic stimuli. Hypertrophic cells show an increase in cell volume, a decrease in the expression of collagen type II and a concomitant upregulation of collagen type X, osteocalcin, alkaline phosphatase (ALP) and matrix metalloproteinases (MMP) involved in cartilage remodelling; this finally results in the production of mineralised matrix [35–37]. Such changes are typically associated with endochondral ossification during fracture repair. Different sources of MSCs have been investigated (bone marrow, adipose tissue, synovium, periosteum, umbilical cord blood, peripheral blood, skeletal muscle and synovial fluid), while different *in vitro* culture systems have been described for mitigating MSC hypertrophic tendency. Other possible candidates for cartilage regeneration are induced pluripotent stem cells and embryonic stem cells that show low levels of hypertrophic markers during differentiation [38,39]. However, their intrinsic tumorigenic potential and ethical concerns have limited their experimentation. Although gene therapy has shown promise [40] and has been approved in Korea for the treatment of OA, it is still at its infancy, with many technical issues and limitations to be tackled, before an off-the-shelf treatment becomes available.

Herein, we first briefly describe the extracellular matrix (ECM) and cellular composition of articular cartilage, as well as chondrogenic mediators. We subsequently focus on all cell sources with the potential to be used in cartilage engineering and to promote cartilage regeneration. Cell culture methods (e.g. biophysical, biochemical and biological stimuli [41–51]) for preventing dedifferentiation and enhancing chondrogenic differentiation are also highlighted (Fig. 1). Although preclinical data are provided, considering that animal models are not representative of human pathology [52], we focus on completed and in progress human clinical trials.

Fig. 1. Schematic representation of cell types and *in vitro* approaches used to induce or maintain chondrogenic phenotype for cartilage engineering. Mesenchymal stem cells (isolated from bone marrow, adipose tissue, synovial membrane, cartilage, periosteum, umbilical cord blood, peripheral blood, skeletal muscle and synovial fluid), pluripotent stem cells and chondrocytes are commonly studied. Media supplementation with growth factors (mainly members of TGF superfamily), use of three-dimensional scaffolds, mechanical stimulation (compression, shear stress and hydrodynamic pressure), appropriate oxygen tension and co-culture systems are extensively studies as means to induce stable chondrogenic phenotype.

2. Articular cartilage and cell populations

Articular cartilage is a specialised type of hyaline cartilage that provides a smooth and lubricated surface for movable joints. It is an aneural, avascular and alymphatic tissue, composed of a dense ECM with a sparse distribution of chondrocytes. Collagen type II is the principal component of cartilage's ECM; it forms a fibrillar network that entraps the proteoglycan (PG) aggregates and provides tensile strength to the joint. Other types of collagen found in articular cartilage are collagen type XI that cross-links with collagen type II to form larger fibrils and collagen type IX that binds in a regular pattern the surface of collagen type II/collagen type IX fibrils, probably mediating interaction with others matrix proteins [53]. Finally, collagen type X gives structural support and is associated with the calcified cartilage layer. PGs form 10–20% wet weight and they consist of a core protein with one or more covalently attached glycosaminoglycan (GAG), a long unbranched polysaccharide consisting of a repeating disaccharide unit. They can be found either in large aggregates, named aggrecans, or as small PGs, such as biglycan and fibromodulin [54,55].

Articular cartilage is typically divided into 4 zones, starting from the joint space to the subchondral bone: superficial zone, transitional zone, radial zone and calcified cartilage zone (Fig. 2). The superficial zone is the thinnest of all layers and is covered with a thin film of synovial fluid. It possesses a high concentration of collagen and a low concentration of PGs and represents the layer with the highest water content. Transitional zone has a high concentration of aggrecans and collagen fibres obliquely arranged, whereas the deep zone contains the largest diameter-collagen fibres with chondrocytes organised in a columnar pattern perpendicular to the joint surface. The calcified zone contains calcified matrix and collagen type X. It provides resistance to compressive forces, given the high PG content, and ensures a cohesive connection to the underlying bone. Cells in this zone show hypertrophic phenotype and low metabolic activity [54,55].

Chondrocytes are the main cellular component of cartilage and they form only 1–5% of its volume. Their place as the sole cell present in cartilage has recently been challenged by studies indicating an even more rare population of chondroprogenitor cells (CPCs) [56]. Chondrocytes with their surrounding pericellular matrix are generally referred as 'chondron', which represents the primary mechanical, functional and metabolic unit of cartilage [57,58]. Characteristic in the ECM of the chondron is the presence of collagen type VI [59], which is involved in mechanical signal transduction, probably by binding cellular integrins or hyaluronic acid (HA) [60–63].

Chondrocytes are round shaped cells with a high metabolic activity and they are responsible for the synthesis of all cartilage ECM proteins. They do not need cell-to-cell contact, so they are sparsely spread within the matrix. In articular cartilage, chondrocytes are found as a heterogeneous population of cells with different properties, capability to synthesise proteins and to respond to mechanical and chemical stimuli. Cells from the middle and the deep zones produce more matrix rich tissue with higher compressive modulus and more GAGs, large aggrecan and collagen, whereas cells from superficial zone show weaker and slower cell attachment and they tend to form cells clusters with little matrix [64]. CPCs reside in the superficial zone of articular cartilage, they share various membrane markers with MSCs (e.g. CD9, CD44, CD54, CD90 and CD166), they express Notch-1, they have higher affinity for fibronectin compared to adult chondrocytes and they possess tri-lineage differentiation potential [56,65]. Recently, 2 different sub-populations of CPCs with different proliferative potential and capacity for telomere maintenance have been found in osteoarthritic cartilage [66]. However, CPCs have been only recently discovered and are not well characterized. Furthermore, after analysis of surface markers of chondrocytes during monolayer expansion, it has also been hypothesised that CPCs are de facto just de-differentiated chondrocytes [67,68].

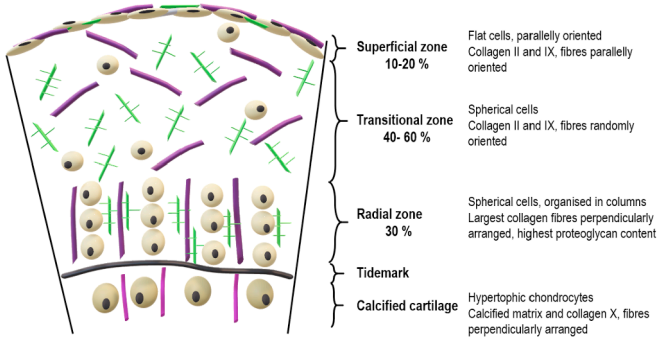

Fig. 2. Organization of articular cartilage, with characteristics of chondrocyte and ECM in the different zones. The superficial zone, which represents approximately 10% of the volume, contains cells and ECM fibres parallelly arranged. The transitional zone, which occupies about 40–60% of the volume, contains spherical cells and fibres obliquely arranged. The radial zone is characterized by spherical cells and large collagen fibres perpendicularly arranged. Calcified cartilage contains hypertrophic chondrocytes, calcified matrix and collagen type X.

3. Mediators of chondrogenesis

Chondrogenesis is a complex process occurring during embryonic and foetal development by which cartilage is formed from condensed mesenchymal stem cells that differentiate into chondrocytes. After chondrogenesis, chondrocytes of the peripheral region of the cartilage template remain as resting cells in the hyaline cartilage, while chondrocytes in the centre undergo proliferation, hypertrophic differentiation and apoptosis. During endochondral ossification, due to blood vessel invasion, osteoclasts and osteoblasts reach the zones of hypertrophic chondrocytes and replace it with bone (Fig. 3) [69].

Central mediators of chondrogenesis are the transcription factors Sex determining region Y box 9 (Sox-9) and Runt-related transcription factor 2 (Runx2). Sox-9 drives mesenchymal stem cells differentiation toward chondrogenic lineage and it is expressed during the first stages of chondrogenesis, whilst Runx2 is involved in the final hypertrophic differentiation and its activity is repressed by Sox-9 [70]. Sox-5 and Sox-6 work in cooperation with Sox-9. Like Sox-9, they possess a high mobility group (HMG) domain, but they lack the transcriptional activation domain and bind DNA as homodimers and heterodimers [71]. Expression of Sox-9 and Runx2 during chondrogenesis is finely regulated by different growth factors, most of them belonging to the transforming growth factor (TGF) superfamily, a large family of cell regulatory proteins comprised of more than forty members. In early

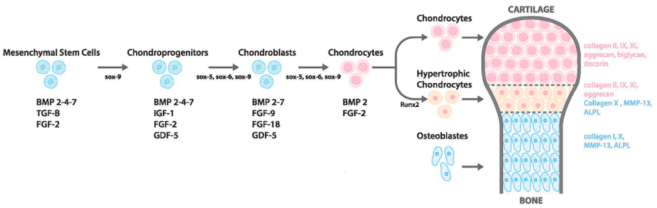

Fig. 3. Schematic representation of chondrogenesis and endochondral ossification during prenatal development. Mesenchymal cells differentiate into chondrocytes through different steps. This process is driven by Sox-9, Sox-5 and Sox-6 and involves different growth factors. Chondrocytes in the periphery of the cartilage template (epiphysis in the long bones) remain as resting cells, while ones in the centre (diaphysis in the long bones) increase the expression of Runx2 and become hypertrophic. A primary ossification centre will form in the central region and bone will gradually replace the cartilage. Resting chondrocytes produce hyaline cartilage whose major component is collagen type II, while hypertrophic chondrocytes produce calcified matrix and share some markers with osteoblasts (collagen X, ALP and MMP-13).

phases of chondrogenesis, they stimulate the expression of Sox-9 and in late phases, they modify the balance between chondrogenic and hypertrophic transcription factors determining the fate of chondrocytes [72,73]. TGF-β, bone morphogenetic proteins (BMPs), insulin growth factor (IGF) and fibroblast growth factor (FGF) are the most studied growth factors involved in chondrogenesis. TGF-β binds a TGF-β RI that recruits and phosphorylates a type II receptor. Depending on the type II receptor recruited, they can activate Smad2/3 pathway or the Smad1/5/8. Strong evidence suggest that Smad1/5/8 pathway cooperates with Runx2 to stimulate hypertrophy and an imbalance between Smad2/3 and Smad1/5/8 pathways has also been observed during OA progression and ageing [72,74,75]. TGF-β proteins also activate the MAP kinase mitogen-activated kinases (MAPK) cascade and they utilise Erks1/2, p38 and JNK as MAPKs. Particularly, p38 activates cartilage nodule formation, whilst Erk 1/2 induces hypertrophy and it regulates the effects of pro-inflammatory cytokines produced during OA progression [72].

BMP family include also the growth differentiation factor (GDFs) subgroup. BMP proteins stimulate MSC condensation, increase chondrocyte proliferation and matrix synthesis, but are also essential for the endochondral ossification [76,77]. They bind to type II receptor, which recruits a type I receptor, finally leading to the activation of Smad 1/5/8 pathway [72,78]. The role of BMP-2, BMP-4 and BMP-6 is controversial, as these growth factors are also well-known inducers of hypertrophic progression [79–81] and of bone formation [82,83]. On the contrary, BMP-7 appears to inhibit hypertrophic progression, probably by regulating the expression of the transcription factor Bapx1 [84,85].

Chondrogenesis takes place in a low oxygen environment (1–6% O_2) [86] that seems to be essential in promoting differentiation. While typically described as hypoxia, the fact that low oxygen is the natural *in vivo* situation the term 'physioxia' is increasingly used [87]. The main mediators of low oxygen are hypoxia-inducible factors (HIFs) that are heterodimeric transcription factors composed by α and β subunits. Under environmental oxygen tension (20% O_2), due to hydroxylation of specific proline residues, HIF α subunits are degraded through the proteasomal pathway. Conversely, when oxygen levels fall below 5%, hydroxylation is inhibited and HIF α subunits can heterodimerise with β subunits and translocate to the nucleus, where they activate Sox-9 expression. Two different α subunits HIF-1α and HIF-2α are involved in chondrogenesis [88,89]. Furthermore, low oxygen activates PKA/Akt pathway that prevents hypertrophic differentiation, presumably by suppressing the expression of Runx2 and inhibits terminal chondrocytes apoptosis by suppressing the activation of caspase 3 and 8 [89].

4. Chondrocytes

4.1. In vitro culture and media supplementation

In traditional ACI techniques, chondrocytes are isolated from non-load-bearing region of articular cartilage. Recent studies have suggested the use of chondrocytes isolated from nasal septum [90] or from auricular cartilage [91] that are easily available with a less invasive surgery. Nasal chondrocytes appeared to possess a higher proliferation and chondrogenic capacity than articular chondrocytes [92,93]. When seeded in collagen I/III matrices, they formed thicker constructs, with a higher GAG content than articular chondrocytes [90]. Further studies are needed to demonstrate the superiority nasal spectrum/auricular cartilage chondrocytes over the articular ones.

Independently of the chondrocyte tissue origin, *in vitro* expansion of chondrocytes is difficult, since they have a low proliferation capacity and they tend to lose their morphology, phenotype and function (Table 2). When cultured in monolayer, they gradually switch from producing aggrecan to producing smaller PG species and from synthesising collagen type II to synthesising collagen type I, resulting in a change of biomechanical properties of the ECM [94]. Furthermore, they lose key chondrocyte markers, such as Sox-5, Sox-6, Sox-9 and increase the expression of hypertrophy markers, such as ALP and Runx2 [95]. The phenomenon of dedifferentiation has been reported as early as the first passage in monolayer culture [96,97] and it represents one of the biggest limitations in cartilage engineering. Methods described to enhance cell proliferation and mitigate dedifferentiation include addition of growth factors to culture media, culture of cells in three-dimensional scaffolds, reduction of oxygen tension and application of mechanical stimuli. Finally, some bioreactors specifically designed for chondrocytes enhance cell proliferation and increase the synthesis of a hyaline-like matrix.

Factors commonly used in chondrocyte culture include members of TGF superfamily, that are the physiological regulators of chondrogenesis and are able to stimulate *in vitro* cell proliferation and synthesis of ECM proteins [98]. TGF-β increased proliferation and stimulates synthesis of collagen type II in chondrocyte culture [99]. Among BMP family members, BMP-2 has been reported to induce chondrogenic differentiation of pre-chondrogenic cell [100] and BMP-7 is known as an inductor of cartilage formation and inhibitor of the terminal differentiation of pre-hypertrophic chondrocytes into hypertrophic chondrocytes [101]. Growth differentiation factor-5 (GDF-5) is another member of the BMP family, often used in combination with insulin. It is able to enhance collagen type II expression, without increasing the expression of collagen type I [102]. IGF-1 and FGF-2 mainly modulate cartilage metabolism [103]. IGF-1 has been shown to prevent chondrocyte apoptosis [104] and to stimulate the expression of type II collagen and proteoglycan in pre-chondrogenic cells [105], whereas bFGF has been shown to elicit dose-dependent effects on chondrocyte mitotic activity and anabolic processes [106]. Insulin, transferrin and selenium (ITS) is often used in combinations with growth factors, as it increases collagen type II synthesis and keeps constant the expression of collagen type I [107]. Coating of culture plastic dishes with collagen type I [108], collagen type II [109] or ECM proteins secreted by synovial cells [110] has been proposed as a method for maintaining phenotype in monolayer culture. However dedifferentiation has been reported also in chondrocytes grown on collagen type I, collagen type II and fibronectin coated plastic dishes [111]. While in traditional ACI technique chondrocyte expansion was performed using autologous serum, the majority of *in vitro* research studies, as well as some variants of ACI [112], used foetal bovine serum [108,113–116]. *In vitro*, human serum, which contains higher levels of EGF and PDGF-β, leads to an increase in proliferation rate up to 7 times compared to FBS [117]. To overcome problems related with the use of FBS, such as the variability in serum quality and potential presence of contaminants, some studies proposed a serum-free culture medium that contained PDGF-β, FGF-2 [118], EGF and dexamethasone [119]. Chondrocytes cultured in serum-free condition showed the same proliferation ability than the ones cultured in FBS [88]. Platelet-rich plasma (PRP) is a fraction of plasma that contains high levels of multiple growth factors [120]. It can be used as an alternative of FBS for chondrocytes expansion, as it been shown to support chondrocyte expansion [121] and matrix synthesis [122]. Human platelet lysate is derived by mechanical disruption of platelets and contains all platelet related factors, used as an alternative to animal serum [123]. However, a few studies have demonstrated that it failed to induce the deposition of typical cartilage matrix components [124,125]. Given the importance of cell-cell contact in preventing dedifferentiation [126], multilayer chondrocyte sheets have been fabricated and have been shown to increase the expression of collagen type II [127,128] and fibronectin and aggrecan [127] and to decrease the expression of collagen type I [127,128] compared to traditional monolayer. However, despite the large variety of conditions tested, there is still no clear consensus on the optimal media supplementation and *in vitro* conditions for expanding chondrocytes.

4.2. Three-dimensional scaffolds

The general trend in tissue engineering is culturing chondrocytes in

Table 2
Changes in protein synthesised by chondrocytes during expansion. Dedifferentiation of chondrocytes is accompanied by loss of most of the chondrogenic markers and upregulation of some of the hypertrophic markers.

Phenotype	Molecule	Changes associated with dedifferentiation	Ref
Chondrogenic phenotype	Collagen type II	↓	[423,424]
Chondrogenic phenotype	Aggrecan	Constant	[53,424]
Chondrogenic phenotype	Sox-9	↓	[53,423]
Chondrogenic phenotype	Sox-5, Sox-6	↓	[53]
Chondrogenic phenotype	IGFR2	↓	[215]
Chondrogenic phenotype	VEGFR1	↓	[215]
Chondrogenic phenotype	CD44	↑	[53,423]
Chondrogenic phenotype	CD151	↑	[423]
Chondrogenic phenotype	CD54 (ICAM-1)	↓	[53,423,425]
Chondrogenic phenotype	VCAM 1	↓	[215,425]
Fibroblastic phenotype	Collagen type I	↑	[423,424]
Hypertrophic phenotype	Collagen type X	Constant	[53,423]
Hypertrophic phenotype	Runx2	↑	[53]
Hypertrophic phenotype	MMP-1, MMP-3	Constant or reduced	[423,424]
Hypertrophic phenotype	ALP	↑	[53]
Hypertrophic phenotype	MMP-13	Constant or reduced	[53,424]
Chondrogenic/hypertrophic phenotype	BMP-2	↓	[215,423]
Chondrogenic phenotype	TGF-β2	↓	[78,426]
Hypertrophic phenotype	VEGF	↓	[215]
Hypertrophic phenotype	FGFR3	↓	[78]

three-dimensional scaffolds, as they have shown more success in retention of phenotype than monolayer systems. Scaffolds can be used for the re-differentiation of chondrocytes that, after monolayer expansion, have undergone dedifferentiation. Culture of cells in three-dimensional scaffolds is believed to inhibit cell spreading on the substrate and the formation of actin stress fibres that probably mediate the dedifferentiation process [129].

Over the past decades, different architectures and materials have been investigated for their capability to maintain *in vitro* chondrogenic phenotype and to provide adequate mechanical properties necessary for the initial *in vivo* support. High porosity scaffolds facilitate chondrocyte attachment [130] and, for this reason, sponges are widely used [131–136], due to their high porosity and interconnectivity degree [137]. Micro- and nano-fibrous scaffolds, although do not provide an actual three-dimensional support, have also been used [134,138–140], as they can provide topographic cues [141] and high porosity [137]. Hydrogels can retain high water content [142] and transmit mechanical loads to encapsulated cells [143], mimicking physiological conditions or porous scaffolds that facilitate cell infiltration [144]. Numerous natural [129,135,145–151] and synthetic [152–156] biomaterials have been investigated in the quest of the ideal one to maintain chondrogenic phenotype *in vitro*. Fibrin contains native arginine-glycine-aspartic acid (RGD) sites for chondrocytes attachment [157,158] and it can support growth and matrix synthesis of chondrocytes [153]. Fibrin gels alone [159] or reinforced with natural [157] or synthetic [153] polymers have also demonstrated enhanced collagen type II and aggrecan synthesis of both articular [153,157] and nasal chondrocytes [159]. HA has been widely used due to its large natural presence in the ECM and was able to induce dedifferentiated chondrocytes to increase collagen type II synthesis and to decrease collagen type I synthesis [147]. Since collagen type II is the main component of cartilage, some studies compared the chondrogenic properties of scaffolds composed of collagen type II and collagen type I. While in one study, no differences in term of phenotype maintenance of goat chondrocytes were observed between collagen type I and II sponges [135], one study showed that collagen type I sponges were not as able as collagen II sponges to induce collagen type II synthesis and to maintain the round morphology of canine chondrocytes [136]. Agarose hydrogels have been shown to maintain chondrogenic phenotype for longer than monolayer culture systems [145]. Culture of human chondrocytes in agarose gels resulted in the formation of 2 regions: a central region with cartilaginous matrix, containing cells with typical chondrocytes morphology and a peripheral region at the interface with the media, containing cells with a more elongated morphology and a higher proliferation rate [160]. Alginate beads were able to preserve phenotype in a long term (up to 8 months) culture [161] and have been used for re-differentiating chondrocytes that have been expanded in two-dimensional culture [162]. Bovine articular chondrocytes cultured for an extended period in alginate beads, formed two different subpopulations: the first was near the surface of the beads and was composed of flattened cells surrounded by a matrix spare in PGs and collagen fibrils, whereas the second was throughout the depth of the beads and composed of round cells surrounded by densely-packed fibrils running tangential to the surface [161]. Decellularised matrices and cartilage derived matrices have been shown to support neocartilage formation in the absence of exogenous growth factors [163]. Appropriate exposure of human chondrocytes to specific ECM proteins enhances synthesis of other cartilage proteins and can be used to produce chondrocytes with various phenotypes [164]. Recently, silated hydroxypropyl methylcellulose hydrogels (Si-HPMC) have been used to maintain chondrogenic phenotype. Rabbit chondrocytes cultured for three weeks in Si-HPMC expressed collagen II and aggrecan at higher levels and collagen I at lower level than ones of freshly isolated chondrocytes [165], whilst dedifferentiated rabbit nasal chondrocytes, reacquired the expression of collagen II and aggrecan and decreased the expression of collagen I, after 4 weeks of culture in these hydrogels [148]. Also, monolayer expanded human nasal chondrocytes cultured for three weeks in these scaffolds in the absence of exogenous growth factors, expressed collagen II and aggrecan, whilst their expression of collagen I was lower than the one of freshly isolated cells [149].

The influence of scaffolds' architecture has also been under intense investigation in order to correlate chondrocyte shape and phenotype. When human chondrocytes were cultured in a non-woven HA web (Hyalograft®C), collagen type I/III fleece (Chondro-Gide®), collagen type I gel (CaRes®) and collagen type I/chondroitin sulphate sponges (Novocart® 3D), the authors found that in all type of scaffolds, except the sponges, 'foot-like' extensions were present and the predominant cell morphology was elongated. Only in collagen sponges, cell extensions were present that physically enwrapped small diameter fibres and cells mainly maintained a spherical morphology. The authors speculated that only in scaffolds with small diameter fibres, chondrocytes can retain their spherical shape and that whilst cell extensions enwrapping the fibres are proper permanent adhesion structures, the 'foot-like' extensions are temporary cell shape alteration that control the de-

differentiation process [134]. However, in this study, analysis of chondrogenic markers and characterisation of ECM synthesised were not performed, probably assuming that cell morphology is a sufficient indicator of cell phenotype. Another study investigated the relationship between fibre dimensionality and chondrocyte phenotype maintenance; the authors found that electro-spun poly-L-lactide (PLLA) nano-fibres, as opposed to PLLA micro-fibres, better maintained morphology and phenotype of bovine chondrocytes in terms of collagen type II, collagen type IX, aggrecan and cartilage PG link protein synthesis [139]. Fibre orientation has also been studied as a factor affecting cell morphology, although there is no clear consensus. In poly(p-dioxanone) (PPDO), polyetherimide (PEI) and co-poly(ether)esterurethane (PDC) electrospun scaffolds of 2–3 μm of size, porcine chondrocytes better maintained their round morphology, when the fibres were aligned. However, collagen type II was synthesised at similar levels, independently of scaffold composition and fibre orientation [140]. However, in another study, bovine chondrocytes on aligned electro-spun poly-ε-caprolactone (PCL) fibres (1 μm diameter size) showed an elongated morphology, whilst on random fibres, they maintained their rounded shape. Despite the differences in morphology, synthesis of collagen type I and II appeared to be similar in both types of fibres, meaning that probably cell morphology is not a sufficient indicator of cell phenotype [138]. A synthetic scaffold with hexagonal pore geometry has been shown to enhance chondrocyte attachment *in vitro*, when compared to scaffold with square geometry [141].

Another study compared the behaviour of porcine chondrocytes in chitosan sponges (with pore size of about 100 μm) and hydrogels (with pore size of about 20 μm) and showed that cells in sponges proliferated faster but had a more elongated morphology. Although the synthesis of collagen type II and aggrecan was similar in the two scaffolds, the synthesis of collagen type I and X was lower in the hydrogels [133]. The authors hypothesised that the smaller pore size and low structural interconnectivity of hydrogel results in a limited diffusion of nutrients that better mimics the native nutrient-poor avascular native microenvironment in which chondrocytes reside [166]. Regarding porous solid scaffolds, the quest for the ideal pore size continues. A study seeded bovine chondrocytes on porous titanium alloys and showed that in smallest pore sizes (13 μm compared to 28, 43 and 68 μm), chondrocytes proliferated faster and the newly-formed tissue was thicker. However, the collagen accumulated per cell was lower [167]. Another study, which used collagen porous scaffolds, identified 150–250 μm as the best pore size for maximum ECM synthesis [168]. Another study showed that rat chondrocytes proliferated faster and expressed collagen type II and aggrecan at higher levels (although also expression of collagen type I and X increased) in porous gelatin scaffolds with a pore size of 250–500 μm, compared to smaller (50–250 μm) pore sizes [169].

Apart from scaffold composition and architecture, the mechanical properties of the scaffolds have been shown to influence ECM production and secretion. It has been shown that by modifying the equilibrium swelling ratio and the compressive modulus of polyethylene glycol (PEG) hydrogels, it was possible to control the localisation of cell-secreted GAGs (pericellular or homogeneous within the hydrogel) and to increase collagen type II synthesis of calf chondrocytes [170]. Despite the large number of studies, there is still no consensus on the optimal system for expanding chondrocytes and probably further investigations on the relation between scaffold architecture, cytoskeleton rearrangement and dedifferentiation are needed for a rational design of scaffolds.

4.3. Oxygen tension

Physiologically speaking, low oxygen tension promotes chondrogenic differentiation of MSCs during pre-natal limb development, whereas adult chondrocytes reside within avascular hyaline cartilage and are bathed in low oxygen synovial fluid. The microenvironment oxygen tension of cartilage is as low as 1% [171]. Both hypoxia (5%) and severe hypoxia (1%) combined with three-dimensional culture, have been shown to enhance the expression of collagen type II, Sox-9 and aggrecan in human chondrocyte culture, although they also increased expression of collagen type I [172]. Furthermore, culture of passaged human articular chondrocytes in 5% oxygen tension in alginate beads allowed restoration of the native chondrogenic phenotype (similar of freshly isolated chondrocytes) in a mechanism mediated by the transcription factor Sox-9 [94]. Furthermore, human OA chondrocytes that showed hypertrophic phenotype and did not express typical chondrogenic markers neither *in vivo* nor *in vitro* reacquired the expression of collagen type I and II and aggrecan at levels similar to those of healthy chondrocytes when they were cultured under low oxygen tension (2% and 5%) in pellet with TGF-β1 [173,174].

4.4. Mechanical stimulation

Perfusion and airlift bioreactors have been used for chondrocyte expansion, since they improve cell growth, maintain the characteristic chondrocyte round morphology, permit cartilage-specific ECM secretion and allow an even cell distribution in the scaffolds [175–177]. Calf and rabbit chondrocytes cultured in these bioreactors synthesised ECM proteins, such as collagen type II, aggrecan and superficial zone protein, even in the absence of exogenous growth factors in the presence of 10% FBS [177,178]. Recently, ultrasound bioreactors have been used for chondrocyte culture [179–181]. In these bioreactors, chondrocytes in three-dimensional scaffolds [179,180] or in liquid solution [181] were forced to aggregate and form agglomerates by acoustic radiation forces. Human OA chondrocytes cultured for 21 days in a serum-free medium containing TGF-β3 and dexamethasone in an ultrasound bioreactor increased synthesis of collagen type II and aggrecan, while the expression of collagen type I and collagen type X were negligible [181].

Bioreactors are also able to provide mechanical stimulation mimicking the native cartilage microenvironment. Articular cartilage experiences a complex combination of compressive deformation, shear loading and hydrodynamic stress during the articulation of synovial joints. Direct compression results from direct contact between joint surfaces and during normal daily activities overall cartilage thickness compresses from 5 to 20%, with the higher compression near the articular surface [182]. Articulation of synovial joints does not result only in compression in the direction of the applied load, but also in expansion in the transversal direction (shear stress) that is highest near the articular surface. Moreover, cartilage is a highly hydrated tissue, due to negatively charged PGs that attract water. During joint loading, water from the synovial fluid is retained within the cartilage resulting in an increase of hydrostatic pressure (HP) that is uniform imparted to chondrocytes. The chondrocyte response to these mechanical stimuli is recognised as an integral component in the maintenance of articular cartilage matrix homeostasis and an important parameter to consider when simulating chondrocyte *in vivo* microenvironment [183]. Regarding hydrostatic pressure, in monolayer culture systems, intermittent application of hydrostatic pressure was beneficial in terms of ECM production, whereas a static pressure seemed to have no effects [184]. Application of intermittent hydrostatic pressure (10 MPa 1 Hz, 4 h/day) to bovine articular chondrocyte resulted in an increasing in collagen type II and aggrecan synthesis and secretion [185]. By contrast, static HP regimens (5 and 10 MPa and 2.8 MPa) have been found to increase GAG and collagen synthesis in agarose culture systems of calf chondrocytes [186] and in collagen scaffolds systems of bovine chondrocytes [184,187]. Dynamic compression (0.1 Hz) of calf cartilage explants up-regulated the expression of collagen type II, aggrecan and transcription factors, such as Jun, Fos and Sox-9 [188]. Dynamic compression (1 Hz) has also been shown to increase ECM deposition of calf chondrocytes grown in agarose hydrogels [189]. Intermittent application of a cyclic sinusoid compression (1 Hz, 1 h/5 h) on human OA chondrocytes seeded in collagen type I hydrogels resulted in an increase of collagen type II and aggrecan expression and a strong decrease in MMP-13 expression [116]. However, it has been proposed that the

duration of load should be limited during the early *de novo* tissue formation [190]. Increases in mRNA expression of aggrecan and collagen II peaked after 2 h of cyclic loading, whereas 8 h per day cyclic load resulted in a lower GAG/DNA than static controls [190]. This is potentially due to the load preventing the newly formed ECM from consolidating into the pericellular matrix.

The low shear stress provided by a rotating bioreactor was sufficient for increasing ECM synthesised by bovine chondrocytes, resulting in the generation of a graft with mechanical properties similar of those of native cartilage [191]. Furthermore, human dedifferentiated chondrocytes and human OA chondrocytes cultured in rotating bioreactor in the absence of exogenous growth factors (with 10% FBS) reacquired the expression of collagen type II and formed a solid tissue during culture [192]. Dynamic shear had a markedly pro-anabolic effect on gene transcription, inducing the upregulation of aggrecan, collagen type II, link protein, fibromodulin, fibronectin, TGF-β of calf chondrocytes [188]. In calf cartilage explants, IGF-1 further augmented ECM deposition and PG synthesis induced by dynamic shear [193]. Some studies observed that bovine cells from the superficial zone and cells from the deep zone respond to compression [194,195] and to oscillatory tensile load [196] differently. Particularly, deep cells, if exposed to dynamic strain of 0.3 Hz inhibited GAG synthesis and when exposed to 1 Hz increased their GAG production. By contrast, superficial cells exhibited a general inhibition of GAG synthesis under compression [194]. Furthermore, superficial cells responded to compression by increasing their proliferation rate, whereas deep cells proliferation rate was not influenced [195]. Oscillatory tensile loading did not influence matrix synthesised by deep cells, but increases both total GAG retained in the graft and released to the media by superficial cells [196]. A bioreactor combining compressive load and shear stress induced higher levels of matrix synthesised by bovine chondrocytes than the single-factor loading regimens [197], suggesting that combination of different mechanical stimuli can further increase chondrogenesis. Recently, a tribological system has been described, where chondrocytes, embedded on a scaffold, were pressed onto a conforming ball that oscillates orthogonally, in a way to reproduce interfaces motion of joint kinematics. Compressive loads were applied along the cylindrical axis of the scaffold, causing a shear force. Bovine chondrocytes seeded onto polyurethane scaffolds and expanded in this bioreactor exhibited elevated levels of collagen type II and cartilage oligomeric matrix protein (COMP) [198]. The majority of the studies on mechanical stimulation in three-dimensional culture systems, however, have been performed with animal cells, so the outcomes need to be further evaluated using human cells before coming to definitive conclusions.

4.5. Preclinical studies

Chondrocytes from different sources have been compared in preclinical studies. Nasal chondrocytes have been implanted in cartilage defects of rabbit [148] and goats [115], in which their transplantation, in contrast to transplantation of articular chondrocytes, did not lead to any increase in subchondral bone area; a sign of onset of OA. The capability of auricular chondrocytes to produce ectopic cartilage has been tested in mice [113,199] and auricular and nasal chondrocytes encapsulated in alginate beads produced higher amount of ECM compared to articular ones [199]. However, another study showed no differences in ectopic cartilage produced by porcine articular, auricular and nasoseptal chondrocytes using PGA scaffolds. Furthermore, independently of cell type, in this study, all the constructs *in vivo* showed some cell-free and some ossified areas [200].

A few studies have proposed to selectively isolate and implant chondrocyte sub-populations that possess a higher chondrogenic potential. A study assessed the ability of CPCs to produce ectopic cartilage [114], while another one showed that subcutaneous implantation of chondrocytes with a higher proliferation rate (selectively isolated through CellTrace™ CFSE) leaded to a higher PG synthesis compared to ones with lower rate (although it was not clear if this was due to a higher synthesis or to a higher number of cells) [201].

Compared to bone marrow mesenchymal stem cells (BMSCs), in one study pellets of articular chondrocytes were able to produce more hyaline ECM, when subcutaneously implanted in mice [202], while in another study, no differences in terms of GAGs and collagen synthesis were observed between cells encapsulated in alginate beads [199]. Discrepancies in these results reflect the importance of finding better cell characterisation methods and adequate *in vitro* pre-culture systems.

In general, pre-expansion of chondrocytes in synthetic [151] and biological [203,204] three-dimensional scaffolds increases their *in vivo* chondrogenic potential [150]. A synthetic scaffold with a hexagonal pore geometry has been shown to increase collagen type II synthesis *in vivo* in rabbit cartilage defects, compared to collagen type I membrane [141]. Also, chondrocytes pre-expanded *in vitro* in alginate scaffolds and then isolated and implanted as scaffold-free constructs have been evaluated in porcine defects and resulted in higher collagen and GAG content and better macroscopic regeneration, compared to non-treated controls [203]. Despite the large number of pre-clinical studies, it is difficult to compare *in vivo* efficacy of different culture systems for chondrocytes, due to the lack of standardisation of designs, controls, outcome parameters and animal models used. The International Cartilage Repair Society (ICRS; Table 3) recommended the use of large animal models, such as goat, sheep and horse, with an adequate skeletal maturity and to avoid the use of species that possess a high auto-healing capability (e.g. rabbits) [205]. Furthermore, since human patients are generally diagnosed and operated after a certain period of symptom progression, ICRS suggested the use of chronic chondral defect models [206] that, compared to acute ones, better mimic the timing, the degeneration and subchondral bone overgrowth of human patients. Guidelines establishing animal models, timepoints and measured outcomes, have also been published by Food and Drug Administration (FDA) and European Medicines Agency (EMA) (Table 3). A study identified 1187 articles on cartilage repair in large animal models published between 1995 and 2015 and estimated that the overall adherence to guidelines ranged from about 32 to 58%, with adherence to measured outcomes even lower than one to study descriptors. It was found that histology was the most reported outcome (97% of the examined studies), despite differences in methods employed were evidenced, with some studies providing semi-quantitative methods, others basic histology and others advanced imaging techniques, such as polarized light. Gross view examination and defect fill were reported by more than 50% of the examined papers, whilst follow-up arthroscopy by less than 8% and gene expression by 11% [207]. Further, a large variety of small [208,209] and large [150] animals and defect models have been used (principally subcutaneous [210,211] and acute intra-articular [208,209]). Particularly, regarding subcutaneous models, one study hypothesised that the production of hyaline matrix may be attributable to MSC migration from adjacent tissues rather than to a chondrogenic phenotype re-acquirement or maintenance by chondrocytes. In this study, human chondrocytes (in collagen matrices, fibrin gels or as aggregates) were subcutaneously implanted inside a permeable chamber that prevented host cell invasion. After 12 weeks, no hyaline matrix was produced in the constructs and collagen and fibrin matrices were not even reabsorbed, contrarily to other studies that, after subcutaneous implantation, had observed a great increase in synthesis of hyaline matrix [212] compared to *in vitro*. However, crucially the authors did not use a control group without the chamber [213].

In addition to the difficulty in comparing results from different *in vivo* models, it is also difficult to translate them to human, since animal cartilage defects have a size much smaller than clinically challengeable and a high involvement of subchondral bone. While in animals, subchondral bone can be of about 80–90% of the whole defect, in human it is usually not affected and it is even believed to act as a barrier that limits the endogenous repair mechanisms [205,214].

Table 3
Recommendation of Food and Drug Administration (FDA), International Cartilage Repair Society (ICRS) and European Medicines Agency (EMA), regarding animal models and measured outcomes for products to repair cartilage defects. NS = Non-specified. Sources: 'FDA Guidance for Industry. Preparation of IDEs and INDs for products intended to repair or replace knee cartilage' [427], 'Preclinical studies for cartilage repair: Recommendations from the international cartilage repair society' [205], 'EMA Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee' [428].

	FDA	ICRS	EMA
Mechanical testing	Compressive strain, aggregate modulus, the shear modulus permeability, dynamic mechanical behaviour of the product including an assessment of the complex shear modulus, failure properties	Indentation and dynamic compression tests, other assays depending on the product tested.	NS
Animal models	NS	- Rodents: proof of principle, clearance, toxicology, safety - Rabbits: proof of principle, developmental, formulation screening - Horse, sheep, goat, pig: pivotal studies	- Small animal model: study of ectopic cartilage formation. - Goat, horse or sheep, pigs or cows: pivotal studies. To avoid the use of immunocompromise large animals, it is suggested the use of autologous animal cells.
Cartilage defect	Location: analogous to patients	- Type: Chronic defects are more predictive than acute ones. - Location: femoral condyle or trochlea. - Size: critical-size defects for pivotal studies	NS
Timepoints	Minimum 1 year, for completion of healing and for assessment of durability of the therapeutic response and of the integrity of the product. Timepoints may vary depending on product characteristics	- 1–30 days to test implant retention - 1–6 month for proof of principle - 6–12 months for pivotal studies - May exceed 12 months for slowly degrading implants	NS
Interim analysis	Arthroscopic assessments, MRI.	NS	NS
Outcomes	MRI, arthroscopy, physical findings from examination of the knee joint. Histology to assess: matrix zonal organization; cell density and cell morphology; collagen types and concentration; aggrecan concentration, size and composition; other proteoglycan concentrations; non-collagenous protein concentrations; inflammatory response. Assessment of synovial fluid samples for cell count, and, as applicable, markers of inflammation and antibody formation. Serological assessments for antibody formation and evidence of inflammation.	Repair tissue histology, gross macroscopic scoring of the whole joint, biomaterial clearance and cell tracking, biomechanical testing (e.g. *in vivo* indentation), MRI, micro-computed tomography.	Macroscopic, histological and MRI assessment of the repair tissue, biomechanical testing. Biodistribution, toxicity depending on the product

4.6. Clinical trials

Some on-going studies are testing different protocols for cell isolation and expansion, for reducing chondrogenic dedifferentiation and improving clinical outcomes (Table 4). A variant of the ACI that has been granted market authorisation by the European regulatory body is the Characterized Chondrocytes Implantation (CCI). The rationale of CCI is that, since articular cartilage is composed of a heterogeneous population of chondrocytes, a selective isolation of cells with a stable phenotype can improve histological quality of substitutes. In CCI chondrocytes with a marker profile predictive of the capacity to produce a stable hyaline cartilage are selectively isolated and implanted [215]. The resulted medical product, named ChondroCelect®, has been compared with micro-fracture, and showed improved clinical outcomes [216,217].

CPCs in collagen scaffolds were implanted in large defects ($> 6\ cm^2$) of 15 patients. One year after transplantation, clinical scores were increased and the defect was fully covered by new hyaline-like ECM in 13 patients, with regular subchondral bone contour in 14 patients [114].

A phase I clinical trial involving 25 patients with full-thickness defects evaluated the efficacy of expanded nasal chondrocytes in collagen type I/III matrices transplantation (NCT01605201). 2 years after transplantation, the self-assessed clinical scores for pain, knee function and quality of life were improved [218]. However nasal and articular chondrocytes have still not been directly compared in clinical trials.

In another recently developed technique named BioCart™, chondrocytes are grown in monolayer in the presence of a variant of FGF and autologous serum, before being seeded onto a fibrin and HA scaffold and implanted. Eight patients undergoing this treatment showed clinical improvement by an increase of clinical scores (Lysholm and IKDC), with no adverse events after one year follow up [219]. An on-going clinical trial is comparing this treatment with traditional micro-fracture in patients with cartilage defects smaller than $5\ cm^2$ (NCT00729716). While in BioCart™, the three-dimensional scaffold is just used as a carrier for delivering chondrocytes, other studies are trying to enhance chondrogenic phenotype maintenance by *in vitro* expansion of chondrocytes in three-dimensional scaffolds. BioSeed®-C is a tissue engineered cartilage graft, composed of autologous chondrocytes grown on scaffolds made from fibrin, PLGA and polydioxanone. A recent study analysed the efficacy of this product in 52 patients with full thickness cartilage defects in the knee, including a subgroup (19 patients) of patients with OA. After 4 years, almost all patients showed moderate to complete filling of the defects, but the maximum strength capacities for knee flexion and extension remained low [220]. NeoCart® is another product made by autologous chondrocytes expanded onto collagen type I scaffolds under hypoxic conditions and exposed to mechanical stimuli in a bioreactor. This product has already passed the phase I clinical trial. A recent 30 patients, 2-years, phase II clinical trial compared NeoCart® treatment with micro-fracture. It reported that implantation of NeoCart® in 3-grade lesions (where defects involve more than 50% of cartilage layer) resulted in a higher score on the knee injury and osteoarthritis outcome score, with a safety similar of that of micro-fracture [221]. Allogeneic chondrocytes, combined with a proportion of irradiated cells transduced to over express TGF-β are currently undergoing phase III trials with the product name Invossa®. HA has also been

Table 4
Cell therapy products, with chondrocytes, proposed for the treatment of focal chondral defects, with clinical trials, defect sizes of patients enrolled and published results. NS= Non-specified. Source: clinicaltrials.gov.

Name		Description	Clinical trial	Phase	Chondral defect size/Control group	Status clinical trial/Published results
ASCROD		Implantation of autologous chondrocytes or ADSCs	NCT01399749	I/II	1–5 cm^2	No results posted
BioCart™II		Chondrocytes grown in monolayer in the presence of a variant of FGF and autologous serum, seeded on a fibrin and HA scaffold	NCT00729716	II	1.5–7.5 cm^2 Control: microfracture	Preliminary results after 1 year, showed clinical improvement (functional scores and MRI) (N = 8) [219]
BioSeed®- C		Autologous chondrocytes grown on scaffolds made from fibrin, PLGA and polydioxanone	N/A	N/A	Grade 3 or 4 No control group	After 2 years, similar functional scores compared to traditional ACI (N = 23) [429]
						After 4 years, clinical improvements complete filling of the defects, but maximum strength capacities for knee flexion and extension remained low (N = 52) [220]
Cartilage Autograft Implantation System (CAIS)		Biodegradable scaffold of PCL and PGA, with particulated autologous articular cartilage	NCT00881023	N/A	1–10 cm^2 Control: microfracture	After 2 years, better clinical score (KOOS) in CAIS compared to microfracture, with lower incidence of intralesional osteophyte formation (N = 29) [430]
CARTIPATCH®		Autologous chondrocytes in an agarose-alginate hydrogel	NCT00945399	III	2.5–7.5 cm^2 Control: microfracture	Preliminary study showed at 2 years, clinical and histological improvement, with higher improvement for lesions larger than 3 cm^2 (N = 17) [431]
			NCT00560664	III	2.5–7.5 cm^2 Control: mosaicplasty	After 2 years, lower score improvement, worse functional outcomes and higher number of adverse events in Cartipatch®, compared to mosaicplasty (N = 58) [432]
Cells Sheet-Autologous Chondrocyte Implantation (CS-ACI)		Autologous chondrocyte cell sheet implantation	NCT01694823	I/II	2.5–5 cm^2 No control group	No results posted
Chondron™		Implantation of fibrin mixed with autologous chondrocytes	NCT01056900 NCT01050816	III	NS No control group	Functional score improvement at 25 months (N = 98) [433]
Chondrosphere®		Chondrocyte spheroids average 59 spheroids/cm^2	N/A	N/S	1–12 cm^2 No control group	Functional improvement at one year (N = 37) [434]
		Chondrocyte spheroids at different doses: 3–10, 10–30, 40–70 spheroids/cm^2	NCT01329445	II	4–10 cm^2 No control group	The treatment was tolerated; no difference in adverse events between the groups (N = 73) [435]
		Chondrocyte spheroids 10–70 spheroids/cm^2	NCT01222559	III	1–4 cm^2 Control: microfracture	No results posted
DeNovo® NT		Particulate juvenile cartilage tissue	NCT01670617	N/A	Smaller than 7.5 cm^2 No control group	Preliminary results showed good defect fill, after 2 years (N = 25) [430]
			NCT00791245	IV	N/A	No results posted
Hyalograft®		Autologous chondrocytes grown on a scaffold made of an esterified derivative of HA.	Case series		Average size 4.4 cm^2	Improvement of clinical scores, with a failure rate of 22.6% after 9 years (N = 58) [436]
INSTRUCT		Autologous chondrocytes and BMSC seeded on a biodegradable porous scaffold	NCT01041885	N/A	NS No control group	At 2 years, defects were filled with hyaline-like ECM and pain reduction (N = 40) [437]
NeoCart®		Autologous chondrocytes expanded in collagen I scaffold	NCT00548119	II	NS Control: microfracture	At one year, better clinical score compared to microfracture. 76% patient responded to NeoCart® therapy, only 22% in the microfracture group [221].
						Clinical outcome scores remained constant at 60 months, but there was evidence of reorganisation of repair tissue and subchondral bone changes (N = 29) [438]
			NCT01066702	III	NS Control: microfracture	No results posted
Nose2Knee	N-TEC	Autologous nasal chondrocytes seeded for 2 weeks on collagen I/III matrix, to give cells the time to produce their ECM	NCT02673905	N/A	2–8 mm^2 No control group	Recruiting
	N-CAM	Autologous nasal chondrocytes seeded for 2 days on collagen I/III matrix				
		Autologous nasal chondrocytes seeded on collagen I/III matrix	NCT01605201	I	2–8 mm^2 No control group	Preliminary results showed improvement of pain, knee function and quality of life at 24 months [218]

(*continued on next page*)

Table 4 (*continued*)

Name	Description	Clinical trial	Phase	Chondral defect size/Control group	Status clinical trial/Published results
NovoCart® 3D	Autologous chondrocytes within a collagen type I sponge	Case series		Larger than 2 cm^2	At 2 years, improvement in symptoms in patients treated [439]
		NCT01957722	III	2–6 cm^2 Control: microfracture	Recruiting
		NCT03219307	III	Patients of NCT01957722 with microfracture failure	Recruiting
		NCT01656902	III	2–6 cm^2 Control: microfracture	No results posted
RevaFlex™	Juvenile human chondrocytes with fibrin glue	Case series		NS	Preliminary results at one year showed improvement of MRI and arthroscopy and no inflammation (N = 12) [440]
		NCT01400607	III	1–5 cm^2 Control: microfracture	No results posted

tested as a scaffold for expanding chondrocytes for clinical applications. Hyalograft® is an implant consisting of autologous chondrocytes grown on a scaffold made of an esterified derivative of HA. A 7-years follow up study involving 58 young patients showed that Hyalograft® is effective for the treatment of isolated cartilage defects of the average size of 4.4 cm^2 [222]. Hyalograft® has also passed phase I and phase II clinical trials that involved 141 patients and had an average follow up time of 3 years. A longer follow-up is needed to decipher whether these new therapies can represent long-term treatments. Furthermore, comparisons of these therapies in patients with same clinical characteristics could help in assessing their efficacy, as factors like differences in age, size of defects, presence of acute or chronic lesions can affect clinical outcomes.

5. Bone marrow mesenchymal stem cells

5.1. In vitro culture and media supplementation

BMSCs are widely studied due to their relative ease of isolation, expansion and their high differentiation potential [223]. In pellet culture, their chondrogenic potential (in terms of GAG synthesis) was higher than adipose derived stem cells (ADSCs), although it was lower than the one of adult chondrocytes [224]. Although such studies need to be interpreted with caution as media used is often optimised for BMSCs. Major chemical factors used to stimulate BMSCs differentiation into chondrocytes include mainly insulin, dexamethasone, TGF-β [199,225] and BMP-2 [226]. TGF-β1 increased collagen type II and aggrecan synthesis [227] and reduced the expression of terminal hypertrophic differentiation markers collagen type X, VEGF, MMP-13 and osteocalcin [228]. TGF-β2 and TGF-β3 induced chondrogenic differentiation of human BMSCs, augmenting the expression of early stage-cartilage proteins, such as fibromodulin and COMP, followed by the expression of aggrecan and collagen type II. Chondrogenesis of human BMSCs mediated by TGF-β was further enhanced by BMP-2 [229] and by dexamethasone [230]. Particularly, dexamethasone enhanced PG deposition [231]. Also GDF-5, in association with BMP-2 and TGF-β1, has been used for BMSCs differentiation, as it enhanced Sox-9 expression, aggrecan and collagen type II synthesis, while increased the collagen type II/collagen type I ratio [226]. The addition of Wnt3A or of its synthetic analogue CHIR can also enhance BMSC proliferation and collagen type II and aggrecan expression [232]. Since during the late stages of chondrogenesis, Wnt3A also increases hypertrophic progression [233], its inhibitors have been used to reduce secretion of calcified matrix in chondrogenically differentiated BMSCs [232]. However, in most studies, BMSCs cultured in the presence of the most commonly used differentiating agents tend to upregulate the expression of collagen type I [234,235] and collagen type X [235,236], resulting in the production of a fibrocartilage/hypertrophic cartilage rather than hyaline cartilage.

Platelet rich plasma (PRP) is the blood plasma enriched with platelets and it has been widely used as a carrier for BMSCs in clinical applications, since it possesses the ability to induce BMSCs chondrogenic differentiation. Medium containing PRP has been compared with medium containing TGF-β1, ascorbic acid and dexamethasone and showed a greater differentiation-inductive capacity. However, media containing PRP also caused upregulation of collagen type I [234]. Since cell-cell contact enhance BMSCs chondrogenic potential [237,238], they are usually differentiated in pellet culture systems or micro-mass [226,227,229]. The micro-mass culture system resulted in lower expression of collagen type I and type X, when compared to pellet culture [239]. BMSCs have been also differentiated as single cell sheets [240,241] or as multicellular layers on permeable membranes [242,243]. The latter resulted in a more homogeneous deposition of collagen II compared to pellet culture [242].

An alternative to biological media supplements approach is based on macromolecular crowding. Macromolecular crowding, the addition

of macromolecules in the culture media, is a biophysical phenomenon, based on the principles of excluding volume effect, that increases biological processes by several orders or magnitude [244–248]. Macromolecular crowding has been used extensively as means to increase ECM deposition in permanently differentiated [249–254] and stem cell [249,255–258] cultures. Although in chondrocyte [259] and in ADSC [260] cultures significant increase in chondrogenesis was not observed (neutral and non-sulphated crowder, cocktail of Ficoll 70 and Ficoll 400, was used), in human BMSC cultures significantly increased chondrogenesis was observed, possibly attributed to the negative charge and highly sulphated nature of the crowder (carrageenan) used [261].

Considering that requirements for media may vary depending on the presence of other chondrogenic modulators (e.g. scaffolds, mechanical stimulation) an ideal combination of media supplements for driving BMSCs differentiation is difficult to identify.

5.2. Three-dimensional scaffolds

Natural [146,199,262,263] and synthetic [262,264,265] biomaterials have been used to enhance BMSC differentiation. Alginate hydrogels enhanced the expression of chondrogenic genes in BMSC culture and induced the characteristic rounded chondrocyte morphology [266]. HA hydrogels have been shown to enhance expression of collagen type II, aggrecan and Sox-9, while maintaining low levels of expression of collagen type I in human BMSC culture, compared to PEG hydrogels [262]. Recently, cartilage ECM-derived scaffolds have been used to induce chondrogenic differentiation of BMSCs [267–271], as they retain the native structure of cartilage and provide BMSCs with signals that modulate behaviour and differentiation [269]. ECM-derived scaffolds have been shown to induce collagen type II synthesis, even in the absence of exogenous growth factors [272] and their composition (in terms of collagen type I/II and aggrecan/biglycan ratios) has been shown to regulate this chondro-inductive effect. In particular, collagen type I enriched ECM-derived scaffolds induced a higher differentiation, compared to collagen type II enriched [271]. Similarly, a study showed that collagen type I coated transwell resulted in a higher chondrogenic differentiation (in terms of collagen II and GAGs synthesis), compared to collagen type II coated transwells [272].

A few studies have investigated how the scaffold architecture influences chondrogenic differentiation of BMSCs. Contrarily to chondrocytes [133], BMSCs in chitosan sponges (with pore size of about 100 μm) proliferated faster and produced higher amount of GAGs and collagen type II compared to ones in chitosan hydrogels with pore size less than 20 μm (although the higher amount of GAGs and collagen type II could be also explained by the higher proliferation of cells in sponges rather than by the higher degree of differentiation). The authors of this study put forward the notion that the better diffusion nutrient capability of sponges (due to their higher pore size and interconnectivity) suits better the metabolic requirements of BMSCs [166]. In another study, rat BMSCs in collagen-HA porous scaffolds proliferated faster, expressed higher levels of Sox-9 and GAGs, and showed a higher ratio collagen type II/I at higher pore size (300 μm compared to 130 μm and 94 μm) [273].

Apart from material and architecture, chondrogenic differentiation of BMSCs has been found to be sensitive to nano-topographical cues [274] and to chemical modification of the substrate [275,276]. In one study, 4 types of PCL films were made by nano-imprinting and coated with chondroitin sulphate. Films with nano-pillars with 250 nm diameter and 500 nm pitch, nano-holes with 225 nm diameter and 400 nm pitch and nano-grills with 250 nm line and 250 nm space were compared to non-pattern films. BMSCs in nano-pillar surface showed spherical morphology with filopodia extrusion, while those on the others showed polygonal or elongated morphology. After 2 and 4 weeks of chondrogenic differentiation, BMSCs on nano-pillar films showed the highest collagen type II expression and synthesis, the lowest collagen type I expression and synthesis and the highest aggrecan expression, although they also expressed collagen X [274]. Finally, functionalisation of surfaces with specific polysaccharides (e.g. 3-sialyllactose) has been shown to increase expression of Sox-9 and aggrecan, even in the absence of any chondrogenic supplements in the culture media [277]. However, despite the large variety of scaffold tested so far, the mechanism by which substrate architecture and topography influence BMSCs differentiation is still not elucidated and this makes difficult the identification of new possible approaches.

5.3. Oxygen tension

Low oxygen tension (3%) has been shown to play a pivotal role on ovine BMSC proliferation, delaying their senescence, and on their differentiation into chondrocytes [263]. Human BMSCs, induced for differentiation under 1% oxygen tension, increased chondrogenesis and showed a lower expression of the hypertrophic markers collagen type X and Runx2 than the ones cultured under normoxia. These cells showed an increase in the activation of PKA/Akt/FoxO pathway that may play an important role in inhibiting the switch to a hypertrophic phenotype [89]. However, an increase in the expression of collagen type X has been reported in sheep BMSCs cultured under low oxygen tension in HA and collagen scaffolds [263]. The effect of low oxygen tension has been recently shown to depend on MSCs differentiation stage. Culture of human BMSCs at 2% oxygen tension resulted in higher chondrogenesis (in terms of increase in GAGs and collagen type II expression) than that of CPCs that possess greater chondrogenic potential (although a greater decrease in the expression of collagen type X and MMP-13 was detected in CPCs) [87].

5.4. Mechanical stimulation

Similar to chondrocytes, BMSCs also respond to mechanical stimulation to increase ECM synthesis. One study found that application of intermittent hydrostatic pressure (10 MPa, 1 Hz, 4 h/day) increased collagen type II and aggrecan synthesis, as well as Sox-9 expression in pellet culture. Administration of TGF-β3 further increased matrix synthesis promoted by HP in human BMSCs [278]. In another study, application of intermittent HP (1 MPa, 1 Hz, 4 h/day) in BMSCs, cultured in a collagen type I scaffold, resulted in increased GAG synthesis and upregulation of aggrecan, collagen type II and Sox-9. However, in this culture system, also collagen type I was upregulated [279]. Cyclic compression was able to enhance TGF-β induced chondrogenesis but, alone, was insufficient to induce expression of collagen type II [280,281] and was able to induce GAGs synthesis only to a lower extent compared to TGF-β administration [282]. However, multiaxial loading (10%, 1 Hz) up-regulated collagen type II and Sox-9 expression in human BMSCs, even in the absence of exogenous growth factors in the media [281,283]. This has been attributed to an increase in the endogenous TGF-β1 synthesised by the cells [284,285], although some differences in cell secretome have been detected during chondrogenesis driven by exogenous TGF-β1 or during chondrogenesis driven by this multiaxial loading system [286]. Activation of TGF-β under physiological load has also been demonstrated and may be related to the stiffness of the repair tissue [284]. Further investigation is needed to fully appreciate the influence/standardise mechanical stimulation.

5.5. Co-culture of chondrocytes and bone marrow stem cells

In co-culture systems, BMSCs are cultured together with adult chondrocytes in an effort to promote differentiation via secretion of paracrine signals and prevention of hypertrophic transition of differentiated BMSCs. Additionally, matrix produced by chondrocytes directs physical cell-matrix interaction and tethers secretory growth factors [287]. Typically, there are 2 different systems of co-culture: the direct co-culture, where BMSCs and chondrocytes are mixed and cultured

together and the indirect co-culture, where the cells are separated with a membrane. It has also been developed a separable-closed co-culture system, that, like the direct co-culture system, allows close cellular interaction between chondrocytes and BMSCs, but, like the indirect co-culture system, allows a physical separation of the 2 groups of cells [288]. The effect of co-culture with chondrocytes in BMSCs differentiation is controversial. In the separable-closed co-culture system, human chondrocytes had a strong impact on human BMSCs' aggrecan and Sox-9 expression. In another study, immortalised human BMSCs co-cultured with immortalised human articular chondrocytes without exogenous growth factors, synthesised collagen type II, without up-regulating the expression of osteogenic markers [289]. In an indirect co-culture system, where bovine chondrocytes and rabbit BMSCs were grown in a PCL scaffold in a flow perfusion bioreactor, chondrocytes increased BMSCs' GAG and total collagen synthesis [289]. However, results of other studies are less encouraging. In one study, researchers performed micro-mass co-culture of human primary articular chondrocytes and human BMSCs and they found that in the absence of exogenous growth factors, chondrocytes failed in inducing chondrogenic differentiation of BMSCs and that in the presence of TGF-β1 and dexamethasone, they failed in preventing BMSC hypertrophy [290].

In general, direct and indirect co-cultures act through different mechanisms and their effect depends on the culture system used [291]. A better understanding of these mechanisms is still needed to assess the efficacy of these co-culture systems, with particular attention paid to relative cell death in direct co-culture models. The state of the chondrocytes is also crucial in this process. The complexity of this approach restricts clinical applications.

5.6. Preclinical studies

While *in vitro* studies generally attempt to identify the most efficient system for inducing chondrogenic differentiation of BMSCs, some preclinical studies aimed at understanding if an *in vitro* pre-differentiation of BMSCs is effective or if undifferentiated BMSCs possess the same regenerative capability. In a mini-pig model, it was observed that *in vivo* signalling molecules and biomechanical stimuli can provide an appropriate environment for BMSCs to correctly differentiate into chondrocytes [292]. The authors concluded that pre-differentiation before transplantation may be unfavourable. However, in rabbit models, it has been observed that undifferentiated BMSCs *in vivo* tended to secrete a fibro-cartilaginous matrix that did not integrate with adjacent matrix and degenerated after several years [21,293]. Another study showed that the *in vivo* regenerative capability of BMSCs was dependent on their differentiation degree. In this study, human BMSCs were differentiated in PGA/PLA porous scaffolds for 2, 4 and 8 weeks before being implanted in a pig model. Constructs that had been differentiated for 4 and 8 weeks resulted in higher deposition of collagen type II and GAGs and a higher histological score [264].

A few *in vivo* studies have compared the regenerative capabilities of pre-differentiated BMSCs with other cell types. In a rabbit model, BMSCs were compared to MSCs from periosteum, synovium, adipose tissue and muscle; BMSCs that were pre-cultured for 72 h on allogeneic demineralised bone matrix with incorporated TGF-β1 possessed the greatest capability to secrete hyaline-like cartilage matrix *in vivo* [294]. However, another study showed that BMSCs possessed lower regenerative capability than adult chondrocytes. In this study, human BMSCs and human chondrocytes were cultured for 7 weeks in pellet in the presence of dexamethasone, ascorbate, insulin, transferrin, selenium and TGF-β3 before being implanted subcutaneously with fibrin glue in mice. While chondrocytes were able to resist vascular invasion and calcification, BMSCs resulted in deposition of mineralised matrix. However, the subcutaneous environment is not very representative of the native cartilage microenvironment [202]. Additionally, the exact monolayer expansion conditions used, including the number of population doublings, is likely to play a role in the final outcome.

BMSCs in different natural [270,295,296], synthetic [264,297,298] or combination of both [299,300] scaffolds have been tested *in vivo* in cartilage defects of small [270,272,299] and large [264,295,296] animals. To investigate the relationship between composition and architecture of scaffolds and chondrogenic differentiation of BMSCs, a study showed that rabbit BMSCs, when grown for 1 week on collagen type I hydrogels, even in the absence of exogenous growth factors, once implanted subcutaneously in rabbit, resulted in a cartilage construct, whilst when they were grown on collagen I and alginate mixed hydrogels they did not [301]. Furthermore, if collagen type I sponges, calcium phosphate ceramic sponges or silk fibroin protein matrix sponges were used (instead of collagen type I hydrogels), no cartilage constructs were produced *in vivo* [302]. Authors of these two studies concluded that only certain materials and certain scaffold architectures possess *in vivo* chondro-inductive properties and that hydrogels may not be suitable for chondrogenesis. However, they did not characterise the scaffolds used. Furthermore, their results are in contrast with other *in vitro* studies that have shown a higher *in vitro* chondro-inductive effect of sponges compared to hydrogels [166,273].

Despite the synergistic action of BMSCs and chondrocytes, which has been described in the *in vitro* co-culture systems, subcutaneous injection of rabbit BMSCs and rabbit chondrocytes in platelet rich plasma gels in mice resulted in a progressive ossification of BMSCs, with increased vascularization and expression of hypertrophic markers [287]. An advantage of using BMSCs may be their immunosuppressive ability and hypo-immunogenic nature, that may be useful in the presence of an inflammatory environment [303]. Recently, a study showed that also chondrocytes differentiated from BMSCs were also not immunogenic (did not expressed HLA-II in response to IFN-γ) [304]. Furthermore, pre-differentiated porcine BMSCs implanted subcutaneously in pigs using a PGA/PLA scaffold reduced infiltration of foreign body multinucleated giant cells and induced M2 polarisation of macrophages (from pro-inflammatory to tissue remodelling activity) compared to chondrocytes [297]. Although M2 macrophages have not been well studied in chondrogenesis, they are usually associated with endochondral ossification [305]. Furthermore, to fully assess the anti-inflammatory activity, further investigations of pre-differentiated BMSCs in chronic defect models, rather than in subcutaneous or acute defects models, should be performed. Further pre-clinical experimentation is needed to assess the efficacy of the most promising culture systems to pre-differentiate BMSCs. If one advantage of using BMSCs is their anti-inflammatory property, this still need to be assessed in suitable animal models.

5.7. Clinical trials

Although significant progress has been made for the *in vitro* differentiation of BMSCs towards chondrogenic lineage, all on-going clinical trials are investigating the therapeutic potential of undifferentiated BMSCs (Table 5). Induction of differentiation of BMSCs before implantation still remain to be tested in humans but its experimentation is limited by the regulatory framework that requires minimal cell manipulation.

A randomised clinical trial that involved 72 patients showed that the injection of autologous chondrocytes or the injection of undifferentiated BMSCs beneath the periosteal flap for cartilage repair resulted in the same clinical outcomes, measured by the International Cartilage Repair Society cartilage injury evaluation package [306]. Additionally, patients that received BMSCs had a greater improvement in their physical functioning assessment, when compared with patients that received chondrocytes. In a pilot study with 5 patients, platelet-rich fibrin glue was used as a scaffold to deliver undifferentiated BMSCs for repairing large-sized cartilage defects, with an average of 5.8 cm^2. After 12 months, magnetic resonance imaging (MRI) in 3 out of the 5 patients showed complete defect filling and complete surface congruity with native cartilage, whereas, in the other 2, incomplete congruity was

Table 5
Cell therapy products with mesenchymal stem cells, proposed for the treatment of focal chondral defects, with clinical trials, defect sizes of patients enrolled and published results. NS= Non-specified. Source: clinicaltrials.gov.

Name	Description	Clinical trial	Phase	Chondral defect size/Control group	Status clinical trial/Published results
ACIC vs MCIC	ACIC: collagen/fibrin gel mixture MCIC: BMSCs implantation in fibrin gel	NCT01984450	N/A	2–8 cm^2 Control group: ACIC	No results posted
ASCROD	Implantation of autologous ADSCs	NCT01399749	I/II	Grade 3 or 4 Control: ACI	No results posted
CARTISTEM®	Transplantation of allogeneic umbilical cells in HA scaffold	NCT01733186	I/II	Larger than 2 cm^2 No control group	After 7 years, functional, clinical and MRI improvement. (N = 7). Treatment-emergent adverse events in 5 out of 7 patients [441]
		NCT01626677 and NCT01041001	III	2–9 $cm^{2.}$ Control: microfracture	No results posted
Hyalofast®	Hyaluronic acid scaffold with bone marrow aspirate	NCT02659215	N/A	1–6 cm^2 Control: microfracture	Recruiting
HYTOP®	Layer of porcine splint-skin and one of collagen fleece, used in conjunction with microfracture	NCT01791062	N/A	Grade 3 or 4 No control group	No results posted
INSTRUCT	Autologous chondrocytes and BMSC in a biodegradable porous scaffold	NCT01041885	N/A	NS No control group	At 2, year, defect filled with hyaline-like ECM and pain reduction (N = 40) [437]
ReJoinTM™	Injection of ADSC and HA gel	NCT02855073	II	2–6 cm^2 with OA Control: injection of HA	No results posted
Application of a layer of fibrin glue, an acellular collagen dermal matrix, undifferentiated ADSCs from IFP and fibrin glue.		NCT02090140	N/A	Smaller than 4 cm^2 Control: microfracture	Recruiting
Four monthly injections of umbilical cord MSC		NCT02291926	I	2–10 cm^2 with OA No control group	No results posted
Injection of subcutaneous fat ADSCs		NCT01799876	N/A	Grade 3 or 4. Control: microfracture	No results posted
Injection of subcutaneous fat ADSCs		NCT02090140	N/A	Smaller than 4 cm^2 Control: microfracture	Recruiting
Autologous BMSCs with periosteal flap		NCT00891501	II/III	1–4 cm^2 No control group	No results posted
Injection of autologous BMSCs or chondrocytes under a commercially available membrane		NCT00885729	I	1.5–6 cm^2 Control: rehabilitation program	No results posted
Injection of autologous BMSCs compared to traditional ACI		N/A	N/A	NS Control: ACI	After 2 years, no differences between the two groups, except for the Physical Role Functioning score, which showed a greater improvement in BMSC group (N = 72) [306]
Injection of BMSCs with platelet-rich fibrin glue covered by a periosteal flap		Case reports	N/A	Mean = 5.8 cm^2 3 out of 5 patients had concomitant inflammatory disease	After 1 year, 3 patients showed complete defect filling; the other 2 (which had concomitant osteochondritis dissecans) incomplete congruity (N = 5) [307]
Transplantation of collagen sheet with BMSC		Case report	N/A	6 cm^2	After 7 months complete defect filling with hyaline-like tissue repair (N = 1) [308]
Transplantation of collagen sheet with BMSC covered by a periosteal or synovial flap		Case reports	N/A	NS	Clinical improvement after 6 and 12 months, but it was dubious whether the tissue repair was fibrocartilaginous or hyaline-like (3 patients with 9 lesions, including 2 kissing lesions) [310]
Transplantation of collagen sheet with BMSC covered by a periosteal flap		Case reports	N/A	12 cm^2 (with chondromalacia) and 4 cm^2	After 1–2 years, the defects were covered by fibrocartilage (N = 2) [442]
Transplantation of collagen sheet with BMSC		Case report	N/A	2.2 and 2.5 cm^2	After 30 months, improvement of clinical scores and pain reduction [309]

evidenced. Differences observed in clinical outcomes can depend on the different potential of BMSCs (possibly, due to a difference in age of patients) or on a possible subchondral pathology [307]. A case report used a collagen sheet for the delivery of undifferentiated BMSCs to a 20 × 30 mm defect in a young patient (31 years old). It was proposed as an effective treatment with a complete filling of the defect with hyaline-like cartilage 7 months after transplantation and a great improvement of the clinical symptoms after 1 year [308]. Implantation of BMSCs with a collagen scaffold in 2 patients with large traumatic cartilage defects of the knee resulted in an improvement of clinical outcomes, complete defect fill, improvement in stiffness and incorporation to the adjacent cartilage, after 32 months [309]. In another study, undifferentiated BMSCs embedded in collagen sheets were transplanted and covered by autologous periosteum or synovium for the treatment of full thickness cartilage defects. The results were dubious: although only 6 months after transplantation the clinical outcomes had improved, the defects seemed to be covered by a fibro-cartilaginous tissue, rather than a hyaline cartilage [310].

BMSC injection has been tested also in patients with OA. In one study, a patient with degenerative knee OA underwent 3 intra-articular knee injections of BMSCs in PBS with platelet-lysate and dexamethasone as differentiating agents. At 24 weeks post injection, statistically significant cartilage and meniscus growth was detected using MRI, as well as increased range of motion and decreased pain score [311]. Undifferentiated BMSCs have been injected in the knees of 5 patients with moderate to severe OA. The injection let to an initial recovery of the clinical parameters, but some of them (e.g. rest time to produce a gelling pain in minutes resting on a chair, walking time in minutes or the number of stairs climbed) underwent to an important decline after 5 years. Additionally, X-ray imaging did not show any improvement of the joint space, not even 6 months after the injection. The authors of this study speculated that the transplanted BMSCs may initially create a microenvironment to repair cartilage and suppress the synovial inflammation typical of OA, but after several months they lose their characteristics, permitting the process of cartilage degradation [312].

Most of the clinical studies are mainly case reports and prospective randomised trials are still not available. Furthermore, to finally assess the regenerative capability of BMSCs, further comparison with chondrocytes with longer follow-up should be performed.

6. Adipose derived stem cells

6.1. In vitro culture and media supplementation

Given the BMSC tendency to produce hypertrophic cartilage rich in collagen type I [235,313,314] and type X [202,313,315,316], alternative MSC sources are under investigation. ADSCs are isolated from lipoaspirates and they are largely studied because they can be obtained in large amount using a low invasive procedure [317]. Major sources of ADSCs are abdominal fat and infrapatellar fat pad (IFP), with the first one being the most investigated [318]. Cells isolated from the two sources exhibited equivalent colony-forming unit (CFU) ability and similar surface markers, although higher expression of both Sox-9 and RUNX2 has been detected in naïve ADSCs from IFP [319]. Compared to BMSCs, subcutaneous ADSCs show a higher proliferation rate and they can undergo a higher number of passages before senescence [317,320]. When exposed to commonly used chondrogenic growth factors, ADSCs tend to upregulate the expression of collagen type I and type X [320], albeit with a lower initial tendency than BMSCs. It is worth noting that a few studies have shown that subcutaneous ADSCs possess a lower chondrogenic potential than BMSCs [147,321–326]. However, in these studies, researchers used identical culture conditions for both ADSCs and BMSCs, despite emerging evidence suggesting that these two cell types respond differently to stimuli and a comparison between them can be affected by the specific culture conditions used [320]. *In vitro*, ADSCs from IFP revealed higher chondrogenic potential and lower hypertrophic tendency compared to ones from subcutaneous tissue [327] and similar *in vitro* differentiation potential than BMSCs [328,329]. Growth factors and chemical substances commonly used for ADSC chondrogenic differentiation include TGF-β1 and TGF-β3, insulin, BMP-6, BMP-7 and ascorbic acid. TGF-β1 alone was not able to induce the formation of cartilaginous matrix [227], but it reduced the expression of the hypertrophic marker alkaline phosphatase [330] and in association with a combination of insulin, transferrin and selenium enhanced both PG synthesis and cell proliferation. The addition of the over-sulphated exopolysaccharide GY785 DRS has been shown to further increase GAG, collagen II and COMP synthesis of ADSCs cultured in the presence of TGF-β1 and insulin [331]. The addition of dexamethasone in ADSCs differentiation media seemed to be unfavourable, since it reduced PG synthesis and deposition [332]. If compared with BMSCs, ADSCs responded differently to growth factors. In particular, they responded less favourably to dexamethasone and TGF-β3 in terms of aggrecan production but responded more favourably to BMP-6 in terms of aggrecan and collagen type II synthesis [320]. In the presence of BMP-2, ADSCs acquired an osteogenic phenotype, while BMSCs differentiated into chondrogenic lineage [330,333]. Similar to chondrocytes, human serum increased ADSCs proliferation rate and expression of Sox-9 [334]. Human platelet lysate enhanced their proliferation rate, but it also induced the expression of the hypertrophic marker collagen type X [123]. ADSCs are commonly differentiated in high-density cell culture systems like pellet [322,323], micro-mass [335], spheroids [321] or multicellular layers on permeable membranes [336]. Spheroid culture has been shown to enhance ADSC expression of HIF-1α, the transcription factor upregulated by hypoxia and responsible for the increase in Sox-9 expression [337].

Despite the large variety of condition tested, ADSCs generally show a lower chondrogenic potential compared to BMSCs, meaning that probably the most suitable culture system has not been identified as yet.

6.2. Three-dimensional scaffolds

Chondrogenic differentiation of ADSCs has been demonstrated in a variety of natural [147,320,323,338,339] and synthetic [340–342] biomaterials. Similar to chondrocytes, rabbit and human ADSCs expanded in three-dimensional scaffolds showed lower expression levels of hypertrophic markers collagen type I and type X than ones expanded in monolayer [107,148]. A study comparing human ADSCs seeded in alginate hydrogels, agarose hydrogels and porous gelatine scaffolds [339] showed that these scaffolds were able to support chondrogenic differentiation similarly, in terms of PG and collagen type II deposition. Another study compared cartilage derived matrix with alginate beads [320] and found that human ADSCs grown in these scaffolds expressed collagen type II and aggrecan at similar levels. One study compared the ability of fibrin glue, PLGA scaffold and alginate hydrogels to induce human ADSCs chondrogenic differentiation and showed that cells grown in fibrin glue proliferated fastest and showed the highest expression of the chondrogenic genes collagen type II, Sox-9 and aggrecan. However, in this system, cells in fibrin also showed the highest expression of collagen type I [343]. A study investigated the effect of biochemical clues (using different compositions of chondroitin sulphate, HA, and heparan sulphate) and matrix stiffness in human ADSCs differentiation. The authors found that the scaffold's stiffness not only influenced the amount of collagen type II, type X and aggrecan produced by the cells, but also the cell response to the biochemical cues [344]. Although various studies have compared the chondro-inductive potential of different materials, the role of scaffold architectures and mechanical properties on ADSCs differentiation needs to be investigated.

6.3. Oxygen tension

Similar to BMSCs and chondrocytes, low oxygen tension (2% and 5% respectively) plays an important role in chondrogenic differentiation [345] and matrix synthesis [323] of ADSCs. Human ADSCs cultured in the presence of TGF-β3 and BMP-2 in atmospheric oxygen tension (20%) augmented both the expression of chondrogenic markers and of hypertrophic markers. However, when they were cultured with the same growth factor but under lower oxygen tension (2%), they exhibited a decreased expression of collagen type X and MMP-13 [229]. In alginate [323] and pellet [346] culture of human ADSCs, 5% oxygen tension increased the synthesis of collagen type II and PG [323], reducing the expression of the hypertrophic markers collagen X and MMP-13 [346].

6.4. Mechanical stimulation

Hydrodynamic pressure, if applied without exogenous growth factors, does not seem to significantly influence ADSCs differentiation. Application of a cyclic HP (7.5 MPa, 1 Hz 4 h/day) in human ADSCs seeded on agarose scaffolds and cultured without exogenous growth factors, resulted only in a low initial upregulation of collagen type II, aggrecan and Sox-9, followed by a decrease in their expression after 14 days [347]. Conversely, human ADSCs cultured in collagen scaffolds in the presence of TGF-β1 and under cyclic HP stimulation (0.5 MPa, 0.5 Hz, continuously for 1 week or 2 alternate weeks), showed an increased expression of collagen II, Sox-9 and aggrecan and a low decrease in the expression of collagen type I and integrin β. However, the expression of collagen type X was also increased over time [348]. Cyclic HP (5 MPa, 0.5 Hz 4 h/day) in pellet culture of human ADSCs in the presence of TGF-β1 and dexamethasone resulted in increased expression of the 3 major chondrogenic markers: aggrecan, collagen type II and Sox-9 [349]. Dynamic HP (17 kPa 12 h/day) increased the expression of Sox-9 and aggrecan of rat ADSCs, even in the absence of exogenous growth factors [350]. Recently, centrifugal gravity, which induced mechanical stress before pre-differentiation of ADSCs, has been proposed as a simple way to increase chondrogenesis, through enhancement of Sox-9 expression [351].

6.5. Co-culture of chondrocytes and adipose derived stem cells

ADSCs could be induced to differentiate into chondrocytes in co-culture systems. In one study, human ADSCs were co-cultured with human articular chondrocytes in the presence of TGF-β1 and dexamethasone in fibrin and collagen matrix scaffolds. They showed only a weak increase in aggrecan, collagen type IX, collagen type II, collagen link protein I synthesis and Sox-9, but the variability was too high to obtain a statistical significance [352]. However, in another study, human OA chondrocytes and human ADSCs co-cultured in hydrogels composed of PEG and chondroitin-sulphate in the presence of TGF-β3 showed a synergistic interaction with enhanced cartilage matrix deposition. The discrepancies between these 2 studies can be explained by the fact that interaction between ADSCs and chondrocytes is highly dependent on the three-dimensional scaffold and soluble factors used [353]. Additionally, in calf chondrocytes and human ADSCs co-culture system, it has been observed that ADSCs chondrogenic differentiation is highly dependent on the intercellular distance and cells distribution in the scaffold [354]. Researchers have also induced oxidative stress injury in rat chondrocytes to mimic OA. These injured cells were indirectly co-cultured with a mixture of rat ADSCs and rat chondrocytes, with ADSCs alone or with chondrocytes alone. After 36 h, only injured cells co-cultured with both ADSCs and chondrocytes expressed aggrecan and collagen type II at physiological levels and decreased expression of collagen type I and collagen type X. Discordances between different studies show that probably further elucidation on the mechanism by which ADSCs interact with adult chondrocytes is needed.

6.6. Preclinical studies

The chondrogenic capability of ADSCs has been tested *in vivo* in small [211,355–357] and large [358–360] animal models. Similar to BMSCs, a preclinical study in rabbits observed that pre-differentiated ADSCs possess a greater capacity to repair cartilage defects than undifferentiated ADSCs, according to histological analysis [356]. However, among all the different culture systems proposed for *in vitro* pre-differentiation, only few of them have been tested *in vivo*.

Although low oxygen tension generally exhibits *in vitro* chondrogenic properties, a study showed that pre-conditioning with low oxygen tension did not increase the *in vivo* chondrogenic capabilities of human ADSCs, probably because the *in vivo* environment overcame this effect. In this study, cells were cultured for 3 weeks in monolayer in the presence of insulin, transferrin, selenium, ascorbic acid, dexamethasone and TGF-β1 at 5% or 21% oxygen tension and then subcutaneously implanted into Si-HPMC hydrogels in mice. Although during the *in vitro* expansion, low oxygen tension resulted in an increased collagen type II and aggrecan expression, 5 weeks after transplantation, no histological differences were observed between the constructs formed by ADSCs pre-conditioned with low oxygen tension and ADSCs cultured under atmospheric oxygen tension. Furthermore, the implantation of rabbit ADSCs pre-conditioned with low oxygen tension (5%) or cultured under atmospheric oxygen tension into rabbit cartilage defects resulted in no significant differences in the histological scores [356]. Compared to a static culture, the use of rotating bioreactor during the *in vitro* pre-differentiation of ADSCs in decellularised ECM resulted in a greater deposition of collagen after the constructs were subcutaneously implanted in mice [342].

An *in vivo* synergistic effect of ADSCs and chondrocytes has also been described. Similar to what observed *in vitro*, a study showed that the composition of hydrogels (chondroitin sulphate, HA and heparan sulphate) and their stiffness (0.67 kPa, 6.90 kPa and 32.70 kPa) modulated the ratio of hypertrophic (collagen type X and type I) and hyaline (collagen type II and aggrecan) ECM-components synthesised by a mixture of human ADSCs and calf neonatal chondrocytes, transplanted subcutaneously in nude mice [211]. In another study, researchers injected a mixture of ADSCs and chondrocytes, chondrocytes alone or ADSCs alone in a rat OA models. At day 42 after transplantation, animals receiving both ADSCs and chondrocytes showed the highest PG content in their knees and the lowest osteoarthritic assessment. These results suggest that a mixture of ADSCs and chondrocytes may be more beneficial than chondrocytes or ADSCs alone, although the molecular mechanism underlying the possible interaction between ADSCs and chondrocytes is not fully understood [357]. Another study, however, showed no difference in term of collagen produced by a mixture of human ADSCS and auricular chondrocytes or auricular chondrocytes alone in Pluronic®F-127 injected subcutaneously in mice (hypertrophic markers were not assessed) [113]. The discrepancies between these studies may be explained by considering the different sources of chondrocytes, scaffolds and animal models used. MSCs naturally possess anti-inflammatory properties [361] that are probably enhanced when they are mixed with adult chondrocytes [357,362]. While in OA models, the beneficial effect of a mixture of MSCs and adult chondrocytes may mainly depend on the increased capability to reduce inflammation, in a subcutaneous model the anti-inflammatory effect may not be required. A direct comparison between pre-differentiated and undifferentiated ADSCs in large animal models and a higher degree of standardisation, when comparing systems for pre-differentiation, are still needed.

6.7. Clinical trials

Similar to BMSCs, all on-going clinical trials are investigating the therapeutic potential of undifferentiated ADSCs, whilst the induction of differentiation of ADSCs before implantation still remains to be assessed

in humans. Very few studies are evaluating the chondrogenic potential of ADSCs in focal chondral defects (Table 5). A study (NCT01399749) is comparing ADSCs with chondrocytes injection for the treatment of traumatic cartilage defects smaller than 5 cm^2 in diameter; another one (NCT02090140) is implanting a collagen dermal matrix with undifferentiated ADSCs from IFP in chondral defects smaller than 4 cm^2; in other two studies (NCT02090140 and NCT01799876) ADSCs from abdominal depots are injected in focal defects. Many clinical trials are in fact evaluating ADSCs injection in patients with OA [363,364], so that results obtained are not easily comparable with ones obtained with other cell types. ADSCs from IFP have been injected in OA knees [363–366] and improvements in clinical and MRI results were maintained for 2 years after implantation, with a positive correlation with the number of cells injected [364]. Injection of ADSCs from abdominal depot also showed beneficial effect in the presence of OA, with chondral defect reduced after 6 months [365], with generation of hyaline-like repair tissue [366].

Since ADSCs can be isolated from different depots, *in vivo* comparison of cells from different sources is needed, as *in vitro* data showed more suitability of cells from IFP compared to ones from abdominal fat [327].

7. Synovial derived stem cells

Synovial cells may be a possible candidate for cartilage engineering since they derive from the same pool of precursor cells as adult articular chondrocytes [367]. Moreover, in mice with osteochondral defects and joint-surface injuries, MSCs from synovium are physiologically involved in wound healing and this means that they may possess the capability of regenerating articular cartilage [367]. Synovial derived stem cells (SDSCs) have been differentiated into chondrocytes using TGF-β3 [368] or TGF-β1 [103] and BMP-2 in pellet culture system [223], even in the absence of serum [103]. Unlike BMSCs, dexamethasone did not influence SDSCs chondrogenic differentiation [230]. An advantage of using SDSCs is their maintenance of plasticity over extended passages. Late passage cells (expanded in the presence of TGF-β1, bFGF and PDGF-BB) exhibited decreased GAG deposition and increased synthesis of collagen type II, so their own secreted matrix resembled native superficial articular cartilage in collagen type II and GAG percentage [368]. Cartilage matrix secreted by synovial MSCs was rich in collagen type II and aggrecan, but not in collagen type I or collagen type X and it was mechanically similar to native articular cartilage [367]. Additionally, the chondrogenic potential of synovial cells was independent of donor age [369], rendering a synovial cells-treatment suitable also for elderly patients. A few studies have demonstrated the capability of synovial cell grafts to promote functional cartilage repair in pre-clinical models. Synovial cells seeded onto a PGA mesh with fibrin glue have been successfully used for repairing cartilage defects in rabbit models [370]. In a swine model, synovial cells injected into the knee were able to repair cartilage defects, as assessed by arthroscopical, histological and MRI analyses [371]. SDSCs from both skeletally mature and immature pigs exhibited same levels of cartilage regeneration (histological assessment) in a pig model independently of maturity of recipients [372]. ECM synthesised by synovial cells injected in cartilage defects of pigs possessed viscoelastic properties similar to those of normal cartilage [372]. However, a study that compared the potential of rat SDSCs sheet and rat chondrocyte sheet in a rat model showed that SDSCs possessed a higher tendency to secrete collagen X and collagen I [373]. Although synovial membrane can be harvested via arthroscopy with minimal complications at the donor site [21], the use of synovial cells may be limited due to the reduced availability of donor material t and the time required to expand their numbers. To date, SDSCs have never been used in clinical trials.

8. Other sources of mesenchymal stem cells

Apart from bone marrow, adipose tissue and synovial membrane, mesenchymal cells can be isolated from articular cartilage, periosteum, umbilical cord blood, peripheral blood, skeletal muscle and synovial fluid.

Cartilage contains a notch 1 positive progenitor cell population that can be isolated on their preferential attachment to fibronectin [65,374]. The cells have great expansion capacity and are resistant to hypertrophy. They have also been shown to be mechano-responsive [375].

Isolation of MSCs from peripheral blood requires a less invasive procedure than the one used for isolating BMSCs. Peripheral blood derived stem cells (PBSCs) have been differentiated towards chondrogenic lineage using TGF-β3 [376,377] or TGF-β1 [378]. The regenerative potential of PBSCs *in vivo* has also been demonstrated: patients with chondral defects first underwent subchondral drilling and 5 days after the operation, they underwent injection of PBSCs in HA solution. Histological analysis showed the filling of defects with hyaline cartilage and minimal adverse side effects were reported [379]. Advantages of PBSCs are the ease and high yield of isolation, but the proliferation rate and chondrogenic capability of these cells still need to be investigated.

Umbilical cord mesenchymal cells, in comparison to ADSCs or BMSCs, exhibited lower isolation efficiency [380] and when exposed to chondrogenic media (TGF-β1, ascorbic acid and dexamethasone), they showed higher expression of hypertrophic markers (e.g. collagen type I) [381]. TGF-β1 and dexamethasone have been used for their differentiation in monolayer culture system and in collagen hydrogels scaffolds [380,382]. A pulsed electromagnetic field treatment has also been described as a means to differentiate them towards chondrogenic lineage [383]. In a preclinical study in rabbits, implantation of human umbilical cord cells seeded on PLLA scaffolds resulted in an improper repair of cartilage defects with inflammation in the synovial membrane [384]. A clinical trial is currently investigating the efficacy of injection of allogeneic umbilical cells in HA scaffold (CARTISTEM®) in patients with full thickness cartilage defects (NCT01041001). Whilst the high availability, painless collection, absence of donor site morbidity, fast and long *in vitro* self-renewal encourage the use of umbilical cord MSCs, their low isolation efficiency and tendency to produce fibrocartilage are the main disadvantages.

Periosteum is known to contain chondrocytes precursors [385]. The periosteal mesenchymal cells' chondrogenesis has been enhanced in the presence of TGF-β3 [386], whilst their differentiation has been enhanced under low oxygen [387]. One of the advantages of using periosteum mesenchymal cells is that they retain their expansion potential even at late passages [223]. MSCs from periosteum have been successfully used for repairing cartilage defects in rabbit models [294,388,389], but they have not been tested in human so far.

Muscle derived stem cells possess the capability to differentiate into chondrocytes in the presence of TGF-β [390] and they have been successfully used for cartilage regeneration in rabbits [391,392] and rats [393]. Their chondrogenic potential seems to be influenced by donor sex: male cells possess higher capacity for chondrogenic differentiation *in vitro* and better cartilage regeneration potential *in vivo* [393]. The limitations in the use of muscle derived stem cells and periosteum cells are the surgical procedure needed for their extraction and the limited availability of donor material.

A population of MSCs has also been isolated from synovial fluid (SFSCs). These cells share similar expression profile with SDSCs [394,395], their number is increased during meniscus [396], anterior cruciate injury and surgery [395] and arthritis and OA [397,398]. They are probably recruited by chemokines in the blood [395] to repair cartilage [398]. The increase in their number is not associated with a change in their chondrogenic capabilities and epitope markers [395].

Table 6
Proposed mechanisms by which *in vitro* modulators induce chondrogenic differentiation and cell types where these mechanisms have been studied.

Modulators	Mechanism	Cells	Ref
Hypoxia	Activation of PKA/Akt pathway	BMSCs	[89,443]
	Increase in stability of HIF-1α	Chondrocytes, MSCs	[443–449]
	Increase in activity of HIF-2α	Chondrocytes	[449,450]
	Increase in activity of HIF-3α	Chondrocytes	[451]
Compression	Signal transduction through integrin	Chondrocytes	[52,452,453]
HP and compression	Calcium signalling probably mediated by primary cilia	Chondrocytes	[454–458]
	Signal transduction via actin cytoskeletal remodelling	Chondrocytes	[459]
Compression	Stimulation of endogenous TGF-β synthesis	BMSCs and chondrocytes	[284,285,460,461]
3-D scaffolds	Signal transduction via actin cytoskeletal remodelling	All cell types	[462–464]
	Pathways associated with cell-cell interaction		[126,237,238]
	Influence the response to mechanical stimulation		[455,465,466]
IGF-1	Activation of MAPKs	Ubiquitous	[72–75,77,78,467,468]
TGF-β	Activation of Smad 2/3 or Smad 1/5/8 and MAPKs		
FGF	Activation of MAPK and STAT1 pathways		
BMP-2, 4, 6, 7, GDF-5	Activation of Smad1/5/8 and MAPKs		

Human SFSCs have been differentiated into chondrogenic lineage in pellet [394,399] and alginate scaffolds [398] in the presence of TGF-β1 and ITS [394,398,399], dexamethasone [394,399], ascorbic acid [398,399] and BMP-7 [399]. Main limitations in the use of SFSCs are the surgical procedure for their isolation and their limited availability.

Perivascular stem cells (PSCs) can be isolated from IFP and represent another source of MSCs, which include pericytes around small blood vessels. When differentiated *in vitro* into chondrogenic lineage, they showed higher chondrogenic capability compared to ADSCs from IFP [319]. However, these cells have been scarcely investigated.

9. Pluripotent stem cells

The tendency of mesenchymal stem cells to undergo hypertrophic differentiation can theoretically be overcome using pluripotent stem cells that possess a higher degree of plasticity. Human embryonic stem cells (ESCs) have been differentiated into chondrogenic lineage in pellet culture and HA hydrogels with TGF-β1, FGF2 and PDGF-BB [38]. After differentiation, they maintained low expression of hypertrophic markers (e.g. collagen type I and collagen type X). Their *in vivo* chondrogenic capability has been demonstrated in rat models [38]. Using different growth factors (BMP-4 or GDF-5), it has been possible to generate 2 different population of chondrocytes derived from mouse ESCs: one with and one without hypertrophic properties [400]. The ethical issues and the risk of immune rejection of ESCs can be overcome using induced pluripotent stem cells (iPSCs). iPSCs are generated by transducing with reprogramming transcription factors (OCT4, SOX2, KLF4 and c-MYC) adult somatic cells, which are generally skin fibroblasts [401], even though also osteoarthritic chondrocytes have been used [402]. iPSCs have been differentiated into chondrogenic-like lineage by exposure to BMP-4 [403] or TGF-β3 [396] or also through transduction with TGF-β1 and co-culture with adult chondrocytes [402]. A combination of FGF-2, BMP-4, follistatin, NF4 and GDF-5 has also been used with a timing that mimics the physiological chondrogenesis [39]. Once differentiated, iPSCs exhibited *in vitro* similar chondrogenic potential to BMSCs, but lower fibroblastic-hypertrophic tendency, in terms of collagen type I, collagen type X and RUNX2 expression [401]. Using different growth factors (TGF-β3 or BMP-4), it has also been possible to generate *in vitro* chondrocytes with hypertrophic or chondrogenic characteristics (in terms of collagen type I, collagen type II and collagen type X synthesis) and these characteristics were maintained even *in vivo*, when the cells were injected subcutaneously in mice [404]. Direct iPSC injection or combination with a carrier (e.g. alginate hydrogel [396], a chondroitin sulphate methacrylate hydrogel [39] or a PLC and gelatin electrospun scaffold [405]) has been successfully used for repairing cartilage defects in rats [396], mice [39] and rabbits [405]. iPSCs have also been used to produce MSCs, whose regenerative potential was then tested in rabbit chondral defects, although it was not compared to the one of other MSCs or with iPSC-derived chondrocytes [406]. Due to their high availability, ease of isolation, high expansion capacity [403] and pluripotent differentiation potential, iPSCs potentially represent a continuous sufficient source of patient-derived chondrocyte-like cells. Their application is further extended by the recent advances in gene therapy, which has also led to the generation, through gene editing, of iPSCs unable to respond to interleukin 1 (IL-1) stimulation and to *in vivo* resistant to inflammation iPSC-derived chondrocytes [407]. Limitations, such as the extensive cell handling and prolonged culture times required, may be overcome by the generation of universal iPSC-derived chondrocyte lines. However, the variation of the populations produced and the risk of tumorigenesis [403] still need to be carefully evaluated, as abnormal differentiation into secretory tumour tissue during iPSC *in vitro* chondrogenic induction has been reported [408]. Further, better comparison with multipotent stem cells, in terms of hypertrophic tendency, needs to be performed.

10. Critique

Although various *in vitro* cell culture approaches (e.g. use of three-dimensional scaffolds, growth factor supplementation, mechanical stimulation, co-culture systems, modulation of oxygen tension, transfection/transduction) have been used to either induce or maintain chondrogenic phenotype in the different cell types, there is still a lack of complete understanding on how these mediators act (Table 6). Considering also the need to minimise cell handling for clinical application, complex approaches are not easily translatable into clinical practice.

With respect to the choice between one cell type over another largely depends on the difficulty in guaranteeing a stable chondrogenic phenotype. Due to their scarce availability, chondrocytes need to be pre-expanded *in vitro*. Although different multifactorial approaches can maintain a chondrogenic phenotype *in vitro*, only a few of them have been tested *in vivo*, making difficult to determine if they can result in stable phenotype. Recent studies suggest that it is possible to selectively isolate CPCs or adult chondrocytes with a more stable phenotype. However, there is no clear consensus on markers for their characterisation and the regenerative potential of these cells has not been clearly compared with the one of heterogeneous population of chondrocytes. Furthermore, the fact that CPCs are normally involved in response to injury may suggest that their regenerative potential is not enough to lead to functional repair [409].

The main advantages of MSCs are that they possess the same regenerative capability as chondrocytes [306] and they are readily available. However, there are still a few issues that need to be addressed. Firstly, it has to be clarified if cells from elderly donors possess a lower proliferation and differentiation capability, as studies provide

contradictory results [410,411]. Secondly, there is no consensus with respect to the pre-differentiation before implantation: whilst some studies have reported that implantation of undifferentiated cells leads to calcification and fibro-genesis [21,293], it should also be noted that *in vitro* differentiation often leads to hypertrophic phenotype [202,287,320]. In the presence of inflammatory diseases, the use of undifferentiated MSCs is encouraged by their immunomodulatory and anti-inflammatory properties [412]. Intra-articular injection of ADSCs attenuated osteoarthritis progression in an experimental rabbit model [361], whilst secretome from BMSCs reduced IL-1 and metalloproteinase synthesis in human synovial explants, while increasing the nitric oxygen production and IL-1 receptor antagonist in human cartilage explants [413]. Undifferentiated MSCs also require minimal handling. On the other hand, a direct comparison between undifferentiated and pre-induced MSCs have been performed only in animal models, in which the acquisition of chondrogenic phenotype could be easier than in human patients [414]. Assuming that pre-differentiated cells lead to better clinical outcomes, numerous studies have compared the *in vitro* chondrogenic potential of MSCs from different sources. However, this comparison is somehow onerous, given that MSCs from different sources may respond differently to the same stimuli and the lack of standardised expansion protocols in defined media, which also leads to dramatic variations [320]. Some studies have also implanted pre-differentiated cells in different animal models, but differences in cartilage thickness, involvement of subchondral bone and self-healing capability of animal models used make difficult the comparison between results of different studies [415]. In general, BMSCs have been described as the MSCs with the highest chondrogenic potential [147,294,321–323], whilst ADSCs have been used since they are readily available and possess a greater expansion capability. However, differences in proliferation and viability of ADSCs from different depot and donor age have also been observed [416–418]. SDSCs have been widely studied, since their chondrogenic potential is independent of donor age and their tendency to hypertrophic differentiation is relatively low. Their main limitation is the reduced availability of donor material and the knee surgery required for their isolation. iPSCs show promising results, possessing a low tendency to undergo *in vitro* hypertrophic differentiation.

To date, only undifferentiated BMSCs and ADSCs have been tested in humans. No differences between BMSCs and chondrocyte treatments have been observed, whilst a clinical trial is currently comparing ADSC and BMSC treatments (NCT02642848). However, the wide spectrum of the clinical requirements of the patients requiring a cartilage substitute makes hard to generalise comparable results obtained. Factors like age, concomitant diseases and defect size affect the clinical outcomes. It has also been evidenced that, due to stricter inclusion criteria, patients commonly enrolled in clinical trials are not representative of patients treated in clinical practice, especially as regard their defect size [419,420]. The limited available clinical data indicate that currently available cell therapies (naïve or engineered cells without pre-conditioning) seem to possess the same capacity to achieve a short-term success, as micro-fracture and osteochondral autograft [421] and they are more expensive and laborious. Further, despite it had been initially developed to prevent the formation of fibrocartilage repair tissue typical of micro-fracture, ACI itself often results in the production of fibrocartilage rather than hyaline cartilage, lacking a long-term regenerative capacity [422]. Thus, it is imperative to conduct more controlled/standardised clinical trials that the reparative potential of pre-conditioned cells would be assessed.

11. Conclusions

Although almost thirty years have passed since the first autologous chondrocyte implantation procedure, cell-based therapies have still the same success rate as other surgical treatments. Whilst different cell types have been proposed as potential candidates for cartilage engineering, significant differences between *in vitro* and *in vivo* cell behaviour, lack of standardisation of pre-clinical experiments, difficulty in transferring results obtained in pre-clinical models to human patients, safety and scalability of the potential technologies and differences in patients' clinical characteristics have made difficult to compare the regenerative capabilities of the various cell types and to bring constantly/consistently cell therapies to market. The growth in newly developed knowledge on how *in vitro* stimuli (e.g. media supplements, three-dimensional scaffolds, mechanical stimulation, oxygen tension, co-culture systems) regulate chondrogenesis in different cell types will help in the rational design of new culture systems, enabling that way realisation of a long-term stable cartilage engineering products and subsequent thereof clinical translation and commercialisation.

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