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Review

Sheep as a model for evaluating mesenchymal stem/stromal cell (MSC)-based chondral defect repair

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SUMMARY

Osteoarthritis results from the degradation of articular cartilage and is one of the leading global causes of pain and immobility. Cartilage has a limited capacity for self-repair. While repair can be enhanced through surgical intervention, current methods often generate inferior fibrocartilage and repair is transient. The development of tissue engineering strategies to improve repair outcomes is an active area of research. While small animal models such as rodents and rabbits are often used in early pre-clinical work, larger animals that better recapitulate the anatomy and loading of the human joint are required for late-stage preclinical evaluation. Because of their physiological similarities to humans, and low cost relative to other large animals, sheep are routinely used in orthopedic research, including cartilage repair studies. In recent years, there has been considerable research investment into the development of cartilage repair strategies that utilize mesenchymal stem/stromal cells (MSC). In contrast to autologous chondrocytes derived from biopsies of articular cartilage, MSC offer some benefits including greater expansion capacity and elimination of the risk of morbidity at the cartilage biopsy site. The disadvantages of MSC are related to the challenges of inducing and maintaining a stable chondrocyte-like cell population capable of generating hyaline cartilage. Ovine MSC (oMSC) biology and their utility in sheep cartilage repair models have not been reviewed. Herein, we review the biological properties of MSC derived from sheep tissues, and the use of these cells to study articular cartilage repair in this large animal model.

Introduction

Disability due to damaged cartilage is a major global health and economic burden[1]. Articular cartilage has a limited capacity for self-repair, leaving it susceptible to degradation over time, resulting in a condition known as osteoarthritis (OA). OA is more prevalent in aging populations, reflecting the impact of cumulative insults on the joint over a lifetime[2]. Early onset of OA is often associated with rapid decay emanating from focal cartilage lesions formed as a result of acute trauma[3]. OA cartilage cannot be repaired, and total joint replacement is required to restore joint function. Focal lesions can be repaired, potentially delaying OA progression and the need for a total joint replacement. Improvements in cartilage tissue engineering are enabling more robust focal defect repair, and future advancements may enable resurfacing of an OA joint.

Selection of a large animal model to study cartilage defect repair

Basic cartilage tissue engineering studies are undertaken *in vitro*. Transition to *in vivo* animal models is influenced by need, appropriateness of the model, and cost. Generally, costs per animal increase with animal size[4]. Early stage research often utilizes small animal models, including mice, rats and rabbits[5,6]. Immune-compromised mice enable characterization of ectopically implanted xenogeneic cartilage-like tissues derived from human cells. While this approach

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enables critical *in vivo* assessment, the microenvironment of ectopic sites differs from joints. Joints in rats and rabbits are sufficiently large for surgical manipulation and these animals are commonly used to study cartilage repair. However, (1) their cartilage is thinner than in human joints, (2) defects may spontaneously heal, and (3) joint biomechanics and loading differ from humans[5].

The need to mimic the dimensions and loading experienced by an adult human joint motivates investment into large animal models of cartilage repair. The most commonly utilized large animals are pigs, goats, horses and sheep[7,8]. Purchasing, handling, housing and experimentation costs are all substantially greater for large animals than for small ones. For example, the purchase costs of sheep and goats are approximately twice that of rats[9], while the cost of pigs and horses is greater still. However, all four large animals offer a pre-clinical stifle joint model that is more relevant in size and anatomy to a human knee joint than a small animal[5]. We quantified published cartilage repair studies that used these four large animals between 1993 and November 2017 [Fig. 1(A)]. The two most common were sheep and pig, with sheep being the most commonly used large animal between 2013 and November 2017 (~5 years).

Standardization of cell therapies has proven critical[10,11], and the focus of limited research resources on a single pre-clinical large animal model could facilitate more rapid advancements toward clinical translation. To date, there does not appear to be a consensus as to which model is best to invest in. An important consideration in model selection is its compatibility with the post-operative care requirements associated with human cartilage defect repair, including immobilization and off-loading. This allows the repair tissue to mature and integrate. Both sheep and goats can be immobilized in a sling following surgery, effectively offloading the repaired joint[12]. However, goats have a propensity to chew and climb fences[13], a characteristic that increases their overall cost relative to sheep, and introduces greater risk of self-inflicted injury. Unlike in sheep and goats, offloading a horse joint is problematic due to the animal's size and weight. Offloading compromises circulation in the offloaded leg, and can cause laminitis in the distal loaded leg[14]. All four large animals are routinely used for the testing of cell-based cartilage repair strategies involving articular chondrocytes (AC) or mesenchymal stem/stromal cells (MSC; Fig. 1(B)). Taking into consideration their anatomy, size and nature, as well as relatively lower associated costs and rising popularity, sheep represent an excellent model for such studies.

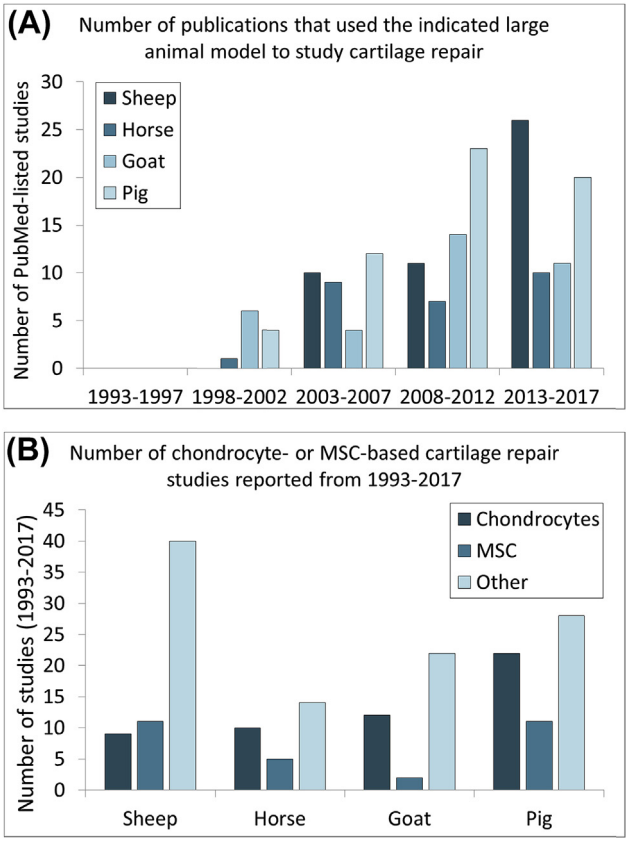

Fig. 1. The prevalence of large animals in cartilage repair studies. (A) The number of studies found through PubMed searches of "sheep cartilage repair [text word]", "horse cartilage repair [text word]", "goat cartilage repair [text word]", "pig cartilage repair [text word]", in 5-year intervals. (B) The number of large animal cartilage repair studies that involved the use of chondrocyte, MSC, or other repair strategies between 1993 and 2017.

Current FDA-approved cartilage therapies utilize autologous AC isolated from articular cartilage harvested from a non-weight-bearing portion of the joint. While data suggest these therapies can enable cartilage repair, the need to create a secondary defect to harvest autologous AC limits the number of cells that can be removed, necessitating monolayer expansion, during which dedifferentiation occurs[15]. These limitations have motivated investment into the development of repair strategies that utilize stem cells in place of AC.

MSC have been historically viewed as a potential cellular panacea for cartilage defect repair[16]. They can be easily isolated from many tissues, are readily expanded in culture, and appear to have the capacity to either directly differentiate into chondrocytes or secrete factors that may upregulate endogenous repair processes. However, despite promise, MSC-based cartilage therapies have not yet achieved the efficacy required for routine clinical use. Despite 20 years of research[17], the field struggles to differentiate MSC into a stable AC population that does not have a propensity to undergo hypertrophy when implanted *in vivo*[16]. Even so, the field has made great strides in understanding MSC biology, as well as standardizing literature definitions and practices. This standardization in reporting is now viewed as essential for facilitating the development of MSC-based therapies[10].

While there have been thousands of publications on human MSC (hMSC) over the past 25 years, our PubMed search identified only a modest 29 studies using MSC to repair cartilage defects in the four indicated large animals [Fig. 1(B)]. Of those, sheep and pig were utilized in 11 studies each. It is clear that pre-clinical testing of cartilage repair strategies will require the use of a large animal model. Ease of handling, relatively lower costs, and anatomical suitability have made sheep a recent forerunner in cartilage repair studies [Fig. 1(A)]. With the view that MSC hold great potential for use in cartilage repair, and that literature assimilation and standardization is essential, we herein (1) discuss the features and anatomy of sheep stifle joints, (2) summarize literature reports on the isolation, culture, and differentiation of ovine MSC (oMSC) and (3) discuss outcomes of studies investigating cartilage defect repair in sheep, including those performed using oMSC.

Sheep stifle joint anatomy

The sheep stifle joint anatomy has been well-characterized and compared with the human knee joint[18,19]. The anatomy and biomechanics of the joint are relatively similar to human knees (Fig. 2)[20]. The average medial femoral condyle (MFC) cartilage thickness is ~1.68 mm in sheep, while this thickness is ~2.35 mm in humans[21]. Bone plate thickness in sheep MFC and trochlea is more than that of human. The bone mineral density at 1 and 3 mm below the bone plate of sheep MFC and trochleas is also greater than that

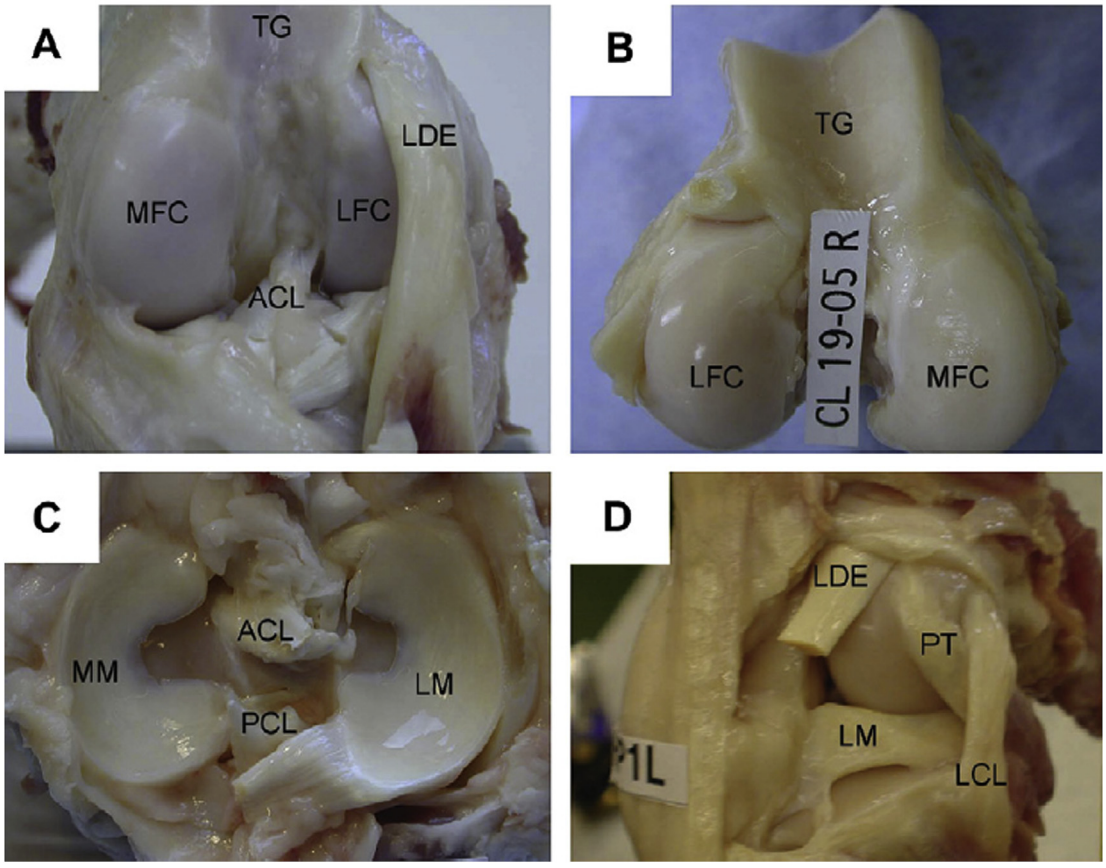

Fig. 2. Anatomy of the sheep knee/stifle joint. This image was originally published by Little and colleagues as part of the OARSI Histopathology Initiative in 2010[21] and is reproduced here with permission. Abbreviations: LCL = lateral collateral ligament; ACL and PCL = anterior and posterior cruciate ligaments; LDE = long digital extensor; PT = popliteal tendon; MM and LM = medial and lateral meniscus; MFC and LFC = medial and lateral femoral condyle; TG = trochlear groove of distal femur.

of humans, and sheep trochleas are more porous than human trochleas[22]. To study acute cartilage damage in sheep, a critical-sized defect (8 mm in diameter), which cannot self-repair, is drilled through healthy cartilage in the knee joint[5,21,23]. Sheep can also function as an effective OA model, as they can develop OA naturally[7].

oMSC isolation, expansion, and characterization

Jessop and colleagues were the first to isolate a bone marrow-derived MSC population in sheep; the cells exhibited fibroblastic morphology and could be induced into adipogenic and osteogenic phenotypes *in vitro*[24]. Since then, others have isolated oMSC from cord blood[25], adipose tissue[26], peripheral blood[27], liver[28], amniotic fluid[29], dental pulp[30], synovial membrane[31], dermis[32], hair follicles[33], and endometrium[34].

In 2006, the International Society for Cellular Therapy (ISCT) published a position paper outlining the three minimal criteria for defining hMSC including: (1) plastic-adherence, (2) positive expression of cell surface markers CD105, CD73 and CD90, and lack of expression of hematopoietic markers and (3) the capacity to differentiate down the three mesodermal lineages (adipogenic, osteogenic, and chondrogenic)[10]. While the cell surface expression profile of culture-expanded hMSC is well-characterized, and human antibodies are readily available and reliable, this is not the case for oMSC. Table I outlines papers identified in our search that characterized oMSC using the classic ISCT hMSC marker profile. Based on these reports, collectively, it appears that oMSC may not consistently express the same markers as hMSC. However, Khan and colleagues identified CD73, CD90, CD105, CD271, and MHC-I monoclonal antibodies that were cross-reactive for oMSC[35]. They also observed positive results with CD29, CD44, and CD166 cell surface markers. Their suggested panel is CD90 and CD105 (>99%), CD73 and CD271 (>95%), and <1% positive for CD117. Even so, Table I demonstrates that no group has identified an antibody combination that can be used to characterize oMSC to ISCT standards; it is critical that further studies identify appropriate antibody clones that enable this characterization.

oMSC media formulations and growth characteristics

The media formulations used for *in vitro* expansion and differentiation of oMSC are similar to those used for hMSC. Expansion and differentiation media formulations are listed in Supplementary Table 1. Expansion media formulation influences oMSC size, proliferation, surface epitope expression, and differentiation potential[43]. Expansion medium is often supplemented with fetal bovine or calf serum. oMSC proliferation is responsive to FBS concentration, with greater proliferation observed with 20% FBS compared to 15, 10, or 5%[28]. One-third of the articles summarized in Supplementary Table 1 augmented medium with basic fibroblast growth factor-2 (FGF-2). FGF-2 increases MSC proliferation and

Table I
Flow cytometry CD markers for human MSC (hMSC) and ovine MSC (oMSC). The results of flow cytometry characterization in selected oMSC studies are compared with the hMSC standard set by the International Society for Cellular Therapy (ISCT). The cell surface marker profile does not consistently align between oMSC studies and the hMSC ISCT-established panel, perhaps due to a lack of antibody cross-reactivity or a differing cell surface marker profile between the two species

ISCT Markers	CD105	CD73	CD90	CD45	CD34	CD14 or CD 11b	CD79a or CD19	HLA-DR
ISCT hMSC minimal criteria[10]	>95%	>95%	>95%	<5%	<5%	<5%	<5%	<5%
Zannettino *et al* 2010[36]	N/A	N/A	N/A	0.30%	N/A	0.70%	N/A	N/A
Mrugala *et al* 2008[37]	Low	N/A	N/A	Very low	N/A	N/A	N/A	N/A
Khan *et al* 2016[35]	99.10%	96.90%	99.00%	39.40%	N/A	N/A	N/A	10.50%
Martinez-Lorenzo *et al* 2009[38]	10.90%	5%	95.50%	N/A	0.80%	N/A	N/A	90.70%
Sanjurjo-Rodriguez *et al* 2017[39]	Did not work	Did not work	Did not work	1.15%	Did not work	N/A	N/A	N/A
McCarty *et al* 2009[40]	99.50%	N/A	N/A	0.60%	N/A	CD14: 28.5%	N/A	N/A
Kalaszczynska *et al* 2013[41]	N/A	N/A	N/A	–	N/A	N/A	N/A	N/A
Gronthos *et al* 2009[42]	N/A	N/A	N/A	0.10%	N/A	0.20%	N/A	N/A
Adamzyk *et al* 2013[43]	0–95%	0–95%	0–80%	<50%	N/A	N/A	N/A	N/A
Caminal *et al* 2015[44]	~25%	N/A	>95%	N/A	N/A	N/A	N/A	N/A
Caminal *et al* 2014[45]	N/A	N/A	93.41%	<5%	N/A	N/A	N/A	N/A
Colosimo *et al* 2013[29]	N/A	N/A	N/A	1.62%	N/A	0.98%	N/A	0.99%
Desantis *et al* 2015[46]	N/A	N/	Low	<5%	<5%	N/A	N/A	<5%
Garza-Veloz *et al* 2013[47]	N/A	N/A	N/A	36.78%	N/A	N/A	N/A	N/A
Godoy *et al* 2014[31]	N/A	N/A	N/A	1.72%	N/A	CD11b: 1.15%	N/A	N/A
Kunisaki *et al* 2007[25]	Low	N/A	Low	N/A	N/A	N/A	N/A	N/A
Locatelli *et al* 2013[48]	N/A	N/A	N/A	<5%	N/A	N/A	N/A	N/A

improves subsequent chondrogenesis[49]. Cryopreservation in 90% FBS/10% DMSO is common with hMSC and has previously been used for oMSC[27,29,50]. A lower percentage of serum (20% FBS/10% DMSO in culture media) can also be used and results in post-thaw viability rates greater than 90%[51].

In vitro tri-lineage differentiation potential is a common characterization assay used for MSC across species, including human and sheep. Differentiation induction medium formulations described in oMSC studies are similar to those commonly used in hMSC studies (see Supplementary Table 1). Adipogenic media is commonly supplemented with indomethacin, insulin, 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone. Osteogenic differentiation media commonly include dexamethasone, ascorbic acid 2-phosphate, and β-glycerophosphate. Chondrogenic media is supplemented with ascorbic acid 2-phosphate, an isoform of TGF-β, dexamethasone, and insulin-transferrin-selenium (ITS).

In humans, the relative number and fitness of bone marrow-derived MSC declines with donor age[52]. When cells were harvested from sheep aged 4 months to 8 years, neither the initial number of bone marrow-derived oMSC obtained nor their subsequent proliferation rate appeared to be age-dependent, unlike the decline in hMSC fitness associated with human aging[53]. The typical lifespan of sheep is 10–12 years, allowing for long-term studies[53]. Like with hMSC, long-term *in vitro* expansion of oMSC is associated with decreased proliferative potential, altered osteogenic potential, and modified surface antigen expression[29]. In sheep, neither breed nor cryopreservation appeared to impact proliferative potential, although donor-to-donor variation has been reported as with hMSC[53]. The above-mentioned similarities between hMSC and oMSC indicate the usefulness of such animal models in pre-clinical studies, though differences in MSC behavior suggest data should be interpreted cautiously.

Ovine MSC *in vitro* chondrogenesis

MSC have been thought to hold great promise for cartilage regeneration either in their undifferentiated or differentiated form. Undifferentiated MSC secrete factors that may encourage endogenous tissue repair and MSC differentiated into chondrocytes could directly contribute to new tissue[54]. While MSC have several benefits relative to autologous chondrocytes, the challenge is differentiating these cells into a stable chondrocyte-like population capable of producing hyaline cartilage[16]. Current approaches include the use of exogenous factors, manipulation of the physiochemical microenvironment, mechanical stimulation, and genetic modification[55,56]. Supplementary Table 1 shows the media formulations used in specific articles. TGF-β1 (used in 6/19 articles) and TGF-β3 (11/19 articles) were the most commonly used chondrogenic growth factors. Although TGF-β isoforms are most commonly used, other growth factors that positively regulate cartilage development, including IGF-1, BMP-2 and BMP-7, have also been described[37,40,57–59].

MSC can be cultured in atmospheric oxygen (20%) and 5% carbon dioxide at 37°C. Culture of hMSC under low oxygen conditions (1–10% O_2) maintained their undifferentiated, multipotent status[60]. As reported with hMSC[61], expanding and differentiating oMSC in low oxygen culture enhanced *in vitro* chondrogenesis[62,63]. Additionally, a mathematical model indicated that low oxygen conferred higher proliferative activity to oMSC, increased colony-forming potential, and decreased senescence[64,65]. Culturing bone marrow-derived cells in low oxygen conditions also resulted in a higher percentage of cells expressing MSC markers[66]. Even when low oxygen-expanded oMSC were subsequently differentiated in normoxia, type II collagen and aggrecan levels were significantly greater than those of oMSC expanded in normoxia[63]. Human stem cell chondrogenesis can be enhanced by mechanical stimulation[67], and similar has been observed in oMSC[68].

The modification of *in vitro* cell culture environments with exogenous growth factors is a powerful tool, but there are limitations including short half-lives, concentration fluctuation, and difficulty in delivering exogenous factors to developing cartilage tissue *in vivo*. Increasingly, gene modification is being used to guide cell fate and is gaining momentum as a viable clinical tool[69,70]. Adenoviral vectors have been used to transduce adipose-derived oMSC with *IGF1*, *TGF-β1*, *FGF-2*, and *SOX9* either alone or in combination[47]. Co–expression of *IGF1* and *FGF-2* improved *ACAN*, *COMP*, and *COL2A1* expression. While *TGF-β1* gene transfer likewise increased *ACAN* and *COL2A1* expression, hypertrophy marker *COL10A1* was also elevated. In other work, oMSC transduced to express *TGF-β1* also facilitated cartilage repair[71].

Ovine cartilage defect models

Expanded autologous AC are routinely used in the clinic, and a recent National Institute for Health and Care Excellence guidance recommended ACI for the repair of cartilage defects greater than 2 cm^2 [72]. However, the use of AC in ACI-type repair approaches is only indicated for treatment of defects, and not for treatment of OA. By contrast, the use of MSC may be broader, but treatments remain experimental for both focal defects and OA. Table II outlines significant details from *in vivo* ovine studies that utilized MSC. In general, implanting cells in a suitable biomaterial or scaffold appeared to facilitate tissue repair. Importantly, scaffolds assist in retaining cells, particularly in shallow defects. It is important to emphasise that results were highly variable within studies and across studies. Table II is structured to describe the use of oMSC to treat (1) OA joint models, (2) chondral lesions, and (3) osteochondral defects, in that order. Each cartilage model reflects a different type of injury or cartilage damage. Thus it could be expected that one treatment method might be more efficacious in one model than in another.

In vitro and *in vivo* comparison of ovine chondrocytes and oMSC

As noted above, expanded AC have been used clinically to repair cartilage defects in humans[84] and in pre-clinical sheep models[85]. Harvesting autologous AC creates a second defect site and the cells typically dedifferentiate during expansion[86]. Like human AC, ovine AC are reported to dedifferentiate during monolayer expansion[87]. It remains unclear whether oMSC or ovine AC are preferred for *in vivo* repair: oMSC produced better cartilage repair in one study[83], while a separate study reported that ovine AC resulted in superior cartilage formation[78]. Nonetheless, techniques using autologous AC are not indicated for patients with OA[88] and this has motivated the field to aggressively investigate MSC-based repair strategies.

In vivo repair with undifferentiated oMSC

It may be possible to repair tissues without differentiating MSC into the desired terminal cell type *in vitro*. Some studies suggest that MSC can home to injury sites, engraft and differentiate into repair tissues, although the veracity of this process has been contested in the field[89]. Alternatively, MSC might be capable of facilitating tissue repair through paracrine signaling and immunomodulation, with direct MSC differentiation likely playing a minor role in some instances[90]. In either scenario, it may be possible for MSC to facilitate cartilage repair without being differentiated into chondrocytes prior to introduction into a patient. If efficacious, such processes would be appealing as their cell culture time would likely be shorter, contributing to reduced total cost.

Some research groups are looking to further reduce cost and complexity through elimination of cell expansion altogether. An example of this is the use of bone marrow and lipid aspirates, minimally manipulated to isolate mononuclear cells, as a treatment for damaged cartilage. Although its efficacy is not clear[91], this minimal manipulation often allows procedures to be exempt from the regulatory oversight that is commonly applied to cell-based therapies and reduces the time, cost, and labor involved in cell preparation. Below, we detail studies in which cells were not expanded and/or differentiated prior to implantation.

Desando and colleagues compared cultured oMSC to fresh bone marrow concentrate (BMC), which is composed of a mixed cell population containing MSC, hematopoietic stem and progenitor cells, monocytes, and other cells[75]. BMC is prepared using density gradient centrifugation, and there is no subsequent culturing of the cells. This yields a heterogeneous cell population but reduces the time and cost associated with *in vitro* culture. Both cell types were seeded in a hyaluronan scaffold and implanted into sheep in which osteoarthritis had been induced. Following 12 weeks, the BMC treatment resulted in superior repair of OA damaged tissue than oMSC treatment. Another group injected expanded oMSC into the knee and compared this to injection of BMC[74]. Superior cartilage repair was achieved using the expanded oMSC. Worth noting is that BMC treatment, which contained very few oMSC, enabled some cartilage regeneration, perhaps indicating that some other cell population in the BMC may be contributing to repair.

In another study oMSC were expanded *in vitro* but not pre-differentiated. Autologous bone marrow-derived oMSC were seeded onto bioceramic β-TCP scaffolds, with empty defects and cell-free scaffolds serving as controls[79]. Following 24 weeks incubation *in vivo* the oMSC group displayed a smooth, integrated tissue with quantities of glycosaminoglycan that reached 89% that of native cartilage. The oMSC group had proteoglycan and type II collagen staining consistent with hyaline cartilage, while the cell-free group appeared to contain more fibrocartilage.

In a model of chronic articular cartilage and meniscal injury, repair with undifferentiated oMSC was compared with an untreated control[77]. In treated groups, there were improvements in many macroscopic and histological parameters. However, articular cartilage and meniscus regeneration were only observed in some animals, and proteoglycan and type II collagen quantities were variable. In a different study published by the same group, autologous oMSC were seeded on PLGA scaffolds and articular cartilage defect repair was evaluated[45]. While neocartilage formation was superior in the cell-seeded group at 4 months post-implantation, the improvement was transient and the macroscopic scores significantly decreased by 12 months. In a follow-up paper, this group utilized undifferentiated autologous cells from cartilage, fat and bone marrow for osteochondral defect repair[44]. Cell-seeded PLGA scaffolds produced variable results in promoting the regeneration of hyaline tissue. Both autologous chondrocytes and bone marrow-derived oMSC demonstrated superior results relative to adipose-derived oMSC and the cell-free scaffold control. The bone marrow-derived oMSC group enabled the best tissue morphology and thickest cartilage.

The potential of cell-free biomaterial-mediated chondral defect repair has also been explored. In one study, undifferentiated bone marrow-derived oMSC were seeded in a PLGA scaffold, with cell-free scaffolds used as the control[80]. Three months after implantation, improved regeneration was observed with oMSC-seeded scaffolds, although histological results were highly variable. The O'Driscoll score was similar between cell-free and cell-seeded scaffolds leading the authors to conclude that the addition of cells was not beneficial in this instance, although high variability was observed across groups.

It may be possible to bypass *in vitro* differentiation if an appropriate growth factor is delivered *in vivo* with the cells. In one study, oMSC were seeded on a chitosan scaffold containing TGF-β3, and directly implanted into defects[37]. After 2 months *in vivo* a well-integrated hyaline-like cartilaginous matrix was observed. Results were superior to either the chitosan scaffold or TGF-β3 alone, suggesting that cells, scaffold and growth factor are required in combination to promote *in vivo* cartilage regeneration.

Studies of intra-articular injections of cell suspensions suggest promise, and the ease of such a procedure is appealing. In a recent (2017) single-blind human study, bone marrow aspirates or a saline placebo control were injected into the contralateral knee joints of patients[91]. Patients reported similar improved outcomes in both joints, suggesting that the placebo effect can confound studies. This highlights a challenge associated with human testing and the value

Table II
Selected *in vivo* MSC cartilage repair studies in sheep. The number of animals used, the type and size of the defect and study length are included. The experimental group and the control group are described, as well as the key findings. osteoarthritis (OA); anterior cruciate ligament (ACL); microfracture (MF), bone marrow mononuclear cells (BMMC); International Cartilage Repair Society (ICRS)

Animals	Defect type	Defect size	Construct(s)	Control group(s)	Study length	Key findings	Reference
Osteoarthritis							
16	OA induction with ACL excision and medial meniscectomy	N/A	Undifferentiated MSC or MSC differentiated with TGF-β3 and IGF1	Media, no cells	6 weeks	Retardation of cartilage destruction in both MSC groups; meniscus repair better in differentiated MSC group	Al Faqeh *et al* 2012[73]
18	OA induction with ACL transection and medial meniscectomy	N/A	Intraarticular injection of either concentrated BMMC or 10 million MSC	Vehicle; untreated	8 weeks	Better repair with MSC however BMMC group also had reduced inflammation markers	Song *et al* 2014[74]
20	OA induction through unilateral partial medial meniscectomy	N/A	Bone marrow concentrate or MSC seeded in hyaluronan scaffold	Scaffold alone; untreated	3 months	MSC or BMC groups repaired tissue better than scaffold-only and no-treatment groups	Desando *et al*[75]
18	OA induction with ACL transection and medial meniscectomy	N/A	Single dose of PHK26-labeled chondrogenically induced adipose-derived or bone marrow-derived oMSC	Culture medium	6 weeks	Both oMSC groups improved ICRS scores compared to control but there was no significant difference between the two treatment groups	Ude *et al* 2014[76]
12	OA induction through bilateral lateral meniscectomy	N/A	Intraarticular injection of MSC	Untreated	12 weeks	At the 12 week endpoint, there was no significant difference in the degree of OA detected by MRI, radiography and post-mortem evaluation between the two groups	Delling *et al* 2015[50]
Chondral defects							
10	Full thickness cartilage defect and tearing of medial meniscus	60 mm^2	Undifferentiated bmMSC	No cells	6 and 12 months	High variability in repair; macroscopic and histological scores of condyles were better with MSC treatment and OA progression was not observed	Caminal *et al* 2014[77]
6	Partial thickness patellar lesions	4 mm diameter	MSC with TGF-β3 and chitosan with fibrin	No TGF-β or chitosan	2 months	Best results with TGF-β-chitosan group suggesting both pre-differentiation and scaffold are supportive of cartilage regeneration	Mrugala *et al* 2008[37]
28	Partial thickness chondral defect on medial condyle	6.2 mm diameter	MSC transduced with TGF-β1	Untreated	6 months	Collagen type II production increased	Ivkovic *et al* 2010[71]
15	Full thickness chondral defect in medial femoral condyle	1 cm^2	Either 1 or 5 million chondrocytes or 5 million MSC	MF	12 weeks	Chondrocytes produced a better repair tissue than adipose-derived non-differentiated MSC	Guillen-Garcia *et al* 2014[78]
28	Chondral defect in medial femoral condyle	8 mm diameter; 4 mm deep	MSC-β-TCP composite	β-TCP construct alone; untreated	24 weeks	MSC group more hyaline-like with good integration while cell-free group more fibrocartilage-like	Guo *et al* 2004[79]
9	Critical size, chondral-only defects in medial femorotibial condyle	7 mm diameter	PLGA scaffold, either alone or seeded with MSC	Untreated	12 months	Transitory improvement of cartilage compared to the control group	Caminal *et al* 2014[45]
9	Full thickness defects in medial femoral condyle	8 mm diameter	PGLA implants alone or seeded with MSC	Cell-free scaffolds	3 months	Large variations in histological results; MSC samples were both fibrous and hyaline-like; O'Driscoll scores similar between cell-free and cell-seeded scaffold groups	Wegener *et al* 2010[80]

(*continued on next page*)

Table II (*continued*)

Animals	Defect type	Defect size	Construct(s)	Control group(s)	Study length	Key findings	Reference
Osteochondral defects							
8	Critical size defect in medial and lateral femorotibial condyles	3.5 mm diameter; 5 mm deep	Porous PLGA scaffolds with autologous progenitor cells from cartilage, fat, or bone marrow (4 mm diameter, 7 mm high)	Cell-free scaffold	6 and 12 months	Scaffolds seeded with either cartilage or bone marrow-derived cells resulted in better repair than fat-derived cells	Caminal *et al* 2015[44]
10	Osteochondral lesions in medial femoral condyle	7 mm	Differentiated or undifferentiated MSC in hydrogel	Cell-free hydrogel; untreated	6 months	Pre-differentiation group displayed superior collagen type II production compared to non-differentiated MSC group	Zscharnack *et al* 2010[81]
10	Osteochondral defects	4 mm (initial diameter); 6.4 mm (6 weeks later)	MSC-seeded triphasic construct with pre-differentiated cells	Osteochondral autografting (OATS)	12 months	A high degree of variability was observed and many of the implants sunk however osseointegration was good	Marquass *et al* 2010[82]
9	Osteochondral lesions in medial femoral condyles	7 mm diameter	Pre-differentiated or non-differentiated MSC in a collagen I hydrogel	Chondrocyte-seeded matrix associated autologous chondrocyte transplantation; untreated control	12 months	Pre-differentiated MSC implanted in a collagen type I hydrogel resulted in better repair	Marquass *et al* 2011[83]
24	Osteochondral defect in medial femoral condyle	6 mm diameter; 8 mm deep	MSC:PBMC 20:1, MSC:PBMC 2:1, PBMC alone; all in GAG-collagen scaffold	MSC in GAG-collagen scaffold	26 weeks	Hypoxic culturing critical for MSC enrichment; MSC-only and PBMC-only groups resulted in thickest neocartilage; no statistical difference in ICRS or O'Driscoll scores	Hopper *et al* 2015[66]

of clinically relevant large animal models that are not susceptible to the placebo effect.

In vivo repair with pre-differentiated oMSC

A few studies have evaluated the repair of osteochondral defects *in vivo* with oMSC that have been chondrogenically induced *in vitro* prior to implantation. These studies are motivated by the notion that *in vitro* pre-differentiation will yield the required cell type, and enable more efficient cartilage defect repair.

In one such study, pre-differentiated oMSC seeded on a type I collagen hydrogel displayed superior type II collagen production compared to non-differentiated cells[81]. While some promising defect repair was observed, this study highlighted the heterogeneity of repair across donors (Fig. 3). In another study, oMSC from both bone marrow and adipose tissue were compared[76]. When induced *in vitro*, bone marrow-derived oMSC displayed higher expression of chondrogenic markers including *COL2A1*, *SOX9*, and *ACAN* than adipose-derived oMSC. When chondrogenically-induced oMSC were injected *in vivo*, there was no significant difference in ICRS scores between the bone marrow- and adipose-derived oMSC groups, though both performed better than culture medium alone[82]. However, another study compared pre-differentiated and non-differentiated autologous bone marrow-derived oMSC in a chronic OA model, where both oMSC groups slowed cartilage degradation[73]. ICRS scores from defects treated with oMSC or induced cells were similar, suggesting that *in vitro* differentation may not be necessary.

In a study conducted by Marquass *et al*., a triphasic construct was seeded with pre-differentiated oMSC and implanted *in vivo*[82]. The construct included a β-tricalcium phosphate osseous bone layer and a type I collagen hydrogel cartilage layer, which were bonded together with fibrin glue. This was compared with the Osteochondral Autograft Transfer System (OATS), similar to mosaicplasty, in which an autologous osteochondral cylinder is harvested and implanted into a defect site. Repair was compromised in 4 of 10 triphasic constructs as they sank into subchondral bone, rather than supporting integration of the cartilage layer at the joint surface. This sinking phenomenon did not occur with OATS controls.

Overall, cartilage repair in sheep appears to be aided by the addition of oMSC delivered in a scaffold. It is not yet clear whether expansion and/or pre-differentiation are beneficial or not. Further studies will be necessary to clarify best practices in this pre-clinical large animal model.

Future perspectives

The field of cartilage tissue engineering could be significantly advanced through the development of a robust large animal model in which to test potential therapies. To date, translation of animal data has been challenging[92]. Further investment and refinement into a single model should lead to fundamental improvements that will facilitate translation; the sheep is arguably an excellent model to pursue. Over the past decade, sequencing of the sheep genome was commenced[93] and completed[94] by the International Sheep Genome Consortium. This new data will facilitate research in sheep models and drive the development of sheep-specific reagents. Study of MSC-based cartilage repair in sheep is in its infancy. There is considerable variation reported within and between studies. The difficulties observed in oMSC studies, such as donor-to-donor variability and conflicting results, parallel those from hMSC experiments. These common challenges could be seen as an indication of the authenticity of the sheep model system, rather than an indictment of it. This is the first review of oMSC isolation,

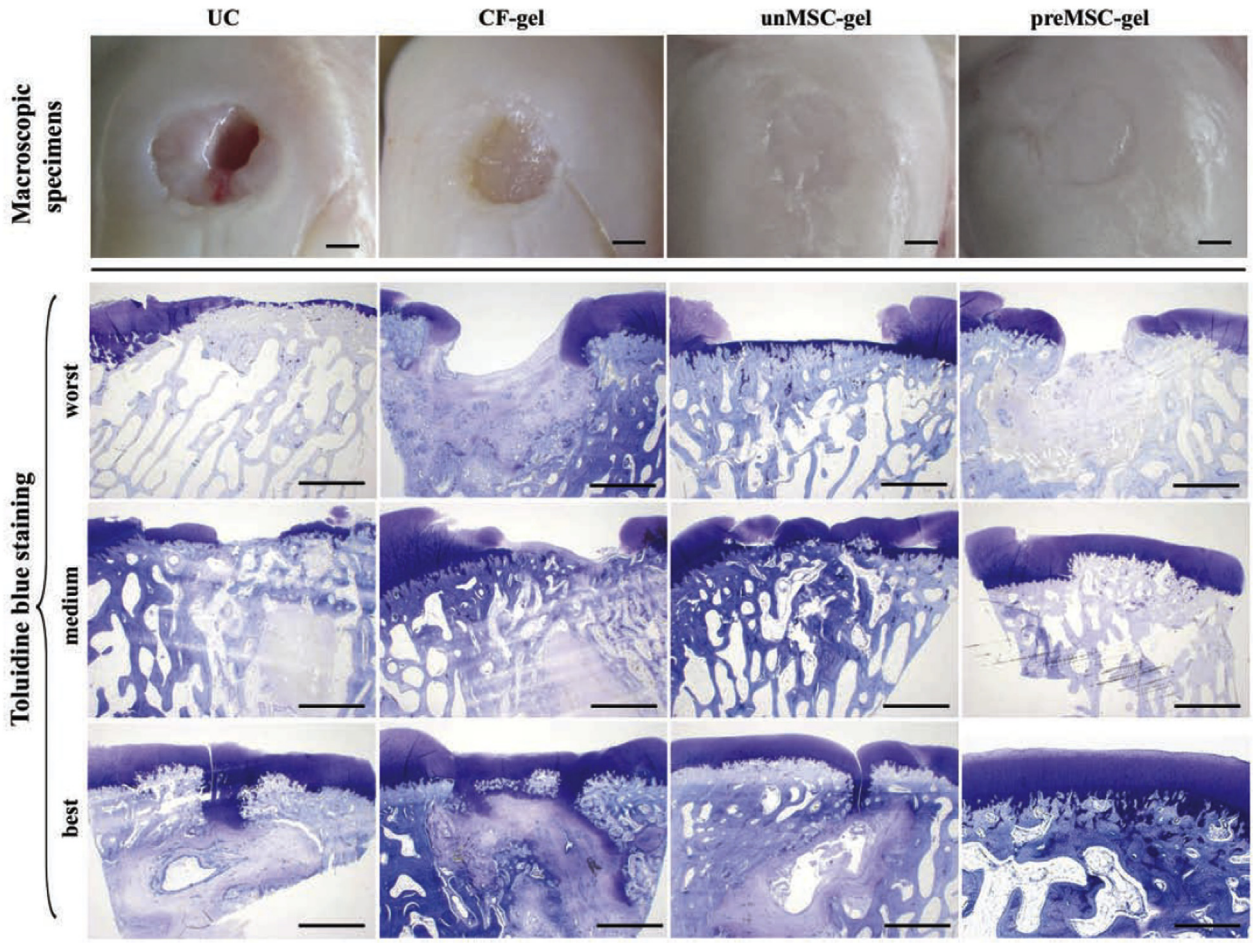

Fig. 3. Relative efficacy of pre-differentiated MSC for cartilage regeneration and result variability. This image was first published by Zscharnack and colleagues in 2010[81] and is reproduced here with permission. Top panel: representative macroscopic images of defect 6 months following treatment. Bottom panels: selected examples of best, medium, and worst results 6 months after treatment. Scale bar = 2 mm.

characterization, and their use in *in vivo* models of cartilage repair. We identify similarities between oMSC and hMSC, and describe the latest sheep cartilage repair outcomes in response to cell expansion, differentiation with specific growth factors, and use of scaffolds. We anticipate that assimilating and building on this data will contribute to cartilage tissue engineering strategies likely to translate to positive clinical outcomes in humans, making investment into this model system a valuable use of research time and funding.

Declaration of author contributions

All authors contributed to the conception and design and drafting of the article, critically reviewed the article for important intellectual content, collected and assembled the data from the available literature, and gave final approval of the article.

Competing interest statement

All authors declare there are no conflicts of interest.

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Supplementary data

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