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Articular fibrocartilage - Why does hyaline cartilage fail to repair?☆

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ABSTRACT

Once damaged, articular cartilage has a limited potential to repair. Clinically, a repair tissue is formed, yet, it is often mechanically inferior fibrocartilage. The use of monolayer expanded *versus* naïve cells may explain one of the biggest discrepancies in mesenchymal stromal/stem cell (MSC) based cartilage regeneration. Namely, studies utilizing monolayer expanded MSCs, as indicated by numerous *in vitro* studies, report as a main limitation the induction of type X collagen and hypertrophy, a phenotype associated with endochondral bone formation. However, marrow stimulation and transfer studies report a mechanically inferior collagen I/II fibrocartilage as the main outcome. Therefore, this review will highlight the collagen species produced during the different therapeutic approaches. New developments in scaffold design and delivery of therapeutic molecules will be described. Potential future directions towards clinical translation will be discussed. New delivery mechanisms are being developed and they offer new hope in targeted therapeutic delivery.
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Abbreviations: AAV, Adeno-associated virus; ACI, Autologous chondrocyte implantation; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs; ALK, Activin receptor like kinase; bFGF, Basic fibroblast growth factor; BMAC, Bone marrow aspirate concentrate; BMP, Bone morphogenetic protein; CD, Cluster of differentiation; Col, Collagen; COMP, Cartilage oligomeric matrix protein; DMOAD, Disease modifying OA drug; DNA, Deoxyribonucleic acid; ECM, Extracellular matrix; EV, Extracellular vesicle; GAG, Glycosaminoglycan; GDF-5, Growth differentiation factor-5; GMP, Good manufacturing practice; HA, Hyaluronan; HGF, Hepatocyte growth factor; IGF-1, Insulin-like growth factor binding protein-1; IL, Interleukin; IL-1Ra, Interleukin-1 receptor antagonist; IHH, Indian hedgehog; mRNA, Messenger RNA; miRNA, MicroRNA; MMP, Matrix metalloproteinase; MSCs, Mesenchymal stromal/stem cells; MV, Microvesicle; NK, Natural killer; NO, Nitric oxide; OA, Osteoarthritis; PBS, Phosphate buffered saline; PEG, Poly-ethylene glycol; PEMF, Pulsed electromagnetic field; PGE2, Prostaglandin E2; PLGA, Poly(lactide-*co*-glycolide); PLGF2, Placenta growth factor – 2; PTHrP, Parathyroid hormone-related protein; RNA, Ribonucleic acid; SDF-1, Stromal cell derived factor-1; SLRPs, Small leucin-rich proteoglycans; TGF-β, Transforming growth factor-beta; TNF, Tumor necrosis factor; VEGF, Vascular endothelial growth factor; xELP, Cross-linked elastin-like polypeptide.

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1. Introduction

The treatment of articular cartilage injuries remains a demanding undertaking, not only because they are notoriously difficult to repair [1], but also because cartilage defects and injuries constitute a leading cause of post-traumatic osteoarthritis (OA). Secondary, or post-traumatic OA results from traumatic injury, anatomical joint deformity or aberration in the cartilage extracellular matrix (ECM), while primary OA is characterised by the absence of known underlying factors or structural abnormalities within the joint. Regardless of the type, the progressive degeneration of articular cartilage is a central feature of OA, the most common form of joint disease. A series of risk factors such as age, genetics, obesity and joint surface defects have been identified as players in the development of this joint disease [2]. To date, no disease modifying OA drug (DMOAD) has been licensed, although a list of failed attempts exists [3]. Difficulties in developing a successful therapy are in part due to the inherent nature and physiology of the tissue [1,4,5]. The location of articular cartilage within diarthrodial joints gives reason for its function as a shock absorbing tissue that provides low friction movement during articulation, protecting the ends of bones and allowing pain-free articulation. The *in vitro* engineering of such a complex, highly organised tissue is still an open challenge, feeding the research on different cell types and sources, materials and delivery methods [1]. Numerous pre-clinical models, both small and large, exist but do not easily translate into clinical studies. This is likely in part due to an overall adherence to the directions provided by the regulatory agencies that, up to 2014, was calculated as below 60% [6].

Reparative and restorative procedures, ranging from bone marrow stimulation techniques to more complex autologous chondrocyte implantation (ACI), are routinely used to treat cartilage injuries based on the lesion size and the level of physical activity [7]. In many cases, a rudimentary repair tissue does form, albeit one that does not possess sufficient mechanical properties to sustain the repeated load cycles applied over the years [8]. The fibrocartilage typically formed degrades with time, leading to a follow up procedure being required. Furthermore, the integration of the *de novo* tissue to the surrounding defect is often poor and leads to an incongruous surface. In other words, the regeneration of articular cartilage to its natural state, rather than repair and substitution with the incorrect type of tissue, represents the real issue behind the intense research on cartilage regenerative medicine. Nevertheless, in a limited number of cases, hyaline cartilage does form during the healing phase. How this occurs, and how we can drive the process more reliably in the hyaline direction is currently unclear. A number of important questions remain when considering therapeutic strategies for cartilage regeneration: should cells be delivered, or can their secretome be used as an alternative [9]? When no cells are implanted, are proteins or nucleic acids more effective? Finally, which is the optimal material to deliver the chosen therapeutic?

This review aims to: 1. Report the challenges that still need to be addressed to achieve the goal of hyaline cartilage regeneration by focusing on the shortcoming of the repair tissue generated after traumatic injury or biological therapy; 2. Discuss the state of the art in cartilage-targeting therapeutic delivery; and 3. Highlight some of the emerging technologies and strategies that the authors believe to hold great promise.

2. Cartilaginous tissues: they are not all the same

Cartilage is classified as a special connective tissue with mesodermal origin characterised by a cellular component immersed within ECM composed of ground substance (polysaccharides), a fibrillar component (fibrous proteins), and interstitial fluid (mainly water). There is no direct supply of blood, lymphatics and nerves; nutrition relies on diffusion from the surrounding tissues. Based on the composition and function, it is possible to distinguish three types of cartilaginous tissues: hyaline, fibro- and elastic cartilage. Here we will focus predominantly on hyaline cartilage, as it is the tissue of clinical interest, and fibrocartilage as this is the tissue typically formed using the current reparative and restorative clinical procedures. In addition, we will also introduce hypertrophic cartilage that naturally functions as transitional tissue during bone growth but during *in vitro* cell differentiation assays it represents the undesired form of cartilage.

2.1. Hyaline cartilage

Hyaline cartilage is the most common type of cartilage, acting as a temporary skeleton during embryo development until it is gradually replaced by bone tissue. Its matrix is rich in ground substance, namely glycosaminoglycans (GAGs) and highly adherent collagen fibres (mainly type II collagen) not visible at the microscope even after routine histological staining (Fig. 1). GAGs create lateral chains covalently linked to a core protein to form proteoglycans, with aggrecan being the largest and the most abundant [10]. Hyaline cartilage is present at the connection between the ribs and the sternum, in the trachea and on the articulating surfaces of the synovial joints, such as the elbow and knee.

2.1.1. Articular cartilage: biology and biomechanical properties

Within articular cartilage, the ECM composition varies within the different zones of the tissue from the more superficial layer in contact with the synovial fluid within the joint cavity to the deepest layer in contact with the subchondral bone. In this direction, the fibrillar and interstitial fluid component both decrease while the ground substance increases.

The upper superficial zone contains minor amounts of type I collagen. Type II collagen, as a major solid component of hyaline cartilage ECM, provides an overall shape of the tissue by constraining the swelling pressure imposed by the negatively charged GAGs. Despite its major contribution to cartilage, its crystal structure is so far unavailable (Fig. 1). In addition, type VI collagen is present in the pericellular matrix and lesser amounts of type IX and XI collagen are deposited in the interterritorial matrix. In particular, type IX collagen interacting with type II collagen functions as a fitting molecule contributing to the biomechanical properties of the tissue. Type XI collagen is present within the fibrils of type II collagen, regulating their thickness. Type X is the only collagen present in the calcified cartilage in contact with the subchondral bone. The collagen fibres display differential organisation in the different layers of cartilage, running parallel to the articulating surface in the superficial layer, appearing rather disorganised in the middle layer and then running vertically across the depth of the transitional zone. This differential

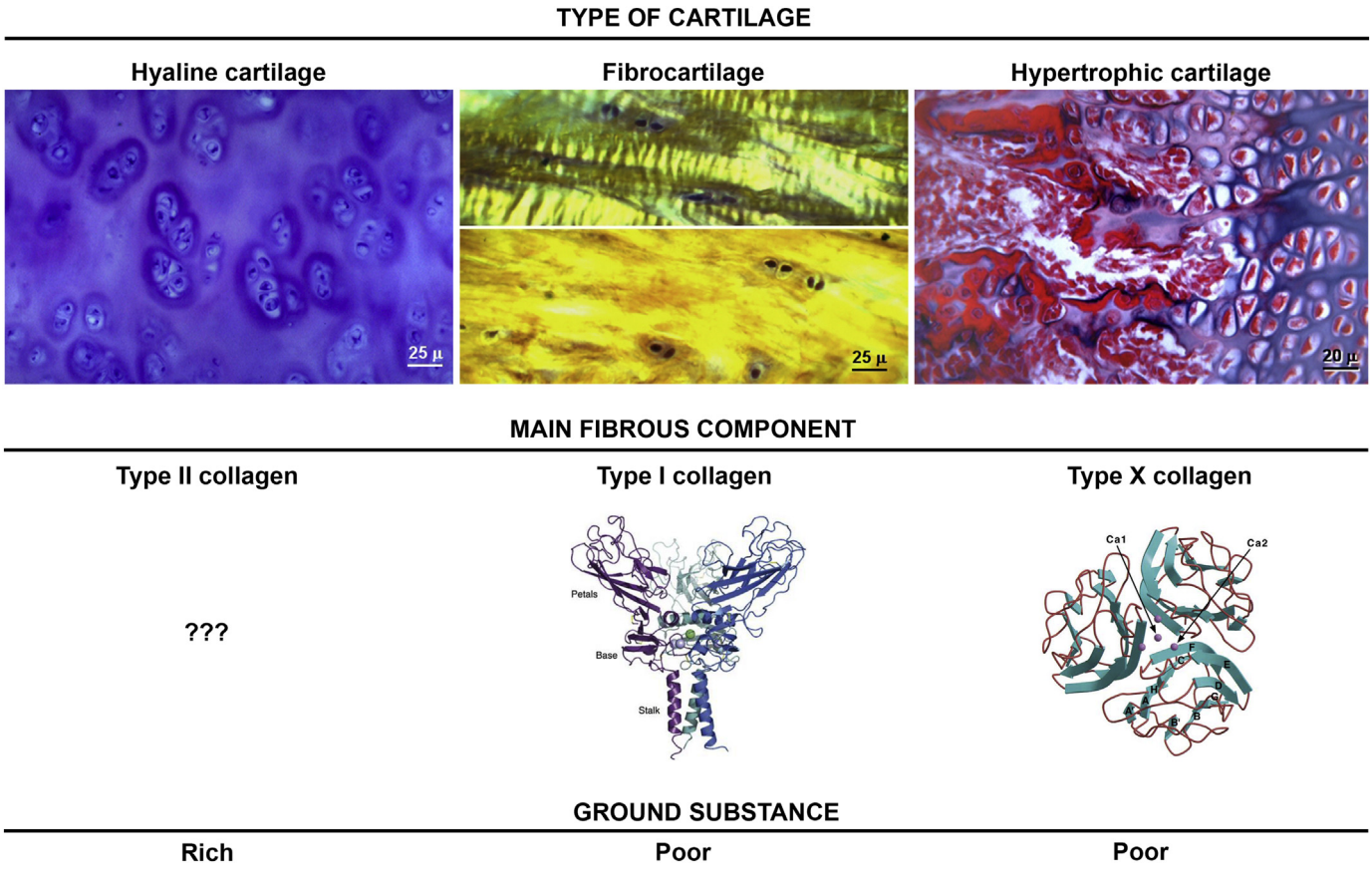

Fig. 1. Hyaline, fibro and hypertrophic cartilage at a glance. The fibrillar component and the ground substance of cartilaginous tissues distinguish hyaline from fibro- and hypertrophic cartilage. The ground substance of cartilage includes GAGs, proteoglycans and small leucine-rich proteins (SLRPs). In the images hyaline cartilage is stained with Haematoxylin & Eosin, fibrocartilage is stained with Haematoxylin and Tartrazine and visualised under polarised light in the upper panel, and hypertrophic cartilage is stained with AZAN trichrome stain. Histology images reproduced from http://anatom-server9.uzh.ch/anatomy/Anatomy.html, with permission of Peter Groscurth, MD, Prof. em. at the Institute of Anatomy, University of Zürich – Switzerland. Type I collagen 3D structure was reproduced with limitation to Fig. 2A of original article by Sharma and colleagues [231] distributed under the terms of the Creative Commons Attribution (CC BY 4.0) licence (http://creativecommons.org/licenses/by/4.0/). Type X collagen 3D structure was reprinted from Insight into Schmid Metaphyseal Chondrodysplasia from the Crystal Structure of the Collagen X NC1 Domain Trimer, Oren Bogin, Marc Kvansakul, Eran Rom, Josef Singer, Avner Yayon, Erhard Hohenester, Volume 10, Issue2, P165–173, © Elsevier Ltd. (2002), with permission from Elsevier [232].

spatial organisation of the collagen fibres depends on the direction of the mechanical stimulation and confers cartilage with tensile properties [11,12]. As previously mentioned, GAGs are negatively charged molecules, which allows the proteoglycans to retain a large volume of water and act as a shock-absorber. When compression is applied to articular cartilage, the water moves from the cartilage to the synovial fluid. When the pressure is removed, the water flows back into the interstitial space between the GAGs, created by the electrostatic repulsion between carboxylic and sulphuric groups within the GAGs. This mechanism explains the resilience of articular cartilage.

Cartilage ECM is produced by chondrocytes, the sole terminally differentiated cell unit, present at different densities throughout the depth of the tissue. Chondroblasts, the precursor cells of chondrocytes, derive from mesenchymal cells and they can divide and synthesise ECM. With the increasing amount of intracellular matrix produced, the cells become separated from each other and embedded within cavities with the matrix known as lacunae. At this stage, chondrocytes with a high division rate are formed with a large endoplasmic reticulum and a highly developed Golgi apparatus, facilitating the intensive protein synthesis. In mature cartilage, chondrocytes within the lacunae organise themselves in isogenous groups derived from the division of the same chondroblast. Isogenous groups are more abundant in the middle and transitional zone compared to the superficial and the deep zone of the articular cartilage. In the deep zone, chondrocytes are hypertrophic and synthesise type X collagen supporting the mineralisation of the cartilage in this zone.

The signalling pathways involved in the development of articular cartilage during joint formation are extremely complex and they have been the main topic of numerous reviews [13–16].

2.2. Fibrocartilage

Fibrocartilage should be accounted as a transitional tissue between hyaline cartilage and dense regular connective tissue such as tendons and ligaments [17]. Its matrix is rich in densely braided collagen fibres (Fig. 1) that make the tissue highly resistant to compression. Chondrocytes are very low in number and are aligned with the thick collagen fibres. In contrast to hyaline cartilage, fibrocartilage contains high levels of type I collagen in addition to type II and only a small component of ground substance. Fibrocartilage is naturally present between vertebral bodies, in pubic symphysis, menisci and at the tendon-bone interface.

2.3. Elastic cartilage

As the name suggests, elastic cartilage is an elastic and flexible cartilage able to withstand repeated bending. Within the ECM the fibrillar component is prevalent, with type II collagen and elastic fibres densely branched in multiple directions. The chondrocytes lying between the fibres are larger, with fewer isogenous groups compared to hyaline cartilage. This cartilaginous tissue provides elasticity to the organs within which it is present: epiglottis, auricle and Eustachian tube.

2.4. Hypertrophic cartilage

Hypertrophic cartilage is characterised by enlarged chondrocytes that have stopped dividing and accumulate glycogen, lipids, and alkaline phosphatase. Hypertrophy occurs at the expense of the ECM production although short-chain type X collagen fibres are produced. Hypertrophic cartilage is also known as the maturation zone within the epiphyseal plate at the junction between the epiphysis and the diaphysis of growing long bones (Fig. 1). It also occurs as an intermediate tissue during bone fracture repair.

3. Cartilage defects, injuries and degeneration

Cartilage composition is regulated by chondrocytes in response to chemical and mechanical extracellular changes [18]. Cartilage turnover is the result of a fine equilibrium between catabolic and anabolic processes. An imbalance between catabolism and anabolism, resulting in the loss of cartilage matrix, is a hallmark of cartilage defects, injuries and degeneration. Cartilage catabolism is regulated by endogenous enzymes including matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS); signs of cartilage catabolism can be detected in the synovial fluid and in the plasma serum. Articular cartilage contains small proteoglycans known as small leucin-rich proteoglycans (SLRPs) including decorin, biglycan and fibromodulin, which play a key role during collagen fibrillogenesis [19] and at the late stage of OA are still synthesised but differentially glycosylated [20]. Fibromodulin and decorin core protein fragments were found to be associated with degenerated human articular cartilage [21]. Another matrix protein of mention is the cartilage oligomeric matrix protein (COMP), which interacts with collagens [22,23] and mediates the chondrocytes-ECM interaction *via* integrin receptors [24,25]. COMP serum level can be used to identify patients affected by OA and correlates with its severity [26]. Specifically, COMP serum levels are higher at the early stage of primary OA when compared to control subjects and decreases with increasing OA duration [27]. It was suggested as a marker to identify patients at risk of faster knee OA progression [28].

Early studies in human OA cartilage [29,30] and experimental OA in dog cartilage [31] showed type II collagen synthesis at the mRNA and protein level. Similarly, the proteoglycan production was shown to increase using radioactive labelling strategies both *in vitro*/*ex vivo* [32–35] and *in vivo* [36]. Interestingly, Aigner and colleagues found a zone-specific response in OA human cartilage. The area of cartilage where new collagen synthesis was detected (middle zone) did not correspond to the area of proteoglycan degradation identified by loss of Safranin O and Toluidine blue staining (upper zone) [29]. Thus, it appears that there is an attempt of the chondrocytes of the middle zone to enhance their metabolic activity to counteract the loss of ECM suffered in the upper zone. This attempt fails at the moment the catabolic events overtake with the result that the articular cartilage loses its matrix framework and consequently its properties. While for post-traumatic OA the injurious event is likely to be the signal that triggers the cascade of events, for primary OA the causative event is still unclear.

A recent study using the carbon-14 (^{14}C) bomb pulse method [37] to quantify the collagen and GAG turnover rates has questioned the current theory of an increased collagen synthesis during the early stages of cartilage degeneration. In this study by Heinemeier and colleagues, it was revealed that while a constant GAG turnover takes place in the adult cartilage, the majority of the collagen matrix is formed before maturity and subsequent collagen turnover is very limited. Both healthy and OA cartilage, sampled from highly and moderately loaded areas, were analysed and no difference in the ^{14}C patterns was observed suggesting that neither a degenerative condition such as OA, nor loading/damage is associated with an increased collagen turnover [38]. This data would support the four hundred years of turnover time for human femoral head articular cartilage, as estimated by Maroudas in 1979 [39]. As the authors of both studies discussed, it cannot be ruled out that a small fraction of collagen with a more dynamic nature exists, although it appears that the greatest amount of collagen is likely produced pre-maturity to last a lifetime.

4. Current therapeutic approaches for articular cartilage repair

While it may initially appear as a simple tissue, the paucity of cells, the high matrix to cell ratio and the lack of direct blood supply in articular cartilage create an environment that is challenging to heal. This has led to repair strategies that aim to increase the local cell number by stimulating endogenous cell migration into the defect using marrow stimulation techniques, or by direct implantation of cells.

4.1. Cell homing

The concept behind a cell homing approach derives from the observation that osteochondral defects involving the subchondral bone have a higher rate of new tissue formation than chondral defects affecting only the cartilage [40]. Clinically, cell homing is commonly employed *via* the various marrow stimulation techniques, such as microfracture, when the chondral defect is not bigger than 2–3 cm^2 [41–43]. During the microfracture technique, numerous microfractures are created 3–4 mm apart in the damaged area. A series of blood clots containing mesenchymal stromal/stem cells (MSCs) from bone marrow will form in the microfracture sites, resulting in the eventual differentiation of MSCs into chondrocytes and production of new cartilaginous tissue. However, typically the tissue derived from the bone marrow MSCs following this technique is mostly described as fibrocartilage expressing type I collagen, rather than hyaline cartilage containing type II collagen [44].

4.2. Cell transplantation: freshly isolated versus monolayer expanded cells

In order to test the feasibility of cell-based therapies for articular cartilage repair, animal models of cartilage injury have been developed [45,46] enrolling different animal species and types of focal defects, with the clear majority of preclinical studies involving monolayer expanded chondrocytes or MSCs.

These studies translated clinically with the ACI technique, first reported in 1994 [47] but with later papers highlighting that the first patient was treated in 1987 [48]. Since then, ACI has established itself as the gold standard for the treatment of large cartilage defects (3–10 cm^2) [49]. As originally described, the transplantation relies on a three-step procedure: 1. Isolation of chondrocytes from a healthy non-load-bearing area; 2. *In vitro* expansion of chondrocytes; 3. Implantation of the chondrocytes into the site of the defect under a periosteal flap. A USA multicentre study evaluated the durability of ACI in a long-term follow-up (up to 10 years) and reported that 69% of the patients improved while 17% failed with 75% of failures registered at the mean follow up of 2.5 years [50].

Hypertrophy and delamination of the periosteal flap led to development of a second generation ACI using a porcine collagen membrane, therefore reducing the intra-operative handling and time. A small study on 23 patients proved superior clinical long-term outcome of the second generation over the first-generation procedure [51]. With the advance of tissue engineering, a third generation of ACI (matrix-associated ACI) was introduced to improve the cell delivery. Autologous chondrocytes are seeded in a 3D porous scaffold that can be cut to fit the defect site and secured using fibrin glue instead of sutures [52]; this provides a support for the cells in absence of a matrix, although there is no compelling evidence to prefer scaffold-based ACI over the first generation approach [53]. Classic and matrix associated ACI were compared in a randomised study and no clinical difference was found up to two years [54]. Results of comparisons between ACI and microfracture are still contradictory, with some studies showing a significantly higher clinical outcome for ACI [55], while others have

reported no significant difference in the clinical outcome [56]. Long-term follow-up trials are warranted to establish whether microfracture performs better than the current gold standard of ACI.

The as yet unsolved disadvantage of ACI is the need for a monolayer expansion. The monolayer expansion step has been shown to be detrimental for the cell phenotype, with chondrocytes expressing a higher level of type I collagen and down-regulating the expression of type II collagen, in a process known as de-differentiation [57]. Indeed, signs of fibrocartilage rich in type I collagen are often reported following ACI [44]. Unlike chondrocytes, the use of monolayer-expanded MSCs has not yet translated to the clinic, where the use of freshly isolated, naïve bone marrow cells is most commonly performed with transplantation of cells collected from bone marrow aspirate concentrates (BMAC), demonstrating promising results as adjuvant or a standalone procedure. Although, as stated by Cotter and colleagues, "Numerous questions remain regarding BMAC usage, including cell source, cell expansion, optimal pathology, and injection timing and quantity" [58].

Studies using monolayer-expanded endogenous human bone marrow MSCs for cell implantation have been reported, but the number of patients is still limited and the follow up too short. Fibrocartilage was observed by arthroscopy when using bone marrow-derived MSC first embedded in collagen gel and then placed on a collagen sheet for the treatment of full thickness chondral defects in two young patients [59]. When the same approach was used to treat the knee cartilage of 12 patients with a diagnosis of OA the results were arthroscopically and histologically promising in the 9 patients undergoing second look operations [59]. A case report of a young athlete showed a three-layer repair tissue, with the first layer being a fibrotic tissue and the middle layer staining positively for both Safranin O, Toluidine blue and type II collagen [60]. MSC-based strategies are attractive because compared to chondrocytes, bone marrow MSCs have the advantage of ease of access, thereby eliminating the need for a first operation, and the greater number of cells obtained during the isolation and following expansion without the risk of de-differentiation. However, the monolayer expansion conditions can heavily influence the phenotype of the cells obtained [61], even when using good manufacturing practice (GMP) [62]. It can be concluded that the difference between the use of monolayer-expanded *versus* freshly isolated cells should not be overlooked when considering the translational aspect of changes in cell phenotype [63].

Despite the apparent advances, a note of caution should be stated that, although the current cell-based therapies have shown symptomatic improvements, so far little or no evidence exists to support a reduction in the risk of developing OA in a joint affected by a focal cartilage lesion.

5. Collagen: the challenge of reproducing the appropriate one

The expression of collagen is a major requirement for connective tissue development and function, with type II, I and X as the main collagen present in hyaline, fibro- and hypertrophic cartilage, respectively. With various tissues expressing different types, collagens can be used as a phenotypic marker to assess functional cell differentiation. For articular cartilage, no matter whether *in vitro* or *in vivo*, the aim is to induce chondrocytes or MSCs to produce type II collagen. When considering that type II collagen is produced during cartilage development and that in the adult or diseased tissue no turnover can be observed, it becomes clear that the challenge is a difficult one.

During cartilage tissue formation, aberrant collagen expression can be observed due to two distinct pathways, resulting in the formation of hypertrophic cartilage or fibrocartilage. *In vivo,* after microfracture, BMAC or even ACI, the most common non-hyaline tissue formed is a mechanically inferior type I/II collagen-containing fibrocartilage [41,44,64]. In the case of microfracture or BMAC, an explanation could be found in the content of the bone marrow. MSCs are the only cell precursor of chondrocytes and their presence within the bone marrow can be as low as 0.001% [65]. Other cell types, such as haematopoietic stem cells and osteoblasts, are also resident in the bone marrow, in addition to blood and the cells carried within it. All these cell types have access to the site of the cartilage defect during microfracture and BMAC, but none of them is ontogenically programmed to produce hyaline cartilage. While the tidemark may transition deeper than normal into the cartilage during marrow stimulation, this is believed to be a bone healing response to the bone damage caused to allow the marrow from the subchondral space to enter the defect. This bone formation can be alleviated by using a finer awl that causes less damage (nanofracture) [42,66]. The influence of the subchondral bone has been shown to greatly affect the outcomes in preclinical animal models of articular cartilage repair, suggesting this is a critical aspect that should be considered when planning suitable and effective animal models [67].

In vitro, when monolayer expanded MSCs are induced to differentiate towards chondrocytes, a common result is the terminal differentiation into hypertrophic chondrocytes expressing type X collagen [68–70], a phenotype typically associated with endochondral bone formation. This is frequently mentioned as the main disadvantage of utilising MSCs for cartilage repair and occurs even ectopically after subcutaneous implantation of MSC-derived pellets [71,72]. Interestingly, when performing chondrogenesis *in vitro* using monolayer expanded chondrocytes, type X collagen expression is limited [73]. An open question is whether the type X collagen observed during *in vitro* MSC differentiation is due to the previous *in vitro* monolayer expansion/selection of the cells or whether the outcome is a reflection of the chondrogenic differentiation protocols used. Monolayer expansion typically leads to the expression of type I collagen, even in the case of fully differentiated chondrocytes, and this expression is generally maintained once differentiation is initiated [57,74]. This type I collagen induction is, in part, an artefact due to the *in vitro* culture of cells on tissue culture plastic, and the mechanism of induction may be different to that seen in *in vivo* fibrocartilage. The commonly reported collagen II: collagen I ratio improves during chondrogenesis, but this is mainly due to an increase in type II collagen rather than a significant reduction in type I collagen [57,75].

Considering that cartilage is tissue with low oxygen tension due to its lack of blood supply, it is important to highlight the fact that monolayer expansion preconditions the cells to abnormally high oxygen levels. This might create complications when implanting the cells into a hypoxic defect site. Genome-wide microarrays of human articular chondrocytes demonstrated that Col2a1, Col9a1, Col11a2, and aggrecan are hypoxia-inducible genes [76]. The effect of hypoxia on the expression of collagen was also demonstrated in a comparison between healthy and OA chondrocytes. In this case, low oxygen tension increased the expression of type II collagen and aggrecan while reducing the levels of type I and X collagen. The latter remained consistently higher in OA chondrocytes compared to the healthy chondrocytes even under hypoxia [77,78]. The impact of oxygen tension during isolation and expansion was evaluated also in sheep-derived bone marrow MSCs. After isolation and expansion under 3% or 21% oxygen, the cells were seeded on a type I collagen sponge or hyaluronic acid (HA) mesh. Chondrogenic differentiation was carried out for 2 weeks using both oxygen tensions. Aggrecan expression at the mRNA level was increased under low oxygen tension, independently of the scaffold. Type II collagen was significantly upregulated under hypoxia only in cells seeded on the collagen sponge. Type X collagen was not affected by the oxygen tension in any of the scaffolds, although it remained consistently higher in the collagen scaffold [78]. Similarly, in another study using bovine bone marrow MSCs seeded onto polycaprolactone (PCL) microfibers mats, hypoxia did not reduce the expression of type X collagen but the co-culture with bovine articular chondrocytes did [79]. Therefore, it is clear that the oxygen tension is important, but it is not the only factor influencing the phenotype of the cells during their expansion and differentiation. For instance, in the work reported by Meretoja and co-authors, hypoxia emphasised the expression of type X collagen in human bone marrow MSCs. Interestingly though, when in co-culture with chondrocytes, the hypertrophy was arrested independently of the oxygen level [79].

The differentiation cocktail used to drive the chondrogenesis of MSCs *in vitro* is also a likely contributing factor and has been shown to induce expression of type X collagen as early as day 7 of culture [68]. The classic differentiation protocol for bone marrow MSCs includes a serum-free medium supplemented with Insulin-Transferrin-Selenium (ITS), non-essential amino acids, ascorbic acid, dexamethasone and transforming growth factor beta (TGF-β) as the main driver of chondrogenesis. The cells are typically cultured at high density for 21–28 days [68]. Since the first description, other protocols have been developed in an attempt to inhibit, or at least reduce, the unwanted synthesis and deposition of type X collagen, and they have been thoroughly reviewed by Studer and co-authors [80]. Classic pathways involved in cartilage development such as Wingless/In (Wnt)/β-catenin, TGF-β/bone morphogenic proteins (BMPs), parathyroid hormone-related protein (PTHrP), Indian hedgehog (IHH) are cited as potential targets to study hypertrophy. A recent study from the group of Ivan Martin took inspiration from the role of BMP receptor I during embryonic cartilage formation [81]. *In vitro*, the inhibition of both activin receptor-like kinase- (ALK-) 2 and 3 allowed the formation of a stable cartilage from adult human bone marrow MSCs, with the inhibition of early and late hypertrophy markers. This was also confirmed *in vivo*, in a subcutaneous model in immunodeficient mice, where remodelling of cartilage into bone was prevented when both ALK2 and 3 activity were blocked but was only delayed when cells were treated with an ALK2 inhibitor.

Although the many attempts to reproduce the appropriate collagen are all valid, the discrepancy regarding the type of collagen produced during *in vitro* (type X) *versus in vivo* (type I) cartilage formation when using MSCs remains an issue. This suggests that the *in vitro* conditions do not faithfully recapitulate the *in vivo* situation, likely explaining the lack of clinical translation of the many promising *in vitro* results to date [82,83]. The use of monolayer expanded *versus* naïve cells may be one of the biggest discrepancies in MSC-based cartilage regeneration. Indeed, naïve and monolayer expanded cells can express different matrix proteins during differentiation and this must be considered when developing new therapies. While strategies aimed at inhibiting the type X collagen expression and the hypertrophy pathway are the major focus of *in vitro* research, the reduction of type I collagen expression should be also a major focus of cartilage repair strategies as it is a major marker of mechanically unfavourable fibrocartilage *in vivo*.

When considering the discrepancy between the *in vitro* expression of collagen X by MSCs and the clinical challenge of type I fibrocartilage, the question is: does hypertrophy constitute an *in vitro* artefact due to the overly simplistic culture models? The typically used single cell type culture models do not recreate the spatial signalling that would be present in an osteochondral defect and the loss of these crucial signalling pathways may result in an unwanted hypertrophic outcome. Alternatively, the monolayer expansion of cells for use in *in vitro* studies may result in a population of cells destined for endochondral ossification. This still needs to be investigated.

It should not go unnoticed that the matrix properties of hyaline cartilage are not solely due to the presence of a specific collagen but to the combination of collagens and proteoglycans present in a specific composition and organisation [84]. The formation of the collagen fibrils themselves is regulated by the presence of SLRPs such as decorin [85–87]. Of note, fibrocartilage and hypertrophic cartilage have a poor ground substance (Fig. 1) and, therefore, it is possible that the differences in collagen deposition may also be driven by the changes in the quantity and type of GAGs being expressed and retained.

6. Cartilage-targeting therapeutic delivery: what is known and what is new

6.1. MSCs as medicinal signalling cells

The actual function of monolayer-expanded MSCs, when placed within a cartilage defect and their specific contribution to the healing response, is itself a subject of debate. Many potential functions have been elucidated (Fig. 2). Most of the early studies have focussed on directing the differentiation of MSCs into chondrocyte-like cells. Increasingly however, the trophic function of MSCs is leading researchers to consider them as therapeutic delivery agents in their own right, with the suggestion they should be renamed as medicinal signalling cells [88,89], because it is their secretome that drives the endogenous response [9].

The MSC secretome has been successfully exploited in a recent clinical safety trial in thirty-five patients with eighteen months follow-up [90]. Young patients with a well aligned knee and showing symptoms due to a full thickness cartilage defect in the femoral condyle or trochlea were enrolled in the study, while OA diagnosis was one of the criteria for exclusion. Chondrons were isolated from cartilage tissue during the initial debriding process, a standard procedure occurring prior to any cartilage repair surgery. Allogeneic MSCs (passage 3) from very young donors (2 and 5 years of age) were mixed in a ratio of 10:90 or 20:80 with the isolated autologous chondrons within a fibrin glue. An initial period of non-weight bearing for three weeks was followed by a progressive increase to full weight bearing at nine weeks. Histological samples from thirty-one patients revealed type II collagen staining, while only two samples showed evidence of type I instead of type II collagen. Crucially, at twelve months post implantation no DNA from allogenic MSCs could be detected in the repair tissue [91]. This indicates that the implanted MSCs provided an initial stimulus, but subsequently died and were cleared from the tissue. However, what the stimulus was and how it signalled is unknown.

It has been shown that MSCs secrete a number of cytokines and proteins [92,93]. Their secretome profile can be manipulated by short term exposure to cytokines [92,94] and by mechanical regulation. Short term exposure to stromal cell derived factor-1 (SDF-1) has been proposed as a mechanism to increase the expression of factors associated with chondrogenesis [94]. In 2011 it was demonstrated that as human bone marrow derived MSCs progress through the chondrogenic differentiation process, there is a gradual increase in MMP7 [95]. The use of a MMP7 sensitive hydrogel dramatically improved the matrix distribution due to the improved diffusion of newly synthesised matrix products into the interstitial space. Mechanical regulation has also been shown to increase the expression of a number of proteins [96], including TGF-β [97,98]. Of note, the application of joint mimicking multiaxial load has been demonstrated to activate the endogenous latent TGF-β, an observation that could have important implications for the design of rehabilitation protocols aimed at cartilage regeneration [97].

6.2. Cell-free cell therapy?

As the secretome of cells has gained further importance, an increasing body of work is focussing on identifying the molecules produced by these cells and effectively delivering them, in the absence of viable cell transplantation. This has the advantage that an off the shelf solution is more easily achieved while being more cost effective. It is well established that cells, including chondrocytes [99,100], are able to communicate using chemical signals, generally proteins or other molecules, in order to influence the behaviour of target cells. Paracrine signalling, involving the nearby cells, is a key feature of MSCs, as demonstrated by numerous *in vitro* studies of MSCs and chondrocytes co-cultures [101–104]. Basic fibroblast growth factor (bFGF) [105,106] is the main MSC-derived paracrine factor responsible for chondrocyte cell survival and proliferation. The immunomodulatory effects of MSCs are being increasingly reported and have led to their investigation in diseases such as graft *versus* host disease (for reviews see [107,108]. MSCs are able to secrete molecules that modulate the local immune response. Examples of paracrine molecules underlying the immunomodulatory properties of MSCs are hepatocyte growth factor (HGF) [109], TGF-β [109,110] and nitric oxide (NO) with effects on T cell activity [111], and

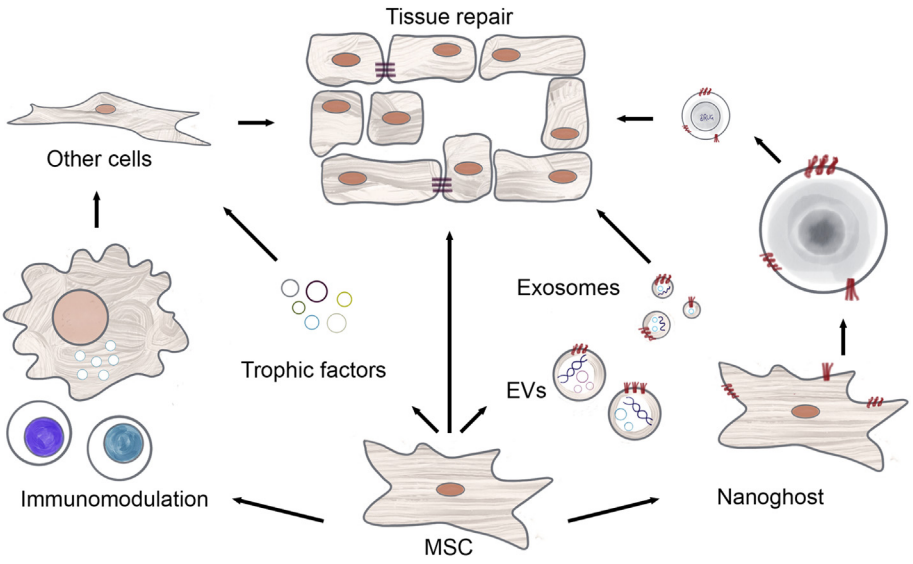

Fig. 2. Multiple applications of MSCs for tissue regeneration. Classically MSCs are studied as a main cell source for tissue regeneration. Alternatively, their trophic and immunomodulatory properties can be used to influence the activity of other cells such as macrophages and fibroblasts during tissue regeneration, their secretome can be exploited in the form of EVs/exosomes, or their whole cell membrane can be used as drug carriers.

prostaglandin E2 (PGE2) for modulation of macrophages [112], natural killer (NK) cells [113], and regulatory T cells [114].

The cell signalling capacity of MSCs is not limited to cells in proximity, since the blood stream and other body fluids, such as the synovial fluid within the joint cavity can act as a reservoir of proteins, which can be used as biomarkers of disease initiation and progression. With this in mind, there has been an increased effort in the identification of serum biomarkers in order to accurately assess an individual patient's healing capacity [for review see [115]]. Correlations of the soluble mediator profiles in patients with differential healing responses have been performed in areas such as polytrauma and delayed fracture healing [116,117]. A particular problem observed with cartilage injuries and degeneration is that they are often asymptomatic, particularly in the early stages, and a diagnosis is generally made once the patient becomes symptomatic. However, since symptoms may appear only when structural changes are already at an advanced stage, the identification of biochemical markers linked to tissue metabolism that are detectable in blood, urine or synovial fluid, represents a useful alternative diagnostic strategy. C-terminal cross-linked telopeptide of type II collagen [118–121], COMP [122–125], GAGs [126–129] and inflammatory cytokines are just some examples of potential markers that would aid early diagnosis. Unfortunately, due to the current incomplete understanding of the biological processes involving these biomarkers in cartilage degeneration and the lack of clinical validation, their clinical usefulness is still questionable [130].

6.2.1. Extracellular vesicles

While protein levels are an attractive marker, their potential to bind ECM molecules, their short half-life and their more general mode of action can make data interpretation complex. Such issues associated with the effective delivery of proteins to tissues has resulted in the search for strategies that protect the protein from degradation/inactivation, thus increasing its half-life and potential efficacy. Nature's way to improve stability has been to encapsulate proteins and RNA in extracellular vesicles (EVs) [131]. EVs are a heterogeneous population of cell-derived lipid vesicles including exosomes and microvesicles (MVs). Currently, the distinction between exosomes and MVs is solely based on the size (30–150 nm for EVs and 100–1000 nm for MVs) and the biogenesis (the endocytic pathway for exosomes and budding from the cell membrane for the MVs). Minimal experimental requirements for the definition of EVs were recommended by The International Society for Extracellular Vesicles and should be adopted and reported by investigators [132] to ensure consistency and improve interpretation and validation between studies. EVs have recently emerged as critical mediators of intercellular information transfer in numerous biological systems [133], including cartilage regeneration [134–137], and they also have been proposed as an off-the-shelf therapy for osteoarthritis [138].

In the *in vivo* work reported by Zhang and colleagues [135], osteochondral defects, created in the trochlear groove of adult rats, were treated with 100 μg of exosomes or with phosphate buffered saline (PBS) (contralateral control). Exosomes, derived from human embryonic-derived MSCs and purified *via* sucrose gradient density equilibrium centrifugation, or PBS were administered weekly for twelve weeks *via* intraarticular injections. Fibrotic tissue, negative for Safranin O and positive for type II collagen staining, was formed in the PBS-treated osteochondral defects. The exosome treatment enabled new tissue formation with typical characteristics of hyaline cartilage: Safranin O staining, lubricin in the superficial layer, the presence of type II collagen and lack of type X collagen. In a further, more recent study, the same group used the same model to confirm the kinetics of the model [137] and demonstrated that exosomes induced the repair of the osteochondral defect with formation of ECM, including deposition of GAGs and type II collagen by two weeks. Synovial fluid was collected at six weeks and demonstrated an increase of M2 macrophage infiltration while a decrease in M1 was detected and associated with decrease in inflammatory cytokines such as IL-1β and TNF-α. However, the IL-6 concentration in the synovial fluid of exosome-treated animals was no different from the PBS-treated animals. *In vitro* data showed that MSC-derived exosomes increased rat chondrocyts proliferation and migration while reducing their *in vitro* de-differentiation, as demonstrated by increased GAG synthesis. The work also revealed the signalling pathways responsible for the observed effects, with both AKT and ERK becoming phosphorylated in chondrocytes with a peak at 6 h after exosome treatment. Inhibition of AKT phosphorylation abrogated the exosome effect on proliferation and migration, whilst the effect on GAG deposition was only partially reduced. Finally, exosome-induced phosphorylation of AKT and ERK was shown to be only partially mediated *via* CD73 and, therefore, other exosome mediators of AKT and ERK phosphorylation must exist.

In their various forms, EVs contain packets of information acting *via* multiple signalling mechanisms, which provide a very stable method of cell-cell communication. Therefore, EVs are an attractive therapeutic

option but further work is necessary to identify the specific growth factors, cytokines and/or proteins involved in their beneficial effect on cartilage repair.

While showing promise, a general lack of standardised protocols for the isolation, purification, characterisation and quantification of EVs is hampering their potential clinical translation [139]. The concentration and characterisation of the harvested EVs is still a major challenge, with many publications using μl/μg of concentrated preparation applied. This makes comparison between groups and reproducibility between studies impossible. As an alternative to the currently used differential centrifugation and commercially available kit-based approaches, newer methods such as asymmetric flow field-flow fractionation [140] or size exclusion chromatography [141] could be used to greatly increase our understanding of the complexity of EVs.

6.2.1.1. miRNA. A major breakthrough was the demonstration that the cargo of EVs included both mRNA and microRNA (miRNA), and that EV-associated mRNAs could be translated into proteins by target cells [142], although it has been proposed that the main mechanism of action is protein-related [143]. One component of EVs that is of particular interest is non-coding miRNA. miRNAs are not converted into protein; they are short nucleotide sequences (20–25 nucleotides long) that bind specifically to mRNA sequences, thereby controlling the level of protein produced. miRNA has been implicated in numerous stages of chondrogenesis and in the development of osteoarthritis [144]. A number of gene silencing approaches are being investigated to promote chondrogenic differentiation during cell-based repair [145]. miRNA-based gene silencing has the advantage that nucleic acid delivery needs to be only into the cytoplasm, removing the need for the more difficult nuclear delivery. Silencing of the anti-chondrogenic factor miR-221 has been shown to promote chondrogenesis both *in vitro* and *in vivo* [146]. Proteins classically associated with musculoskeletal regulation have also been shown to exert effects by way of miRNA regulation. BMP2 regulates miR199a*, which then inhibits early chondrogenesis by inhibiting Smad1 signalling [147]. In a recent work, the role of miR-140-5p has instead been associated with delay of OA in a rat experimental model. Intraarticular injections of exosomes derived from synovial MSC overexpressing miR-140-5p prevented cartilage damage caused by the OA model (Fig. 3), providing great hope for future clinical applications of MSC derived exosomes [136]. Both miR-140 and miR-221 have also been implicated in chondrocyte proliferation, and this feature may play a role in the effects highlighted above [148,149]. miR-221 and miR-222 have been identified using microarray analysis in the anterior weight-bearing superficial zone and the authors proposed this as a mechanotransduction response in areas with higher stress [150]. Whether this is due to its effect on proliferation is yet to be elucidated. miRNA has also been utilised to protect cells from the negative effects of inflammation. Silencing miR-101 had a protective effect on chondrocytes exposed to IL-1 [151]. miR92a-3p has been shown to protect cartilage from OA-related damage by enhancing the expression of Wnt5a both *in vitro* and in a mouse OA model when the miRNA is applied contained in exosomes [152].

A number of targets have been identified to enhance chondrogenic differentiation of MSCs. While some, such as miR-140, have been associated with directly driving chondrogenesis [153] and protection from OA [154], some of the more promising targets are miRNA that have been shown to inhibit chondrogenesis. Silencing of miR-221 [146], miR-222 [155] and miR-1305 [156] have been shown to be particularly powerful inducers of chondrogenesis. This would suggest that the delivery of antagomirs is a promising approach to induce chondrogenesis during marrow stimulation techniques.

6.3. Modifying inflammation

Cartilage catabolism is stimulated by a series of inflammatory mediators among which IL-β and TNFα are the main drivers. IL-1β has been shown to negatively affect aggrecan synthesis at mRNA level [157], to suppress collagen II synthesis [158] and instead increase MMPs expression [157,159] and ADAMTS activation [160]. It is clear that NF-κB activation decreases chondrogenic differentiation and this can occur with low dose exposure to IL-1β and TN-α [161]. Therefore, any environment which has an inflammatory component would reduce chondrogenesis, thereby reducing collagen II synthesis. Due to the NF-κB driven mechanism, direct inhibition of NF-κB allowed for a recovery of chondrogenesis [161]. With this in mind, a number of strategies are being developed to control inflammation during cell therapy strategies. Hydrogels that are specifically activated by a localised inflammatory response have been used to deliver drugs on demand during an inflammatory flare [162] (Fig. 4).

The potent inhibitory effect of IL-1β has made it a significant therapeutic target. The naturally occurring IL-1 receptor antagonist (IL-1Ra) has been extensively investigated as a mechanism to decrease IL-1 signalling. Typically, it is cloned into a viral delivery system and applied locally to the joint. This has been performed clinically in patients using a retrovirus as a proof of concept [163] and more recently has been showing great promise in a horse OA model using a safer adeno-associated virus (AAV) [164]. Sustained intra-articular delivery of IL-1Ra from a cross-linked elastin-like polypeptide (xELP) drug depot demonstrated positive effects in a post-traumatic murine OA injury model [165]. Notably, the same study demonstrated that inhibition of TNF-α alone, or in combination with IL-1, led to deleterious effects in bone morphology, articular cartilage degeneration, and synovitis in the same model. Alternatively, sulphation of hydrogels such as alginate has been proposed as a mechanism to sequester IL-1 ligand, thus reducing cytokine triggered gene expression [166].

Increasingly, the role of the immune system on the direct control of the regeneration process is becoming apparent. Macrophage infiltration has classically been associated with a proinflammatory response. However, the transition from a proinflammatory M1 macrophage to a pro regenerative healing M2 macrophage is a mechanism required for healing [167].

Dexamethasone is a glucocorticosteroid that is commonly used clinically to control inflammation, which it does by using a combination of transactivation (increased gene transcription) and transrepression, which is a result of direct binding to NF-κB, leading to its inactivation [168]. The application of dexamethasone is also required for *in vitro* induction of bone marrow derived MSC chondrogenesis [68], yet its exact role in the differentiation process is unclear. Thus, its application in the joint would potentially have dual benefits, of increasing chondrogenic differentiation, while decreasing inflammation and its associated negative effects on chondrogenic differentiation. *In vitro,* it has been demonstrated that longer term exposure to dexamethasone leads to an improved matrix deposition, potentially due to its repression of proinflammatory signals [169]. However, long term exposure of the joint *in vivo* to corticosteroids is associated with decreased collagen synthesis and increased structural damage [170]. This has ramifications for *in vivo* cartilage repair, where the injury and the surgical intervention would lead to an inflammatory response. MSCs have been shown to modify immune responses by way of exosome signalling [171], further emphasizing their critical role in homeostasis and repair.

6.4. Physical modulation of cell phenotype

Cartilage has the unique advantage that it is heavily mechanically regulated by forces including hydrostatic pressure [172], shear, compression, and a combination of the above [173]. This can provide localised activity dependent activation triggers for drug depot activation. It can also provide signals for drug activation. Similar load can be applied during patient rehabilitation protocols after surgery and the significance of the modulating effect of mechanical stimulus should not be overlooked when assessing new biomaterials and growth factor release systems. This is leading to a new area of research, regenerative

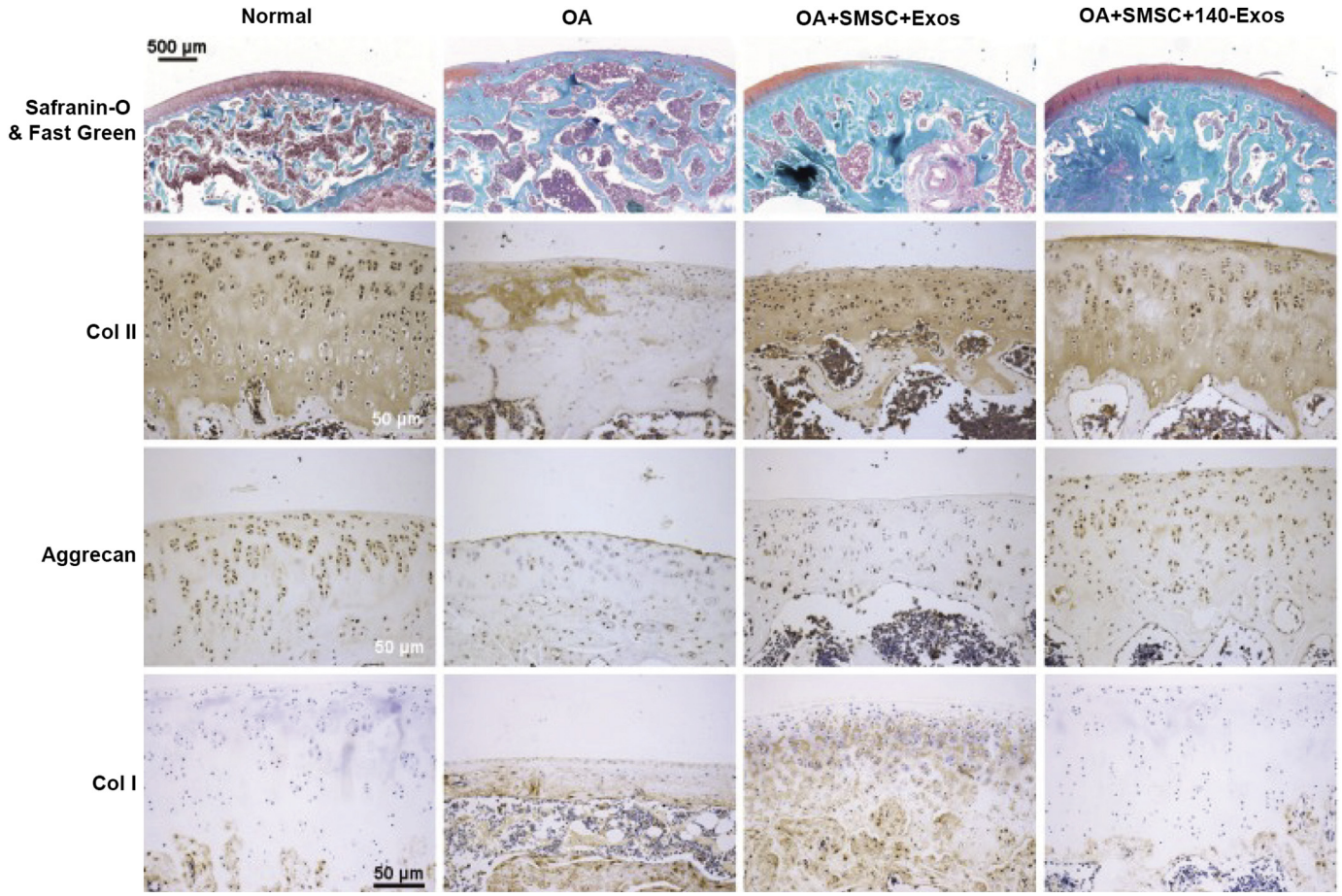

Fig. 3. Cartilage damage is prevented by synovial derived MSCs overexpressing miR-140-5p. Femoral condyle section of a rat model of OA including a control group without injection and receiving saline injections (normal), OA experimental model receiving saline injection (OA), OA experimental model receiving articular cavity injections of synovial MSCs-derived exosomes (OA + SMSC-Exos) and OA experimental model receiving articular cavity injections of exosomes derived from synovial MSCs overexpressing miR-140-5p (OA + SMSC-140-Exos). Figure reproduced with limitation to part A from the article of Tao and colleague [136] distributed under the terms of the Creative Commons Attribution (CC BY-NC 4.0) licence (https://creativecommons.org/licenses/by-nc/4.0/).

rehabilitation, that specifically investigates the synergistic effect of the rehabilitation protocol [174,175]. Dynamic tissue shear has been shown to increase cartilage matrix production [176], yet when combined with exogenous IGF-1, a synergistic effect is observed [177]. Similar results have been observed with hydrostatic pressure [178].

Endogenous cells also have the ability to produce secretory molecules that can enhance the repair process and as such should be considered advanced therapeutic delivery agents in their own right [9]. Thus, direct mechanical stimulation of the regenerative cells should be considered as an approach for growth factor delivery. The commonly performed clinical treatment of microfracture allows stromal cells from the marrow to penetrate into a chondral defect leading to a fibrocartilage repair tissue [41]. Once the cells are in the defect, the mechanical load experienced by the cells has been shown to modify the final phenotype obtained. Cyclical uniaxial load has demonstrated benefits in the enhancement of chondrogenic matrix [179]. The response observed is dependent on the actual load applied and in the case of chondrocytes, may be dependant of the presence of a pericellular matrix. Uniaxial load is beneficial for chondrocytes and this is likely to be more effective once a pericellular matrix is present [180]. This is also true for MSCs in the presence of exogenous TGF-β. TGF-β can be released both *in vitro* and *in vivo* using biomaterial-based delivery systems. Controlled release of TGF-β over the first five days has been shown to be sufficient to enhance chondrogenesis [181].

The requirement for exogenous TGF-β can be partially alleviated by the application of multiaxial load, where shear is superimposed over compression [98]. Additionally, hydrostatic pressure has been shown to increase TGF-β expression in rabbit MSCs [182]. Multiaxial load has been shown to induce TGF-β production and physically activate latent TGF-β into its active form, a process that involves shear [97,183]. The resultant chondrogenic induction has been shown to be dependent on the shear component [184], which is able to activate the latent endogenous TGF-β that is produced by the cells [97]. As the production and activation of the TGF-β is related to the stiffness of the material, it provides valuable insights into design criteria for novel materials for cartilage regeneration. Utilising mechanics to enhance endogenous repair can further be improved by redistributing cells in a way that increases the fraction of the cells at the superficial zone [185]. The use of relevant kinematic load during *in vitro* testing of novel materials for cartilage repair greatly improves the accuracy of the data obtained and may offer a more representative system to predict the *in vivo* outcome.

Attempts have also been made to activate local delivery using external non-invasive devices. Pulsed electromagnetic fields (PEMF) lead to an enhanced *in vitro* chondrogenic response of human MSCs [186]. Magnetic particles coupled to receptor binding ligands have been shown to be able to active pathways *in vitro*, such as the Wnt/β-catenin pathway, remotely [187]. Shockwave devices have been employed, resulting in localised mechanical activation both *in vitro* and in rat and rabbits where it enhanced cartilage healing [188,189]. Such technology allows for extracorporeal activation of implanted materials on demand. Materials are being developed to utilise these remote activation processes to fine tune the temporal regulation of the healing response. Near infrared light has been used to trigger activation of nanoparticles, releasing

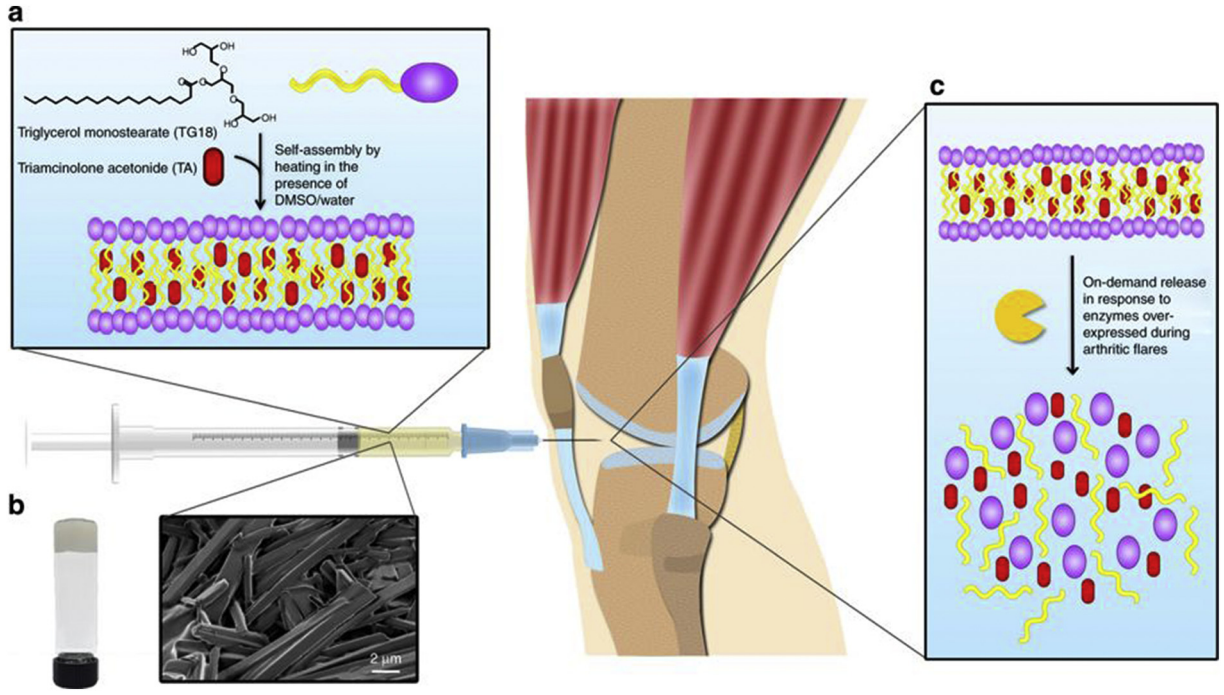

Fig. 4. Flare-responsive intra-articular drug release. TA is encapsulated within the lamellar structure created upon heating and following cooling of TG-18 in DMSO/water (a); high resolution scanning electron microscopy of fibrous network created by the lamellar structures within TG-18 hydrogel (b); the lamellar structure of the TG-18 hydrogel is disassembled by enzymes overexpressed within the joint during arthritic flares and the TA is released within the joint (c). Figure reproduced from the work of Joshi and colleagues [162] distributed under the terms of the Creative Commons Attribution (CC BY 4.0) licence (https://creativecommons.org/licenses/by/4.0/).

kartogenin leading to chondrogenic differentiation of human MSCs [190] (Fig. 5).

6.5. Advanced materials

The joint offers the advantage of localised delivery by injection. However, the charged and dense nature of cartilage makes it less permeable to injected molecules, which in the synovial fluid can be rapidly cleared by the lymphatic system [191,192]. In this sense, materials could help in retaining the drug and, therefore, increasing the time available to penetrate the cartilage ECM. Both natural and synthetic materials have been employed in cartilage tissue engineering in the form of porous scaffold or water-based hydrogels. How the design of new materials takes or should take more inspiration from biological processes is thoroughly discussed in a previous review from this group [4].

Drug releasing materials are being designed with increasing level of complexity. Initial materials relied on a burst release, whereby the factor of interest is simply mixed with the material carrier. The size and charge of the molecule then plays a significant role in the release kinetics. Small molecules are rapidly released, potentially resulting in unwanted side effects in the local area. A clinical example is type I collagen scaffolds releasing BMP-2 that led to numerous side effects such as ectopic bone formation [193]. This is believed to be more a result of the delivery system, rather than the BMP-2 itself, with the 12.8 kDa BMP-2 protein rapidly leaching out of the collagen scaffold. As molecules increase in size, their delivery slows and this has been utilised to increase retention of the encapsulated therapeutic. For instance, it has been shown that adenoviral vectors (90–100 nm) are more effective, leading to a more persistent expression while using a lower viral dose, when encapsulated in hydrogels and can be well retained within the gel [194].

In order to increase localised concentrations of growth factors, the scaffold can be modified with a growth factor retaining peptide sequence [195,196]. A composite of the self-assembling peptide RAD16-I and heparin sodium salt has been shown to enhance the specific binding and release of growth factors with heparin binding affinity such as vascular endothelial growth factor (VEGF)165 [195]. Additionally, a hyaluronic acid-based gel has been functionalised with branched macromolecules containing BMP-2 or TGF-β1 affinity binding peptides [197]. These materials have been combined with dendrimer structures that allow for a fine tuning of growth factor combinations. Proof of concept has been demonstrated for both BMP-2 and TGF-β1 [197].

Another approach is to tether growth factors to hydrogels, which are then released upon cleavage by the required endogenously produced enzyme [162,198]. MMP-sensitive poly-ethylene glycol (PEG)-based hydrogels were used to encapsulate chondrocytes, leading to a more diffuse, less cell surface-constrained cell-derived matrix in the chondron, and increased type II collagen and GAG synthesis [198]. In a proof of concept study using a mouse inflammatory model, the group led by Jeffrey Karp demonstrated flare-responsive intraarticular drug release [162]. The corticosteroid triamcinolone acetonide (TA) was encapsulated within the lamellar structure created upon heating and following cooling of triglycerol monostearate (TG)-18 in DMSO/water. The lamellar structure of the TG-18 hydrogel was disassembled by enzymes overexpressed within the joint during arthritic flares (Fig. 4). Using near infrared dye (DiR), the TA release could be monitored by *in vivo* imaging and the intensity of the inflammation was demonstrated to control the TA release.

An alternative approach to improve release characteristics has been to modify the molecule of interest, rather than the material delivery system. A number of growth factors, such as VEGF, platelet derived growth factor (PDGF), FGF, BMP and TGF are known to possess matrix binding peptide sequences that lead to entrapment and decreased diffusion. Their binding affinity varies for different matrix proteins, allowing for a degree of specificity [199]. A screen of twenty-five growth factors and their binding characteristics to various natural materials demonstrated that placenta growth factor-2 (PLGF-2) possessed strong matrix binding properties, while PLGF-1 did not. This was attributed to a matrix binding domain within PLGF-2 ($PLGF2_{123–144}$) that strongly and promiscuously binds ECM protein sequences [199]. IGF-1 has been modified to contain a heparin binding domain, a feature that enhanced its localization to cartilage after intraarticular injection in rats [200].

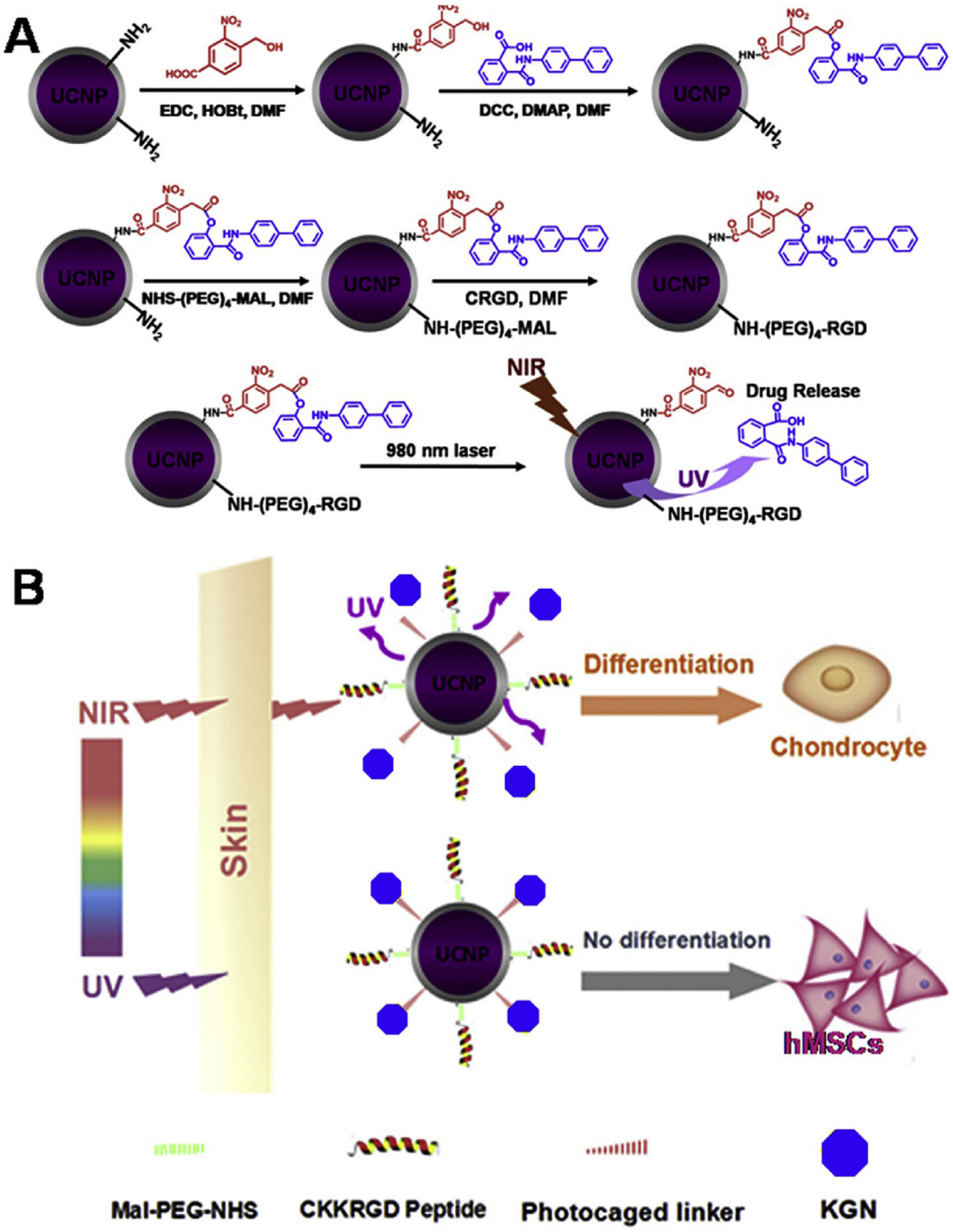

Fig. 5. Near infra-red light-controlled release of small molecule inducing chondrogenesis. The scheme depicts (A) the synthesis of upconversion nanoparticles (UCNPs) and the release of kartogenin from the nanoparticles following near-infrared light triggered cleavage of photolabile linker and (B) the principle behind the *in vivo* application of a controlled release of kartogenin to stimulate the differentiation of MSCs. Reprinted from Near-infrared light-triggered release of small molecules for controlled differentiation and long-term tracking of stem cells *in vivo* using upconversion nanoparticles, Biomaterials 110 1–10, Jinming Li, Wayne Yuk-Wai Lee, Tianyi Wu, Jianbin Xu, Kunyu Zhang, Dexter Siu Hong Wong, Rui Li, Gang Li, Liming Bian, © Elsevier Ltd. (2016), with permission from Elsevier [190].

Ideally, a therapy would be off-the-shelf, with a long shelf life. This has led to the interest in gene activated matrices, where nucleic acid is encapsulated within a scaffold material and the cells are exposed to the vector as they infiltrate the scaffold [145,201,202]. There are a number of complex materials that are being functionalised in order to enhance the biological outcome. The Guilak group have developed a woven scaffold that enables tailoring the mechanical properties to those favourable for cartilage [203]. The structure can be produced to have an anisotropic weave and the resurfacing of whole joints is feasible. This has been further functionalised using a lentivirus that overexpresses TGF-β3 [204]. Delivering the gene in this way leads to chondrogenesis comparable to that obtained using consistent application of exogenous TGF-β3, yet the scaffold could in principle be delivered as an off the shelf approach. Alternatively, the scaffold was tailored to express IL-1Ra for additional anti-inflammatory benefits [205]. Combining layers where various genes, *e.g.* TGF-β and BMP2 are included allows for structured osteochondral implants with localised gene expression control [206].

Mechanically responsive particles offer a particular advantage within the joint, as it allows release of molecules based on localised

strain patterns. A mechanically responsive liposome-in-hydrogel system was demonstrated to lead to load dependent release of an encapsulated dye [207]. A similar approach has been applied in the cardiovascular field using shear-stress activated vesicles [208].

The use of decellularised tissue, both as a scaffold and as a powder has been shown to provide an enhanced endogenous repair response. Matrix has been isolated from porcine urinary bladder matrix [209] and small intestinal submucosa [210] and has been tested *in vitro*. The tissue has been shown to modulate the macrophage response, reducing the damaging M1 proinflammatory macrophage response while enhancing the pro-reparative M2 macrophage response. The preparation of the tissue influences the efficacy, and this is believed to be in part due to the retention of exosomes/MVs in the matrix. These exosomes have been shown to contain miRNA species that regulate macrophage polarity, leading to the M1/M2 transition observed [209–211]. The role of matrix derived nanovesicles for cartilage regeneration still has to be elucidated *in vivo*.

7. Conclusions and future perspectives

Articular cartilage is a stiff, elastic and low friction tissue. To date, there is no industrial material able to reproduce these mechanical properties. Considering that the mechanical properties derive from its composition and organisation, revealing why *in vivo* chondrocytes or MSCs would produce type I rather than type II collagen is of paramount importance. The basic biochemistry of cartilage has a lot of missing information and likely this is one of the reasons we are still writing and discussing about cartilage repair. The quest for the reproduction of the right collagen should therefore look into the signalling pathways controlling the synthesis of different types of collagen, into SLRPs as regulators of this process [212] and into the molecular organisation of collagen [213]. Protocols should be strengthened based on the results of developmental biology studies. In light of the fact that cells have memory of their tissue of origin and carry with them characteristics of that tissue, it should not be surprising that bone marrow-derived MSCs do not possess the temporal and spatial clues necessary to recreate articular cartilage but rather fibrocartilage and hypertrophic cartilage as the basis for bone formation. The reproduction of the right type of collagen should also be searched in different type of cells such as synovium-derived MSCs. Synovium-derived MSCs, with their common origin from growth differentiation factor 5 (GDF5) expressing cells shared with the articular cartilage [214] are already attracting the attention of different research groups. These cells have been proven with a higher chondrogenic potential when compared to bone marrow [215], although digestion using enzymes of animal origin is still required for isolation of these cells [216], hindering their clinical translation.

Using cells as delivery agents has progressed into the clinic. In 2017, INVOSSA™ (Kolon TissueGene) was approved for clinical use in South Korea [217]. The allogeneic cell therapy has a fraction of cells that are first transduced with a retrovirus overexpressing TGF-β and then irradiated to alleviate any risks of insertional mutagenesis. A portion of the genetically modified cells are then mixed with the original unmodified population of chondrocytes, therefore providing a combination of cells expression of TGF at therapeutic levels and cells to effect a repair.

Due to the financial restraints associated with translating a therapy into the clinic, an off-the-shelf solution will have advantages [224]. This allows for easier safety testing and reduces costs associated with complex cell culture.

The idea of a possible cell-free therapeutic application is already fuelling the current research on EVs as delivery systems, both naturally produced and synthetic. In this sense, artificial EVs offer the potential to produce clinical grade therapeutics with defined, reproducible characteristics, without the risk of disease transmission [218,219]. Being able to combine the various components and artificially produce EVs complete with the required cargo would lead to a truly cell-free delivery system. Vesicle stability within the synovial joint and the interaction with different cell types of the joint environment will be subject to future investigations. New techniques to monitor the biodistribution will be constantly developed to increase accuracy [220]. This would have repercussions on the analysis of *in vivo* biodistribution of the EVs. The labelling of the EVs, necessary for tracking the EVs upon administration, can itself interfere with the biodistribution. Intravenous injection of EVs derived from murine melanoma cells and collected by ultracentrifugation were cleared from the blood stream with a half-life of 2 min [221]. Spleen and liver are the first organs of distribution when using intravenous injection [220].

In recent years, researchers also looked into cell membrane properties and a way to concurrently use it as a shell and a targeting system and for the first time the creation of ghost cells, the so-called nanoghosts, was described as drug carriers using human and rat MSCs and human smooth muscle cells [222]. A short burst release was reported to be followed by a linear sustained release very similar to synthetic liposomes. Also, the cell origin and the species showed no effect on the nano-ghost size, charge and morphology. More recently, cell uptake in a tumor cell line was shown to increase when monocyte membrane was used to coat Doxorubicin-PLGA nanoparticles [223]. Although a very limited number of work targeted to cancer therapy has been published, a question on the possible future application of such technology to target cartilage injuries arises and waits for an answer. Overcoming the negatively charged cartilage matrix by utilizing a positively charged nanocarrier of the correct size has been proposed as a mechanism by which cartilage can act as a drug reservoir [227]. Avidin has been proposed as a molecule that dramatically improves drug penetration during OA treatment [228,229].

At the end, it should not be overlooked that an untreated cartilage defect leads to OA. The heterogeneous nature of OA aetiology is complicated by the well-established role of cartilage, synovial membrane and subchondral bone in the disease pathogenesis, making OA a disease of the whole joint [225,226]. Despite all the research that has been performed, there are no drugs or treatments that effectively prevent or treat OA and current therapies focus mainly on pain treatment.

The rising prevalence of cartilage injury and disease in particular with knee OA prevalence doubled since the mid 20th century [230] is leading to a dramatic, life changing, societal burden that will only amplify with the increasing elderly population. New treatments are desperately required to alleviate these conditions and they should aim to prevent cartilage degeneration in the long term to justify their potentially higher initial costs. An improved understanding of the underlying biological mechanisms can only derive from an interdisciplinary approach that does not focus on one aspect at the time but rather comprise multiple ones by sharing knowledge and expertise. This research strategy combined with more clinically relevant preclinical models, will be a major step towards the goal of articular cartilage regeneration.

Acknowledgements

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