Review

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Advanced cell therapies for articular cartilage regeneration

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Advanced cell-based therapies are promising approaches for stimulating full regeneration of cartilage lesions. In addition to a few commercially available medicinal products, several clinical and preclinical studies are ongoing worldwide. In preclinical settings, high-quality cartilage tissue has been produced using combination strategies involving stem or progenitor cells, biomaterials, and biomolecules to generate a construct for implantation at the lesion site. Cell numbers and mechanical stimulation of the constructs are not commonly considered, but are important parameters to be evaluated in forthcoming clinical studies. We review current clinical and preclinical studies for advanced therapy cartilage regeneration and evaluate the progress of the field.

Current advanced therapies for cartilage regeneration

Articular cartilage is an avascular tissue with a highly complex structure that has only limited capacity for self-repair because it mainly consists of chondrocytes encapsulated in a dense matrix of proteoglycans and collagens. Focal chondral or osteochondral lesions in the knee caused by traumatic injuries often result in pain and swelling, frequently developing into larger, degenerative lesions and osteoarthritis (OA). Severe focal injuries in cartilage are currently treated by one of the three main types of surgery: bone marrow stimulating techniques, mosaicplasty, and cell based therapies.

The first generation of cell based therapy, autologous chondrocyte implantation (ACI), was introduced in 1987 by Brittberg and published in 1994 following FDA consent for clinical studies [1]. In October 2009, the first cell based product to obtain market authorization from the European Medicines Agency (EMA) as an Advanced Therapeutic Medicinal Product (ATMP) was ChondroCelect from Tigenix (Belgium). In the USA, Carticel from Genzyme is the only FDA-approved cell therapy product for regenerating articular cartilage. ChondroCelect and Carticel therapies may eventually involve the use of a type I/II collagen hydrogel patch, such as ChondroGide (Geistlisch) or CaReS (Arthro kinetics), instead of the periosteal flap to confine the *ex vivo* expanded chondrocytes to the lesion site. Histological comparison of tissue formed after using periosteal or Chondrogide® during ACI pointed towards the superiority of the latter [2].

In addition, clinical trials are underway to evaluate hydrogels as matrices for supporting autologous chondrocyte implantation. This strategy involves culture of chondrocytes on a matrix, followed by implantation at the lesion site, a procedure known as matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI). MACI™, a proprietary version of the technology from Genzyme, has been approved for clinical trials by the FDA. Several proprietary biomaterials, such as Novocart 3D [3], Neocart [4], and Chondron [5], which are approved in clinical trials for use without cells, are being considered by their respective companies for implantation with autologous chondrocytes according to the MACT strategy (Table S1 in the supplementary material online). In a different approach, Chondrofix [6] and DeNovo NT [7], hyaline cartilage allografts for treatment of severe and initial articular cartilage lesions, respectively, are being considered as tissue engineering (TE) products. As recently reviewed [8], these products have great potential because they can enable multifactorial mimicry, which has not yet been achieved using man-made biomaterials.

In January 2012 the Korean FDA approved the manufacture and sale of Cartistem as an allogeneic stem cell drug for the treatment of OA. This product uses mesenchymal stem or progenitor cells (MSC) isolated from umbilical cord blood (UCB). Despite great interest from the worldwide advanced therapy community, no results have yet been reported in peer-reviewed journals.

Clinical trials using advanced therapies

Clinical translation of cell based therapies is challenging not only because it requires costly and reliable cell manufacturing but also because delivery to the patient is complex. Strategies consisting of a single ATMP are essentially those in which cells, a matrix, or vectors carrying genes are injected intra-articularly or are loaded into the lesion site. Combination strategies consider the simultaneous use of these elements before implantation: cells embedded in a hydrogel or previously cultured on a scaffold (Figure 1A), genetically modified cells (Figure 1B),

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a matrix embedded with gene vectors (gene activated matrix) (Figure 1C), or a combination of all three elements (Figure 1D). Most of these strategies are now in clinical trials for cartilage regeneration (Table S1), with the exception of the implantation of a gene-activated matrix on the lesion site, which has been evaluated only in preclinical settings [9]. Over the past decade, several authors have systematically reviewed the results of randomized controlled trials (RCT) or Level IV studies using ACI [10–12], both ACI and MACT [13], and stem cell therapies [14–16].

Ideally, any therapy should be as simple as possible. However, in the case of articular cartilage lesions, especially

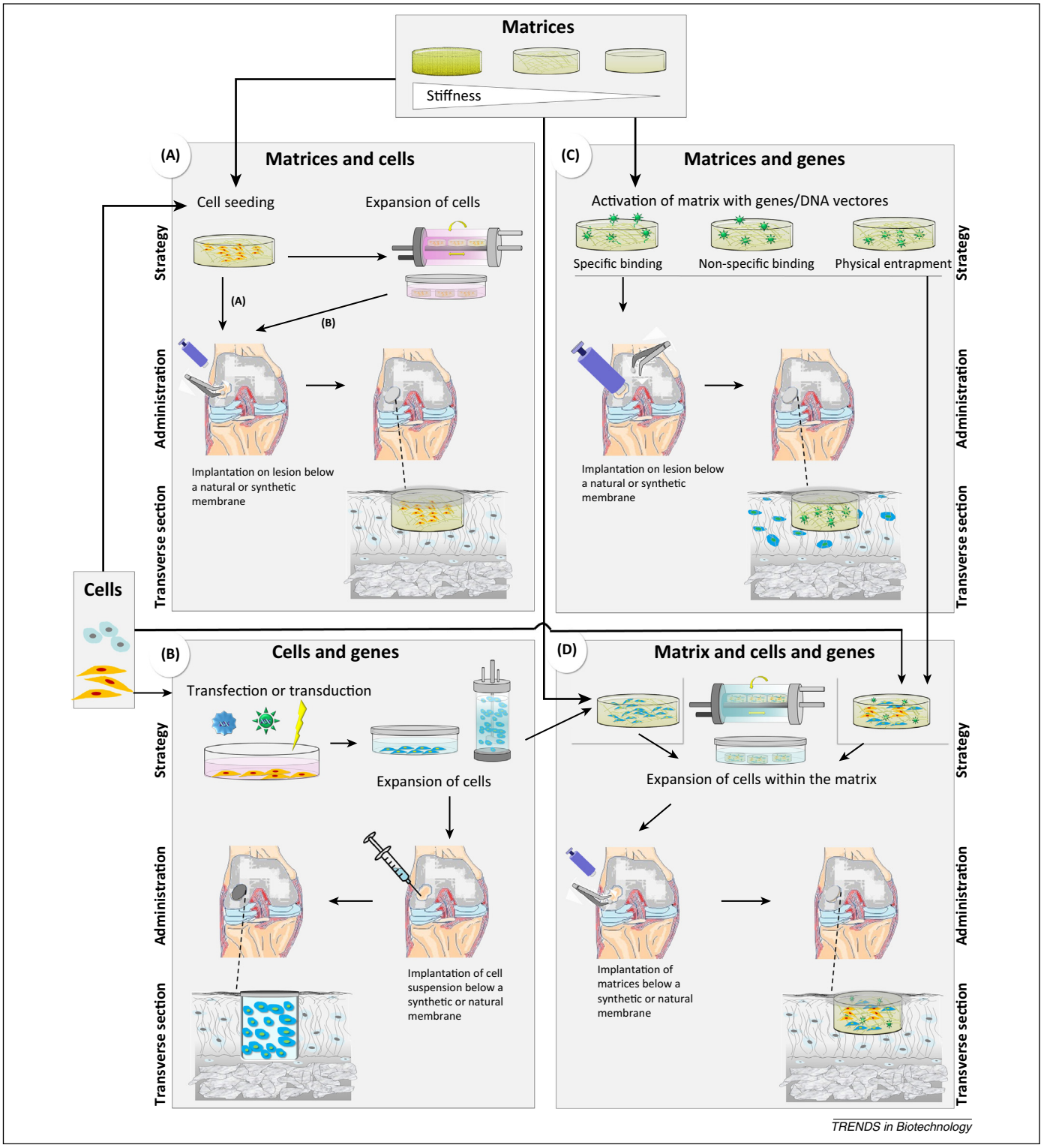

Figure 1. Combination strategies under consideration for cartilage regeneration in clinical and preclinical studies. **(A)** Matrices and cells. (a) Freshly isolated cells are mixed with a hydrogel and implanted at the lesion site. (b) Cells are seeded on a matrix and expanded under static or dynamic culture before implantation. **(B)** Cells and genes that are implanted at the lesion site below a natural or synthetic membrane. **(C)** Matrices and genes implanted after specific or non-specific binding. **(D)** Matrices and cells and genes. *In vitro* physical stimuli during cell expansion can be considered in strategies (A), (B), and (D).

severe ones, a single strategy might not be enough. Compared to bone, cartilage regeneration demands additional approaches in view of its complex structure. Recent findings have pointed out towards the successful use of combination strategies [17–19]. Combination products may be evaluated by the FDA either as a device or as a biological agent, respectively by the Center for Devices and Radiological Health (CDRH) or by the Center for Biologics Evaluation and Research (CBER) [20], whereas the EMA considers these products to be ATMPs, to be regulated by the Committee for Advanced Therapies (CAT).

Cells

Chondrocytes

ACI was first introduced as a two-step procedure [1]: chondrocytes are isolated from small biopsies, expanded *in vitro*, and implanted at the lesion site. A periosteal flap is then surgically affixed to cover the defect (first-generation transplantation). Complications such as periosteal hypertrophy and difficult surgical handling prompted the use of a collagen membrane instead of periosteum (second-generation transplantation). A 14 year follow-up evaluation of ACI in 23 patients (Level IV) provided evidence that first-generation ACI resulted in a substantial improvement in all clinical outcome parameters, although a small deterioration in some parameters was noticed between intermediate and final evaluations [21]. Concerns about the losing the chondrocyte phenotype during 2D expansion led to the cultivation of isolated cells on 3D matrices, thus establishing a third generation of MACT chondrocyte transplantation. This strategy is being considered in 10 of the 17 current clinical trials (Table S1) and has shown promising results [22,23], but these are still surrounded by controversy [24]. Several alternative scaffolds have already been commercialized or are in clinical studies, but further well-designed RCT and long-term evaluation will be necessary to confirm the superiority of these treatments over conventional or ACI-based strategies [25,26] and conventional treatments [12].

Through the past decade, several synthetic and natural biomaterials, and combinations of both, have been evaluated as scaffolds for culturing chondrocytes and implantation at lesion sites according to the MACT strategy [27]. Importantly, recent preclinical studies have provided evidence that properly designed biomaterials can trigger immunomodulatory effects on seeded chondrocytes [28]. In addition to the specific type of biomaterial employed for the scaffold, key parameters for achieving a native-like tissue in the preclinical setting include the number of seeded cells, whether there was sustained release of growth factors during chondrocyte culture, and the presence of biomechanical stimuli. Implantation of high cell densities ($2–50 \times 10^6$ cell/cm^2 [1,29]) is crucial for achieving high-quality cartilaginous tissue, but it is difficult to maintain the chondrocyte differentiation state upon 2D expansion or circumvent their OA phenotype.

The administration of expanded chondrocytes encapsulated in microspheres has recently been evaluated [29–32]. One major advantage of this strategy is microspheres could potentially be exploited to achieve sustained release of growth factors [33]. Because the chondrocyte niche is hypoxic, *ex vivo* chondrocyte cultivation under low oxygen tension should also be considered. Although further studies are required, a combination strategy involving hypoxic conditions, growth factor delivery, and COL1A1 (collagen type 1, α1) silencing of chondrocytes was found to increase their differentiation on collagen scaffolds [34].

Mesenchymal stem cells

Strategies based on the isolation of autologous chondrocytes have been hampered by difficulties in achieving a high cell density and in ensuring that the cells maintain their differentiation state, prompting the quest for other cell sources. Stem cells have been considered as a potential alternative in clinical and preclinical studies, particularly mesenchymal or progenitor stem cells (MSC). These cells can be isolated from bone marrow (BM), adipose tissue (AT), synovium, periosteum, skeletal muscle, skin, amniotic fluid, or cord blood matrix. They have common immunophenotypic characteristics, high proliferative capacity, and immunomodulatory characteristics [35].Currently, 20 interventional studies worldwide are open and recruiting patients suffering from articular cartilage lesions to test the efficacy of MSC in cartilage regeneration. MSC isolated from umbilical cord matrix (UCM), AT, or BM have been used, either injecting or implanting autologous or allogeneic cells to take advantage of the immunomodulatory properties of this stem cell type [35]. Importantly, no evidence of malignant transformation was observed in cells from hundreds of patients worldwide who were intra-articularly injected with expanded autologous BM-MSC [36]. Stem cell based therapies for human cartilage defects have recently been reviewed and, in the absence of RCT, it was suggested that there is an urgent need for control-based studies, as well as for the harmonization of study designs, follow-up methods, criteria reporting, and evaluation [14–16]. When using allogeneic adult stem cells or BM cells directly implanted or injected in the lesion, the patient only undergoes a single round of surgery to the knee (one-step), whereas the use of expanded autologous stem cells requires a two-step procedure. Veronesi *et al.* analyzed 11 case series and reports that used autologous BM-MSC on 1–48 patients over the past 10 years; and they concluded that larger cartilage defects can be repaired by the two-step technique than by the one-step strategy [14], most probably because of the higher cell numbers achieved by the expansion procedure.

MSC chondrogenesis demands the use of high cell density and, despite a few reports that state the total number of cells employed, to the best of our knowledge few if any studies specify the number of cells as a function of lesion area or volume (cells per cm^2 lesion area or per cm^3 volume). For chondrocytes, Brittberg suggested the implantation of 2×10^6 cells/cm^2 below a periosteal or collagen membrane sealed with fibrin glue [1], whereas for MSC a systematic review recorded a range of $4–11 \times 10^6$ cell/ml for *ex vivo* expanded BM-MSC [14]. In some clinical studies BM-MSC are not expanded; instead, after isolating MSC from BM, the cells are mixed with collagen or hyaluronic acid and are implanted into 2 cm^2 lesions at a depth of 0.4 cm [37,38]. However, only seeding high densities of MSC in hyaluronic acid hydrogels produced engineered

cartilage with native tissue properties [39]. Although few clinical studies disclose the number of cells or the area of lesion, an inconsistency remains between the cell density values used in clinical and preclinical studies. Regarding the implantation of expanded MSC within a matrix, a dose of 1–2.5 $\times$ 10^6 MSC/cm^2 has been used in clinical studies [40–42], whereas in preclinical studies a dose of 8.0 $\times$ 10^5–8.0 $\times$ 10^7 cell/cm^2 has been used [43–45], without any evident rationale according to the type of animal model used.

The most common *in vivo* model for testing ATMP and observing cartilage regeneration is the rabbit; porcine, equine, caprine, and ovine models have also been used, but more rarely. Cartilage thickness at the femoral condyle in different mammalian species ranges from 90 to 3000 μm and bears a negative allometric relationship to body mass [46]. Moreover, cellular density decreases with increasing body mass, although gross biochemical composition remains constant [46]. These results emphasize the need to use appropriate *in vivo* models in translational research.

Because of the inherent immunosuppressant properties of MSC, synovium-derived mesenchymal stem cells (SMSC) can be harvested from allogeneic synovial tissue, which is discarded in most knee operations, and could be used for cell therapy and TE protocols [47]. The use of combination strategies, such as cultivating cells on suitable biomaterials [48,49] and/or genetic engineering [17,50], and biomechanical stimulation [51], might improve the quality and stability of the neocartilage formed. Moreover, the addition of biological products such as platelet-rich plasma (PRP) is other possible route to improve the therapeutic potential of stem cells for articular cartilage repair [52].

Interestingly, in two recent clinical trials, ongoing in European countries, chondrocytes are combined with stem cells and implanted on the lesion site. This strategy is based on the finding that engineered cartilage derived from co-culture of these cells had reduced hypertrophy and enhanced functional properties [53]. Nevertheless, there is evidence that chondrocyte differentiation is downregulated during non-contact co-culture of the two cell types, even in a 3D culture system [54]. The effects of using different biomaterials for the assembly of these co-culture systems on the cell responses that promote the generation of new cartilage tissue remain to be evaluated in preclinical settings.

Clear evidence has been provided about the effect of scaffold properties on MSC chondrogenesis [55]. Oriented ligand presentation during MSC chondrogenesis in a 3D environment is necessary to trigger the appropriate cellular and molecular events for robust neocartilage formation [48]. Hyaluronic acid was also better for stimulating MSC chondrogenesis than either alginate, pluronic, or chitosan [19].

Notwithstanding the current ongoing clinical trials using MSC, the process of cartilage formation using these cells remains in its infancy. There is still room for improvement once the effects of countless combinations of biomaterials, growth factors, oxygen tension levels, mechanical stimulation, number and source of MSC are systematically evaluated for the formation of native-like cartilaginous tissue.

Biomechanical stimulation

Preclinical studies have provided clear evidence that, under specific culturing conditions, physical stimuli is a promising approach for obtaining hyaline-like cartilage. Hydrostatic pressure, direct compression, and high and low fluid shear stress are the type of forces that can be applied to cells [18]. Several encouraging results have pointed towards the relevance of applied force in the field of cartilage engineering.

A growing body of research has highlighted the role that mechanical forces play in the activation of latent growth factors in biological tissues [56], combating cartilage destruction [57], and lubricin metabolism *in vivo* [58]. Pulsed electromagnetic fields, for example, limit the catabolic effects of inflammatory cytokines and reduce degradation of the construct in the surgical microenvironment [59,60]. Dynamic compressive loading significantly reduced the expression of hypertrophic markers by human MSC, and suppressed the degree of calcification in MSC-seeded HA hydrogels [61]. Moreover, mechanical and electromagnetic stimulation of 3D-seeded chondrocytes resulted in improved extracellular matrix (ECM) production [62]. Mechanical compression, even with excessive stress, can activate Smad2/3P signaling, which is protective for articular cartilage and blocks chondrocyte terminal differentiation [60]. However, major advantages might be gained by adjusting loading regimes during cartilage TE [50]. The development of integrated systems for the expansion and differentiation of stem cells that include *ex vivo* mechanical stimuli [47] might increase costs and logistics, but these results still highlight the importance of mechanical loading for improving neocartilage properties and maintaining the cartilage phenotype during stem cell based therapy for cartilage repair.

Tissue engineering

The TE strategy applies the principles of biology and engineering to the development of functional substitutes for damaged tissue [63]. There are two basic approaches [64]: (i) *in vivo* TE, in which the construct is implanted with or without prior partial *in vitro* cultivation, and is allowed to mature *in vivo* for tissue repair and regeneration, and (ii) *ex vivo* TE, in which the tissue is generated entirely *in vitro* with full functionality before transplantation. In both approaches, the three components that crucially influence the outcome of a TE construct are: responsive cells, appropriate biomaterial, and an appropriate environment.

Four ongoing clinical trials are using hyaline cartilage tissue affixed with a sealant to the lesion. These studies are being carried out in Singapore, Taiwan, and the USA, and have been considered by the FDA as either biologicals or devices (Table S1). The DeNovo NT allograft utilizes minced juvenile articular cartilage that is maintained in storage medium at 19–26 °C for up to 49 days [7]. The Cartilage Autograft Implantation System (CAIS) is a similar technique but uses autologous tissue. Short-term studies of both TE strategies demonstrated their safety, feasibility, and efficacy, with evidence of clinical improvement [65]. However, RCT will be necessary to generalize these outcomes to the clinical setting. Recently, in a preclinical study, goat frozen allografts displayed signs of

failure at 6 months, with cartilage softening, loss of cells and matrix, and/or graft subsidence, supporting the importance of maintaining cell viability during allograft storage, and suggesting that outcomes at 6 months may be indicative of long-term (dys)function [66].

A prospective study (Level IV) showed that when the Hyalograft C implant was used to treat initial small cartilage defects the clinical improvement was excellent, whereas, when used in patients with prior anterior cruciate ligament reconstruction, less clinical improvement was observed [67]. However, Hyalograft C was recently withdrawn from the European market owing to a lack of evident positive benefit/risk ratio [68].

Biomaterials are powerful tools for regeneration of articular cartilage by providing microenvironmental cues including scaffold architecture as well as geometric, mechanical, and adhesive cues, soluble cues, and regulators [69]. In most clinical and preclinical studies that use only biomaterials for cartilage repair, these are combined with standard microfracture surgery to allow the migration of stem cells from the BM. Moreover, the inclusion of chondroitin sulfate in the scaffold formulation has clearly improved the quality of cartilaginous tissue [70–72].

Recent progress in the development of 3D printers for TE purposes [73] will be pivotal for the assembly of novel scaffolds with different properties and compositions. The development of novel biomaterials that entrap lubricants [74,75] and therapeutic biomolecules (proteins or genes) [33] for generating functional cartilaginous tissue is also important. Moreover, these constructs should be evaluated by tribological methods [76] as well as in terms of their degradation profiles and biomechanical properties [77].

Gene therapy

The transfer of genetic material into cells is not straightforward, and generally a viral or non-viral vector is needed to facilitate transport through cell membranes into the cytoplasm, and ultimately into the nucleus. Viral vectors give rise to high levels of gene transfer into cells, but their use is still associated with immunogenic episodes [78]. Nevertheless, in cartilage regeneration, viral vectors have been used to transfect cells under *ex vivo* conditions without major disadvantages [79,80]. Three clinical studies use transduced allogeneic chondrocytes (TissueGene-C) for TGF-β1 overexpression in patients with severe osteoarthritis of the knee joints, a condition which is refractory to existing non-operative therapies [81]. Several other genes have been identified as suitable for stimulating neocartilage formation by autologous cells, including IL-10 [82], fibroblast-like growth factor (FGF-2) [83,84], transcription factors such as SOX9 (SRY-related HMG box) [85], or specific bone morphogenetic proteins (BMPs) [86]. Viral vectors containing such genes have been injected not only into small animal models but also into the joints of large mammals [87].

Combined constructs involving the use of cells, matrices, and genes can be prepared by two approaches: (i) genetically engineering cells and culturing them on a matrix or embedded in a hydrogel, and (ii) culturing cells on or in a matrix that is pretreated with DNA vectors that will be released and enter the cells. Viral and non-viral vectors can be released from the matrices and, in both cases, native-like cartilage was attained [9,88–90]. Recently, sustained transgene expression and ECM formation by MSC was achieved by using lentiviral vectors immobilized on a biomechanically functional scaffold [91]. Moreover, studies involving the coexpression of genes [9] or smart polymers [88] gave rise to clear regeneration of cartilage compared to the use of each strategy in isolation. Indeed, understanding the role of secreted factors during embryonic development, and transposing this system into adult settings, will be another relevant step forward in the regeneration of a tissue as complex as cartilage.

When using non-viral vectors, minicircles, miniplasmids, or plasmids with S/MAR (scaffold/matrix attachment region) sequences are currently viewed as promising alternatives to conventional plasmids, circumventing their tendency to triggering cell death and/or gene silencing, and prolonging their maintenance inside cells [92]. By contrast, several miRNAs have already been identified as regulators of cartilage matrix proteins; these might provide an alternative to the use of genes [93] or could eventually be used as biomarkers for cell differentiation of *ex vivo* constructs before implantation [18]. The most expressed miRNAs in human cartilage are miR-140, miR-675, miR-27b, miR-146 [18], and miRNA-1 [94]. These small RNA molecules are more unstable than plasmid-based vectors, but do not need to be delivered into the nucleus, which simplifies the delivery process.

Over the years, several studies have identified the key factors that govern cartilage development [93,95], and the appropriate sustained release of these factors from smart biomaterials, together with mechanical stimulation [96,97], might constitute a more efficient strategy for driving differentiation of endogenous or exogenous cells towards chondrogenesis.

Concluding remarks and future perspectives

Regeneration of cartilage using advanced therapies is rapidly evolving towards strategies that integrate a combination of different cell sources, such as adult stem cells, with novel biomaterials in conjunction with sustained release of biomolecules. The foremost effort of these therapies, given the high costs associated, is complete regeneration of the tissue. Nevertheless, commercially available advanced therapies have not shown clear morphological and functional recovery of the tissue. Indeed, given the complexity of cartilage structure, the regeneration of focal lesions might demand a more complex strategy that not only involves the use of stem cells but also their previous controlled expansion and differentiation on appropriate matrices under dynamic conditions, even though this strategy will requires the use of good manufacturing practice (GMP) facilities. Until reaching this point, an endless number of combinations between novel biomaterials, cells, and genes, as well as physical stimuli, have yet to be assessed for the production of cartilage tissue in preclinical settings. Outstanding questions are listed in Box 1. Large animal studies that relate the quality of cartilaginous tissue to the number of cells applied per cm^3 of lesion should be required before clinical trial consent. Randomized clinical studies using stem cells are lacking, and it will

Box 1. Outstanding questions

- What is the range of mesenchymal stem cell numbers versus volume of lesion that triggers the formation of native-like cartilage tissue?
- How could we integrate a combination strategy with biomechanical stimulation *ex vivo*?
- How long would it be appropriate to differentiate MSC *ex vivo* before implantation of the construct?
- For regenerating cartilage, what is the best animal model to use before clinical trial consent?
- How far are we from elucidating the appropriate biomarkers to assess the quality of *ex vivo* formed cartilaginous tissue?

be essential to harmonize the techniques, biomarkers, and outcomes used to characterize the authenticity of cartilage generated from precursor tissue generated *in vitro* or *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tibtech.2014.11.003.

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