Multi-Disciplinary Approaches for Cell-Based Cartilage Regeneration

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ABSTRACT: Articular cartilage does not regenerate in adults. A lot of time and resources have been dedicated to cartilage regeneration research. The current understanding suggests that multi-disciplinary approach including biologic, genetic, and mechanical stimulations may be needed for cell-based cartilage regeneration. This review summarizes contents of a workshop sponsored by International Combined Orthopaedic Societies during the 2019 annual meeting of the Orthopaedic Research Society held in Austin, Texas. Three approaches for cell-based cartilage regeneration were introduced, including cellular basis of chondrogenesis, gene-enhanced cartilage regeneration, and physical modulation to divert stem cells to chondrogenic cell fate. While the complicated nature of cartilage regeneration has not allowed us to achieve successful regeneration of hyaline articular cartilage so far, the utilization of multi-disciplinary approaches in various fields of biomedical engineering will provide means to achieve this goal faster. © 2019 Orthopedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

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Articular cartilage does not heal itself in adults.[1,2] A lot of resources and efforts have been devoted to cartilage regeneration research.[2] The first cell-based therapy for cartilage repair was autologous chondrocyte implantation (ACI), which was first described in clinical reports 25 years ago.[3] As this method has revealed numerous limitations, alternative strategies have been developed.[4] Stem-cell based cartilage regeneration represents one of those approaches. It has been investigated extensively with a view to clinical application. However, our incomplete knowledge of developmental and regenerative processes and stem cell biology has meant that promising in vitro data have not often translated into favorable in vivo results.[5] The current understanding suggests that multi-disciplinary approaches including biologic, genetic, and mechanical stimulations may be needed for cell-based cartilage regeneration. In this review, updates in multi-disciplinary approaches for cell-based cartilage regeneration including the cellular basis of articular cartilage chondrogenesis, gene-enhanced cartilage regeneration, and physical modulation to divert stem cells to chondrogenic cell fate are summarized.

CELLULAR BASIS OF CHONDROGENESIS FOR CARTILAGE REPAIR

Current Methods to Repair Focal Cartilage Defects

Cartilage pathologies are the most common cause of chronic disability among adults. Early intervention to repair focal defects is key to restoring tissue integrity before chronic degeneration.[6–8] Unfortunately, successful surgical repair remains a challenge as resultant tissues are usually fibrocartilaginous and cannot meet the functional demands of the joint.[9] Current clinical choices for attempting repair include marrow stimulation through microfracture of the subchondral bone, cell-based strategies such as ACI or osteochondral (OC) grafting. Of these approaches, only OC grafting results in reasonably consistent hyaline cartilage repair.[10] To treat larger defects, mosaicplasty was developed, involving the transfer of a number of small plugs to produce a congruent joint surface. However, its use is limited due to the technical difficulty of the procedure, poor integration, and donor site morbidity. While allografts can overcome the latter problem, challenges associated with maintaining cell viability and the limited donor pool remain significant issues. However, the relative success of OC autografting to treat small defects suggests that engineering scaled-up, patient-specific tissues that mimic the complex extracellular matrix of AC could significantly advance regeneration of large defects. The unanswered question is what is the best cell type to use to create such a tissue?

Isolation of Articular Cartilage Progenitor (ACP) Cells

Brunger et al.[11] recently described the use of CRISPR/Cas9 genome engineering to create stem cells that antagonize IL-1 or TNF-α-mediated joint inflammation with autoregulated feedback-control to mitigate effects of pathological levels of the cytokines. They modified murine-induced pluripotent stem cells (iPSCs) for this purpose and showed that they were still capable of chondrogenic differentiation post-modification after the genome engineering. However, iPSC-derived chondrocytes have inconsistencies depending on cell sources that provide challenges for their use in regenerating AC lost in such diseases.[12] In addition, they will express the endochondral phenotype as mesenchymal stem cells (MSCs) do,[13] something not necessarily desired in the production of stable AC. Thus, alternative sources of cells would be advantageous for novel therapeutic techniques like the one published by

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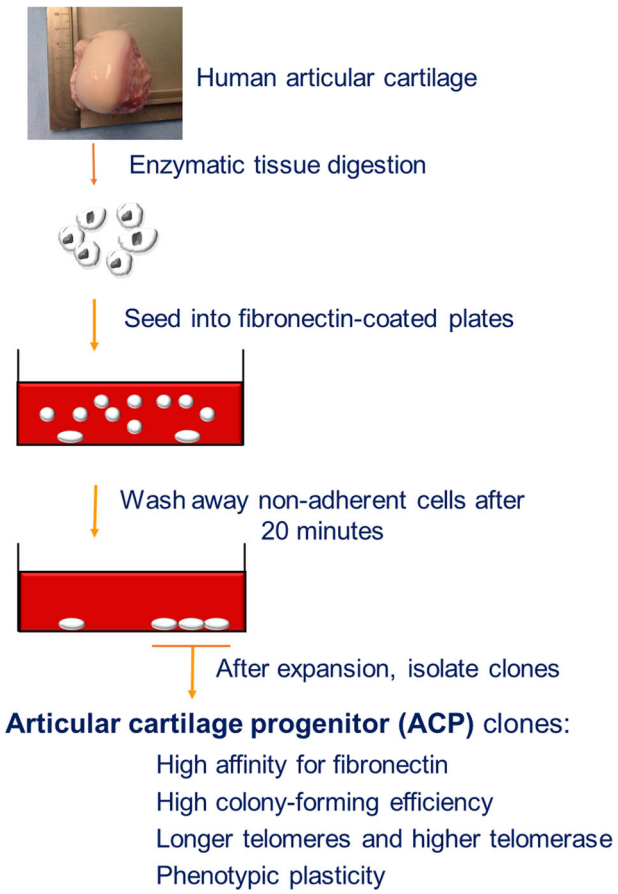

Figure 1. Isolation of articular cartilage progenitor clones. Articular cartilage is digested with enzymes to free cells. Those cells with high affinity for fibronectin adhere to fibronectin-coated plates quickly, which can then be cloned after expansion. Work with the human articular cartilage progenitor (ACP) clones has demonstrated that they have longer telomeres, telomerase[14], and phenotypic plasticity.[15,16] [Color figure can be viewed at wileyonlinelibrary.com]

Brunger et al.[11] To this end, we have isolated and characterized ACPs directly from human AC (Fig. 1).[15,17] These cells have been established as not only clonable but also able to differentiate into chondrocytes with the stable cartilage phenotype,[14–20] which makes them a potentially superior cell type to use for therapies such as those suggested by Brunger et al.[11]

However, we have also found clones that will consistently undergo endochondral chondrogenesis.[14] In a recent study, we tested human ACP clones from multiple biological replicates for their chondrogenicity in pellet culture at physioxia (defined as 2–5% oxygen for human articular cartilage) and hyperoxia (20% oxygen).[15] As reported by Archer's group,[14,16,19,20] the majority of clones appeared to make cartilage of a stable phenotype, expressing hypertrophic markers (COLXA1, MMP13) only at or below the level of detection. Physioxia decreased any detectable hypertrophic markers even further.[15,17] However, we did find clones in some biological replicates (~5–10%) that consistently underwent endochondral chondrogenesis in the same manner as MSCs do in the assay.[21] Having distinct clonable cartilage progenitor cell types allows us to propose a research plan to interrogate their respective transcriptomes to determine differences that contribute to their differentiated phenotype. The range of clones allow us to explore the mechanisms that control the production of stable versus endochondral human AC. They may also allow construction of articular cartilage implants with the necessary depth-dependent anisotropy.

Subpopulations of ACPs

Radiolabeling and BrdU studies of Archer's group and Hunziker,[22,23] along with the more recent prg4 lineage tracing experiments of Kozhemyakina et al.,[24] all strongly suggested that the mechanism of AC growth was appositional rather than interstitial.[25] Thus, progenitor cells should be localized in the upper regions of the tissue, in a manner similar to growth plate. However, a very recently published study from Decker et al.[26] has cast doubt on the interpretations of prg4-labeled cell tracing experiments in mice. Using R26-Confetti and single color reporters, they found that cartilage growth and thickening occurred through formation of non-daughter cell stacks and cell rearrangement, with matrix elaboration and zone-specific increases in cell volume, and little contribution from proliferation. In other words, interstitial growth from progenitors distributed throughout the tissue. Of interest, both the Decker et al.[26] and Kozhemyakina et al.[24] suggest that the progenitor cells of the deep zone of cartilage form calcified chondrocytes. Are the endochondral human ACPs we isolate responsible for this, perhaps representing a separate lineage of progenitor cells from stable cartilage ACPs? Where do they diverge from ACPs higher in cartilage? Answers to these questions remain unclear. An intriguing hypothesis presents itself based on the work of Schwartz et al.,[27] who demonstrated that in the embryo, mouse joints form from a continuous influx of progenitor cells. Moreover, their work suggests differentiation signals exist that contribute differently to various joint tissues, facilitating lineage divergence. If this is correct, then we may be able to define different markers for the diverged subpopulations of ACPs.

Tissue Engineering with ACPs

How can we best exploit the human ACP populations in AC? It may be possible to stimulate native ACPs to repair and regenerate cartilage in situ although we do not currently have the right stimuli, or know the best way of exposing the ACPs in the tissue to them. An alternative is to attempt tissue engineering with ACPs. To this end, we created scaffold-free ACP-derived cartilage.[17] To engineer discs of cartilage from ACPs required use of a fibronectin-coated substrate to prevent contraction of the ACPs into large pellets. As we isolate ACPs using their high affinity for fibronectin binding, this was a logical step. It may also be relevant that fibronectin is the earliest expressed extracellular

matrix molecule during cell condensation in embryonic development of limb cartilage.[28,29]

The creation of scaffold-free cartilage discs allows the investigation of the effects of altered environmental conditions on the content and organization of the tissue created. The intent is to study those factors responsible for facilitating the creation of the anisotropic organization of mature AC. This anisotropy occurs not only from the surface of AC to its deep zone but also from the chondrocytes outward, with the formation of pericellular, territorial, and interterritorial matrices. In our initial studies, we found that culture in physioxia improved the collagen expression profile relative to hyperoxia in the human ACP discs.[17] While there was a maintenance of collagen II expression, collagens I and X were decreased throughout the tissue. Furthermore, ACP-derived cartilage had collagen VI localized pericellularly after 28 days, whereas in discs formed from articular chondrocytes collagen VI was not localized in this manner. As these proteins are distributed throughout the matrix in neonatal cartilage and subsequently localized to the pericellular matrix with maturation.[30,31] The result suggests that ACP-derived cartilage is more mature. This more mature matrix presumably contributed to the superior mechanical properties of the ACP-derived cartilage over that created from chondrocytes. The compressive equilibrium modulus of the ACP-derived cartilage was significantly higher than that of chondrocyte-derived cartilage for each strain ramp introduced, and with strain-stiffening behavior in physioxia.[17] In summary, human ACP clones could be manipulated to produce cartilage that is mechanically superior and more mature than that produced from chondrocytes. One advantage is that ACP discs can be derived from a single cell following clonal isolation and expansion, allowing the choice of superior clones for the purpose. Use of human ACPs also avoids the problem of replicative aging that occurs in expanded chondrocytes,[32] which presumably also contributes to the lower quality of tissue produced.

GENE-ENHANCED CARTILAGE REGENERATION

Need for Gene-Enhanced Cartilage Regeneration

Gene transfer is a method to introduce genetic material into cells to cause a change in cell structure and function at molecular level.[33] During in vivo gene transfer, a vector that contains the therapeutic gene is introduced into the recipient individual directly. In ex vivo gene transfer, transgenes are transferred into cells in vitro before implantation in the recipient. Most investigations of gene therapy in cartilage regeneration reported to date have used ex vivo gene transfer.[34] While it can be debated that gene therapy should be used for nonlethal conditions including cartilage defect or osteoarthritis (OA), the rationale for gene-cell therapy is that the transfer of therapeutic gene would enhance the effect of cell therapy.[2]

Cartilage defects or the consequential OA are principally local pathologies involving a single or a small number of joints and relatively discrete enclosed environments. Local therapy such as intraarticular (IA) injection or arthroscopy can be easily utilized to deliver therapeutic materials. On the other hand, when implanted, small molecules diffuse out through subsynovial capillaries, while macromolecules and particles leave through the lymphatics. Therefore, it is very difficult to achieve sustained, therapeutic concentrations of delivered materials in joints.[35,36] The idea to use gene or cell therapy for cartilage regenerations is also based on the need to resolve this problem

Vectors for Gene Transfer

As DNA molecules with negative charges do not penetrate the mammalian cell membrane, a vector is essential to transfer the therapeutic gene into the cells. Vectors are classified into nonviral and viral types. The principal advantage of viral gene transfer is high efficiency in carrying genetic materials into target cells. However, safety issues are a great concern despite modification of viral genomic structures to eliminate pathogenicity.[37] In order to solve this issue, physical and chemical methods have been developed to transfer DNA into cells as alternatives to viral gene transfer. Nonviral vectors are generally less efficient than viral vectors, having a short duration of transgene expression. This may not necessarily be a problem depending on the gene being delivered, as a burst of expression of a certain growth factor (GF) may actually be advantageous. Regardless of this possibility, the transfer efficiency of nonviral vectors has been greatly enhanced with recent advancements in technology.[38]

Cell Sources and Vectors in Gene Cell Therapy for Cartilage Regeneration

Chondrocytes from AC and MSCs have been extensively studied for implantation targeting human AC defects.[2,5] In addition, fibroblasts, perichondrial cells, periosteal cells, and muscle-derived cells have been also investigated as possible cell sources for cartilage regeneration. Both viral and nonviral methods have been employed for gene transfer to target cells. Nonviral, adenoviral, retroviral, and adeno-associated virus (AAV) vectors have been used to deliver genes to AC defects using ex vivo approaches.[2] While most studies were performed in small animals such as rats and rabbits, data from large animal (sheep or horse), which would be more predictive of human results are generally lacking.[39]

Gene Transfer of GFs for Cartilage Regeneration

Gene transfer of GFs makes transduced cells secrete active peptides that can produce a more favorable environment for cartilage regeneration. Genes that would stimulate chondrogenesis or inhibit AC degeneration can be used with this type of gene therapy.[2]

Transforming growth factor-β (TGF-β) superfamily of GFs can induce the chondrogenesis of MSCs and

maintain the chondrogenic phenotype of differentiated cells.[40] TGF-β1 is a crucial component of well-known chondrogenic differentiation medium[40] although TGF-β3 is now increasingly used for this purpose.[41,42] TGF-β-1-tranduced chondrocytes (Invossa TM, Kolon Bioscience, Korea) were developed into cell therapeutics to treat OA. As a retrovirus was used for the transduction, the transduced cells were irradiated before IA injection to patients so that the injected transduced cells disappear from the joint shortly after injection. Immunomodulatory and anti-inflammatory actions were reported to be main mode of action rather than enhancement of chondrogenesis. Clinical trials using this cell therapeutic reported significant pain relief while the structural improvements were not pronounced.[43–45] This gene-cell therapeutic was later withdrawn from the market after it was confirmed that TGF- β-1-tranduced cells were actually HEK-293 cells.

Bone morphogenetic proteins (BMPs) are also members of the TGF-β superfamily. As BMP-induced chondrogenesis is thought to be an intermediate step leading to bone formation, BMP application for chondrogenesis needs to be finely tuned with the multiple other stimuli involved in the process.[46,47] IGF-1 promotes proliferation and differentiation of AC chondrocytes.[48] IGF-1 stimulates the synthesis of collagen type II and proteoglycan as well as cell proliferation simultaneously.[49] Fibroblast growth factor (FGF)-2 is a mitogenic GF that can increase the pool of cells responsiveness to chondrogenesis. Collagen type II expression and individual parameters of chondrogenesis such as cell morphology and architecture of regenerated tissue are enhanced with FGF-2 gene transfer.[50]

Several factors that possess complementary mechanism of action may be used to enhance chondrogenesis. Gene transfer of IGF-1 and FGF-2 targeting anabolic function and cell proliferation, respectively, may be applied simultaneously to promote chondrogenesis.[2]

Overexpression of Transcription Factors (TFs) for Cartilage Regeneration

TFs act inside cells. They are not secreted from cells unlike GFs. Sox-9 is a key chondrogenic TF that binds directly to the collagen type II and aggrecan promoters, activating the expression of these genes.[51] Sox-9 has been transduced into cells using adenovirus, retrovirus, AAV, and lentivirus vectors.[2] Sox-9 together with its cofactors Sox-5 and Sox-6 are called the Sox trio. Our group has used microporation to transfect MSCs from bone marrow and adipose tissue with the Sox trio, showing high transfection efficiency (approximately 70%). Chondrogenic differentiation of those cells was significantly enhanced following Sox trio co-transfection. Collagen type II expression was enhanced while collagen type X expression decreased.[52,53] Our previous studies have also shown that retroviral Sox trio transduction to rat ASCs significantly enhanced the in vitro chondrogenic differentiation of rASCs.[54] We have also devised a chondrogenic scaffold system, consisting of a poly(lactic-co-glycolic acid) (PLGA) scaffold loaded with plasmid DNA (pDNA) containing the Sox trio genes. pDNA was slowly released and transfected ASCs seeded in the scaffold (Fig. 2). Enhanced cartilage regeneration was demonstrated in an in vivo OC defect model in rabbits when the composite seeded with ASCs was implanted into the defects for 8 weeks.[55]

Sox genes may also be transfected into cells via nanoparticles. Our group has designed nanoparticles comprising dexamethasone-conjugated polyethylenimine complexed with plasmid harboring *Sox duo* (*Sox-9, -6*) and

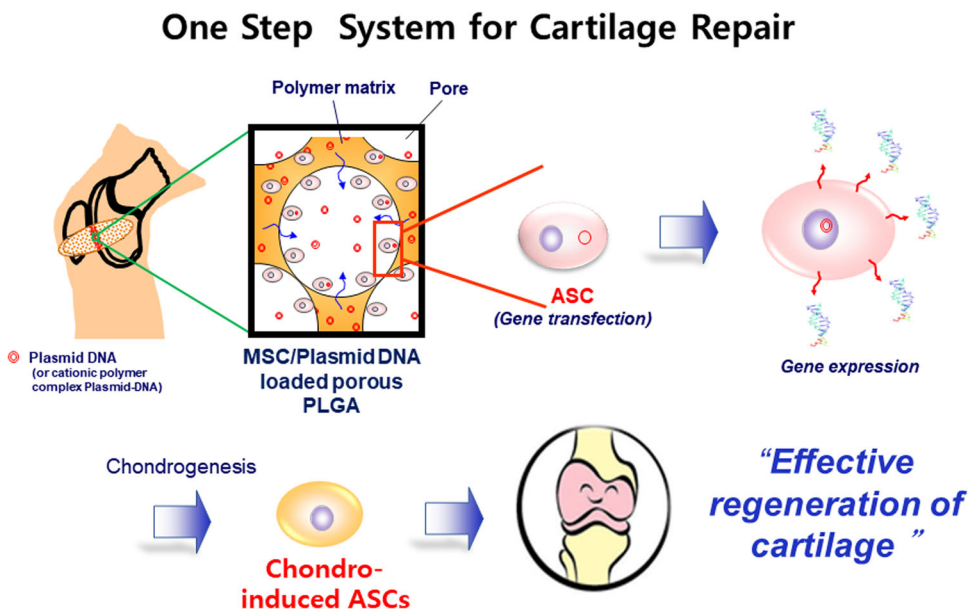

Figure 2. Chondrogenic scaffold system for one-step cartilage repair. Poly(lactic-co-glycolic acid) (PLGA) scaffold loaded with plasmid DNA (pDNA) containing the Sox trio genes. pDNA was slowly released and transfected human adipose stem cells (ASCs) seeded in the scaffold.[55] [Color figure can be viewed at wileyonlinelibrary.com]

ANGPTL4 small hairpin RNA in the expectation that transfection of these nanoparticles would enhance chondrogenesis of stem cells and suppress inflammation in OA. ASCs transfected with Sox gene-harboring nanoparticles significantly enhanced chondrogenesis, showing higher expressions of the collagen type II gene and protein and suppression of matrix metalloproteinase 3 and 13 genes compared with control plasmid-harboring nanoparticles. In vivo experiments using surgically induced OA in rats showed that Sox gene-harboring nanoparticle transfected ASCs arrested the progression of OA in that model.[56]

As a preclinical study to test the feasibility of Sox gene-based gene cell therapy to treat OA, the authors have developed *Sox-6*, *9*-transfected human adipose stem cells ($^{SOX\text{-}6,\ 9}$ASCs). These were tested for their effectiveness in arresting OA progression when IA injected in a surgically induced OA caprine model. In vivo tracking of injected $^{SOX\text{-}6,\ 9}$ASCs in rats demonstrated that these cells disappeared from the joint cavity within 2 weeks, suggesting a paracrine mode of action. In a surgically induced goat model of OA, IA $^{SOX\text{-}6,\ 9}$ASCs at a dose of 0.6×10^7 best preserved AC and produced significantly better macroscopic and microscopic scores than negative controls in femoral and tibial articular surfaces.[57]

Reprogramming

Direct reprogramming or direct conversion means converting cells into other lineages without undergoing full reprogramming into pluripotent stem cells (PSCs). It has the potential advantage of skipping complicated differentiation processes needed for PSC conversion into the desired lineage. Direct reprogramming has been reported to generate cells of various lineages from somatic cells. Tsumaki et al used retroviral transduction of TFs Sox-9 Klf4 and c-Myc to reprogram fibroblasts into chondroprogenitor cells. By combining PSC-inducing reprogramming TFs (Oct-4, Klf4, C-Myc, and Sox-2) with the chondrogenic TF Sox-9, murine dermal fibroblasts were directly reprogrammed into chondrocytes.[58] This combination also induced direct chondrocytic conversion of human dermal fibroblasts.[59] While the use of oncogenic factors such as c-myc raises concerns for the clinical application of direct reprogramming, the concept of direct reprogramming per se can evolve into a major category of gene-cell therapy in regenerative medicine if managed properly. Particularly, the use of safer transduction methods including nonviral techniques and exploration of other direct reprogramming factors may bring this concept within the scope of clinically applicable technology.[2]

In Vivo Gene Therapy to Treat OA

IA injection of genetically modified cell suspensions is unlikely to achieve long-term transgene expression because injected cells are cleared from joints within days to weeks. These limitations of ex vivo gene delivery including short-term cell survival and failure to engraft has prompted the possible use of IA in vivo gene delivery to treat OA. Safety issues are even more of a concern when contemplating human application of in vivo gene therapy for OA, but AAV has already been used for in vivo IA gene delivery in two human trials.[60,61]

It has been reported that particles larger than 10 nm have difficulty to pass through the dense extracellular matrix of normal AC.[62] Larger viruses including adenovirus or lentivirus, both of which are approximately 100 nm in diameter, therefore, cannot penetrate AC and transduce chondrocytes. AAV, which is approximately 20 nm in diameter, can transduce chondrocytes throughout the full thickness of normal AC. Notably, efficient in vivo transgene expression after IA injection has been reported with AAV serotypes 2, 2.5, 5, and 8 in various mammals.[63]

While more synoviocytes than chondrocytes may be transduced with IA gene delivery, it is not necessarily a concern if anti-inflammatory factors, rather than chondrogenic factors, are used. Contrarily, it may rather offer an advantage considering that chondrocytes are dysfunctional in OA. In vivo gene delivery of interleukin-1 receptor antagonist (IL-1 Ra) that blocks IA actions of IL-1 has been developed to treat OA using delivery with recombinant AAV.[63] Unlike data from athymic rats, a rapid or extensive decline of gene expression was not observed in equine joints.[64]

PHYSICAL MODULATION TO DIVERT STEM CELLS TO CHONDROGENIC CELL FATE

Importance of Pericellular Matrix in Response of Chondrocytes to Mechanical Stimuli

The role of mechanics on the musculoskeletal system has long been appreciated. Numerous studies have investigated the specific role of mechanical stimulation on cartilage development and maturation.[65–73] A large number of studies have investigated the role of uniaxial compression on hydrogel encapsulated chondrocytes. In each case cyclical load increased anabolism and matrix production. However, during early stages of culture, isolated chondrocytes are less responsive to the load applied, and the responsiveness increases as a pericellular matrix develops.[74] A similar effect has been observed using bovine MSCs.[75] This occurs more rapidly in young animal cells compared with adult human cells, which affects the timing of the response. Therefore, the chondrocyte plus matrix (chondron) is more responsive to load than a chondrocyte in the absence of extracellular matrix. For chondrocytes, the improved responsiveness to load once a matrix has formed is less of an issue as they have a natural tendency to form chondrogenic matrix when encapsulated in 3D hydrogels, likely due to their epigenetic memory.[76] Therefore, under nonloaded conditions the cells will naturally start to produce a chondrogenic matrix, albeit of varying quality.

The Role of Mechanical Strain and TGF-β in Chondrogenesis of MSCs

Adult MSCs do not have a natural tendency to form cartilage-specific matrix after 3D encapsulation in the

absence of specific chondrogenic stimuli. Thus, providing early stimuli is crucial if the desired fate is to be obtained. During the differentiation process of MSCs, the application of mechanical strain has the potential to modify the cell phenotype. It is Increasingly becoming clear that the response to the load applied is context-dependent and is greatly influenced by the differentiation state of the cells under investigation. As monolayer expanded human MSCs tend to express aggrecan and produce glycosaminoglycan, detection of an increase in GAG synthesis as a response to stimuli may be a general increase in anabolism rather than a chondrogenic-specific stimulus. In our hands, collagen II is a more robust marker, although a larger panel of markers needs to be investigated to establish the extent and specificity of differentiation. Collagen II is a critical component of the cartilage matrix and is often not expressed in monolayer expanded MSCs but is expressed in chondrogenically stimulated cells. Uniaxial compression of MSCs from various species in the absence of exogenous TGF-β has differing effects regarding collagen II expression.[77–80] In part, this is confounded by a potential for young animal cells to spontaneously undergo chondrogenesis over time after encapsulation, particularly rabbit cells.[80] The load could then be enhancing this spontaneous response, rather than providing the initial trigger. Uniaxial load of adult human MSCs does not lead to chondrogenic differentiation when considering collagen II as a primary outcome.[81] To overcome this, some groups prime the cells with TGF-β in 3D culture to trigger a chondrogenic response and then stimulate the cells mechanically to promote or consolidate the cartilage matrix production.[82] These studies have demonstrated that cyclical compression can enhance TGF-β-induced chondrogenesis of MSCs. However, differences in species and materials used to encapsulate the cells must always be taken into consideration. Considering naïve MSCs require TGF-β stimulation prior to being mechanically loaded, the question of TGF-β source in vivo arises.

Mechanical Activation of Endogenous TGF-β

Within fracture repair, and in some cases of cartilage repair using marrow stimulation techniques, a chondrogenic differentiation of MSCs is possible. Particularly in the case of fracture repair, this is known to be strongly regulated by mechanics. Cyclical mechanical strain across a fracture gap leads to a chondrogenic intermediate (endochondral ossification) rather than direct osteogenic differentiation (intramembranous ossification). We have studied the application of mechanical stimulation of adult human MSCs in the absence of serum and endogenous GFs (namely TGF-β).[83,84] While uniaxial load in the absence of TGF-β does not lead to chondrogenic differentiation, compression with superimposed shear, that mimics the kinematic joint, does lead to collagen II production and a chondrogenic differentiation.[81] Increasing exogenous TGF-β concentrations in the medium decreases the responsiveness to load.[84] As the loading protocol increases TGF-β production and activation,[84,85] adding concentrations of exogenous TGF-β greater than that generated endogenously by the cells can mask the response to load, thus decoupling the biological response to the load applied. Whether the shear component is mechanical or fluid shear has yet to be determined. Our working hypothesis is that interfacial shear activates the TGF-β protein by removal of the non-covalently bound latency associated peptide (LAP) and the compression allows for enhanced penetration of the active TGF-β into the 3D encapsulated cells below. The mechanical activation of TGF-β in synovial fluid by shear has also been shown.[86] This effect can be enhanced when the cells are asymmetrically seeded, demonstrating that under loading conditions cell location has an influence on outcome.[87] When the material is stiff enough, the application of shear superimposed over compression can lead to mechanical activation of TGF-β in a cell-free scaffold by removing the non-covalently bound LAP.[85] This offers a material testing strategy, whereby a novel material can be tested under cell-free conditions using compression and shear with latent TGF-β included in the culture medium. After a few hours load, the presence of active TGF-β in the medium can be assessed and using as an initial readout measure of how the material will impact the biological responses in vivo during rehabilitation after cartilage repair treatment.

One consideration that has been established by the work discussed above is the context dependency of the cells undergoing the loading protocols. The transition from progenitor cell, to chondroprogenitor, to chondrocyte, and finally chondron offers different challenges and opportunities when considering the type of load to apply. The sensitivity to load and the ultimate response is dependent on the species, age, and maturity of the cell itself, and the maturity and composition of the surrounding extracellular matrix (Fig. 3). Chondrocytes are often quoted as being able to survive harsh environments, yet early tissue engineering attempts often described an inner core where cell viability and matrix synthesis were reduced, suggesting it is the chondron that is robust. A mature chondrocyte in the absence of matrix is less sensitive to load and this can be overcome once a rudimentary pericellular matrix has been established.

SUMMARY AND FUTURE PERSPECTIVES

The ability to produce large-scale implants of a defined geometry from ACPs may eventually allow us to generate tissues that can meet the functional demands required for articular cartilage regenerated tissue. However, it is a challenge to create scaffold-free implants of the dimensions and zonal anisotropy needed for human joints. Layering zonal cells for cartilage tissue engineering has been attempted by others,[88] and has not been limited to the use of chondrocytes. Recently, Levato et al.[89] combined ACPs with MSCs to try

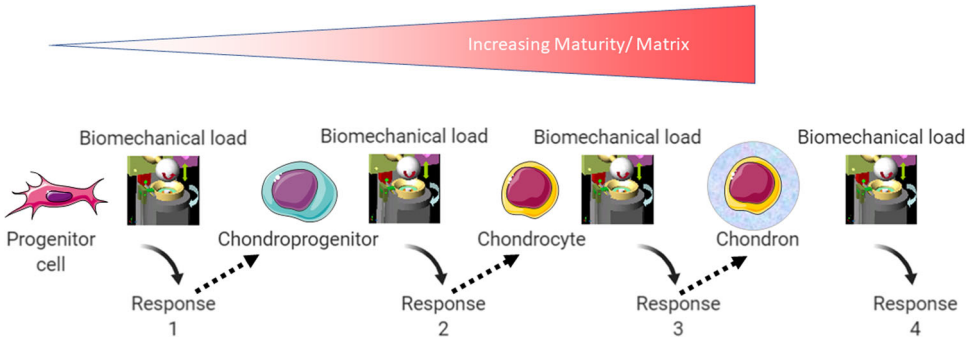

Figure 3. Difference response to load with differentiation. As the differentiation state increases, the response to load differs. Under certain conditions, the load may drive differentiation toward the next stage (dotted arrows). [Color figure can be viewed at wileyonlinelibrary.com]

to define the deep zone as the ACP clones they used form only non-hypertrophic cartilage. Use of ACP clones with differing capabilities would presumably alleviate the need for the second cell source. In summary, clonal ACPs represent a promising cell source for use with modern 3D bioprinting techniques and the technologies for cell manipulation, as described by Brunger et al.[11]

It is very important for the clinical applications of gene transfer for cartilage regeneration and OA treatment to ensure the safety while guaranteeing effectiveness of the gene-cell therapeutics. Viral gene delivery methods with improved safety features show enhanced potential for clinical application. On the other hand, nonviral delivery, which lagged behind in clinical application with transfection rate, have greatly advanced in gene-transfer efficiency. When we take into consideration the nonlethal character of cartilage defects or OA, the safety should precede the efficiency. A recent report of unexpected inclusion of HEK-293 cells in Invossa^{TM}, a first approved gene-cell therapeutics to treat OA, has demonstrated the difficulty and caveats in translating an idea based on research data into commercially viable therapy. Quality control issues that inevitably follow cell therapeutics can be made more complicated with the addition of genes to cells. Also, IA injected cells were shown to undergo death within a short period of time (2–3 weeks) without engraftment into cartilage defects.[57] In those circumstances, a brief paracrine effect before cell death will be all that can be expected from a gene-cell therapeutics. It should be considered whether gene transfer is still necessary when injected cells survive for such a short period of time and limited effects are expected from the transgene. If long-term existence of implanted cells is necessary for wanted regeneration of cartilage, methods to enhance the engraftment and survival of administered cells should be devised.

A greater understanding of the underlying mechanisms that drive chondrogenic differentiation and matrix deposition will allow for future rehabilitation protocols to be evidence-based. The articulating joint offers a unique opportunity to provide defined stimuli at defined times after surgery. This area of research is coming together under the umbrella of regenerative rehabilitation[90,91] and will lead to a more holistic treatment approach that combines surgery with patient-specific rehabilitation protocols.

In conclusion, there are both hopes and caveats in all three approaches for cell-based cartilage regeneration introduced here. While the complicated nature of cartilage regeneration has not allowed successful regeneration of hyaline AC so far, the utilization of multidisciplinary approaches, with understanding of various fields in biomedical engineering, will provide means to get us closer to the achievement of this goal. On the other hand, from clinical point of view, the increasing complexity of regeneration technology can add up to the difficulty in passing through regulation agency as well as the cost of therapy that is considered important by healthcare providers. In this regard, strategies that employ more simplified approach and utilize only the essential part of a devised regeneration technology may be necessary at the same time.

AUTHORS' CONTRIBUTION

All three authors equally contributed to this work in drafting the paper and revising it critically. They all read and approved the final submitted manuscript.

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