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Viruses: Friends and Foes

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Abstract

In this chapter, we will review how viruses can be used to positively affect joints and cartilage of their hosts. Many viruses are arthrogenic, and cause persistent and debilitating arthritis. Even those viruses that are not typically arthrogenic can also cause bone lesions as secondary pathogenesis. Some of these foes include members of the alphaviruses, like chikungunya and Ross River viruses, the rubiviruses, such as rubella, and erythoparvoviruses, like parvovirus B19. Some more uncommon viruses, which can occasionally have detrimental effects on their hosts' joints, include herpes simplex virus, varicella zoster, mumps, human cytomegalovirus, avian orthoreovirus, and caprine arthritis-encephalitis virus. Despite some viruses having negative impacts on cartilage and joints, others have been used as an effective means of gene therapy for bone and cartilage repair. We will take an in-depth look at the current therapeutic strategies for treating arthritis using various viral vectors.

Keywords: viral gene therapy, cartilage and bone healing

1. Introduction: viral peptides/vectors used as gene therapy for joint repair

Viruses have long been used as vectors for gene therapy. Some of the more popular viral vectors include retroviruses, oncolytic viruses, lentiviruses, adenoviruses, and adeno-associated viruses to name just a few. They are used in a wide variety of fields and are able to treat a diverse range of diseases, including Parkinson's disease, many cancers, amyotrophic lateral sclerosis, genetic disorders, cardiovascular diseases, hemophilia, and central nervous system CNS diseases and disorders [1–5]. In recent years, there has been an increase in the development of viral vectors to treat the musculoskeletal system, including the joints [6].

Articular cartilage damage can result from a variety of insults, either from over usage, diseases and disorders, or accidents, and often leads to different types of arthritis including

osteoarthritis (OA) [7]. Articular cartilage damage can cause swelling, pain, and subsequent loss of joint function. Due to its structure, cartilage does not usually regenerate after injury or disease, thus leading to loss of tissue and formation of a defect [8]. Cartilage is devoid of nerves, lymph, and blood supply, thereby explaining the limitations to self-repair. Current therapies targeted at treating articular damage have demonstrated variable results. These therapies include oral administration of a variety of components of the extracellular matrix, such as glucosamine or intra-articular injections of corticosteroids, biological agents (e.g., infliximab, etanercept), analgesics, and autologous blood products [9, 10]. Many approaches have also been investigated to help heal cartilage damage, including the use of viral peptides/vectors as a means of gene therapy for joint repair. These strategies mostly rely on overexpressing therapeutic factors or suppressing genes involved in joint destruction. In this chapter, we will examine the use of the severe acute respiratory syndrome (SARS)-coronaviruses (CoV), recombinant adeno-associated, and adenovirus vectors as well as retroviruses and lentiviral vectors for the treatment of joint repair.

2. SARS-coronavirus peptides

Coronaviruses (CoV) are potentially lethal viruses of the *Coronaviridae* family. They are positive-sense enveloped RNA viruses, which infect humans and animals. Two virulent strains, HCoV-229E and HCoV-OC43, were first identified in the 1960s from patients who presented with coryzal symptoms. Due to increased surveillance of CoV disease prevalence, other strains circulating in the population have recently been identified, including HCoV-NL63 and HCoV-HKU1 [11, 12]. Also, since 2003, more pathogenic strains of coronaviruses have been discovered, including severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS), which predominantly infect the lower respiratory track and cause lethal pneumonia [13, 14].

Despite being pathogenic, coronaviruses have been used as both viral vaccine vectors and gene therapy vectors [15–18]. The uses of CoV as vectors range from delivering immunostimulatory cytokines and antigens to treatment of feline infectious peritonitis. A recent publication has shown the potential of using CoV vectors for the treatment of arthritis [19]. In this study, the authors demonstrated that the use of a small synthetic peptide (MG11, 11 amino acids in length) derived from SARS-CoV fusion protein was able to reduce inflammation in a collagen-induced arthritis (CIA) mouse model. Furthermore, MG11 also was shown to protect mice against bone and cartilage damage. A 14-day treatment regimen with a dose of 25 mg/kg was considered efficient at reducing arthritis in this common autoimmune animal model of rheumatoid arthritis. Histological analysis showed treated mice had no or very minimal inflammation and minimal cartilage damage in the joints of the paws. Knees and ankles also had limited inflammation, or no inflammation, and synovial membrane thickening did not differ from normal limits. The findings suggested that the decreased pathogenesis is due to the ability of MG11 to inhibit cytokine and growth factor production mediated by inflammatory T cells. This study is interesting and paves the way for potential usage of CoV peptides as a novel therapeutic to alleviate rheumatoid arthritis.

2.1. Recombinant adeno-associated virus vectors (rAAV)

Adeno-associated vectors (AAV) are frequently used as viral vectors for gene therapy. They are small nonpathogenic members of the *Parvoviridae* family and the genus *Dependovirus*. These members are nonenveloped viruses with a single-stranded DNA genome (≈ 4.7 kb) [20] and only about 20–25 nm in size [21]. They are safe to use as viral replication (the lytic stage) can only occur in the presence of a helper virus, either adenoviruses or herpesviruses. AAVs were first isolated from stocks of human and simian adenoviruses and thought to be contaminants [22]. AAVs are of interest since they have the ability to specifically integrate into host genomes and establish latent infections. Furthermore, more great advantages are that the preparations are stable and can be produced at titers of more than 10^{12} particles per ml [23].

Many clinical trials have commenced looking at the use of rAAV for treating a variety of conditions including but not limited to Pompe disease, cystic fibrosis, Parkinson's disease, muscular dystrophy, α -1 antitrypsin deficiency, and hemophilia [24–28]. Europe has even approved a rAAV drug manufactured under the name Glybera, which is the first gene therapy, to treat a very rare disease called lipoprotein lipase deficiency [29]. Despite the efficacy, the staggering cost of such a treatment has hindered the commercial success and use of this drug. There are currently a few other gene therapy drugs in the pipeline, including Amgen's FDA-approved drug IMLYGIC, which is a genetically modified oncolytic virus (Herpes simplex virus type 1) with proposed usage for melanoma cancers.

Previous work had shown that cell proliferation actually increases rAAV transduction, thereby making arthritis a candidate disease to be treated by rAAV [30]. Arthritis is not only accompanied by local influx of immune cells but also proliferation of cells in the synovial lining. The first *in vivo* experiment to examine the use of rAAV for the treatment of arthritis was done in the late 1990s. The authors chose to use a rat model of acute arthritis by intra-articular injection of lipopolysaccharide (LPS). The rAAV vector contained the *Escherichia coli* β -galactosidase gene regulated by the cytomegalovirus (CMV). The paper goes on to show efficient and stable gene delivery by rAAV and similar to previous *in vitro* findings, inflammation or disease state seems necessary to facilitate gene delivery. There is a clear enhancement of gene expression during the inflammatory process and the severity of the arthritis. At peak disease, 95% of the synoviocytes expressed high levels of the transgene, whereas when the arthritis subsided at 30 days post-LPS treatment, only basal levels of expression was seen. Interestingly, the study supports the feasibility of a preventative treatment approach, since rAAV responds to the disease state of the target tissue.

Since that study, rAAV vectors have demonstrated a great efficiency at transducing a variety of joint/articular cells, both *in vitro* and *in vivo*, including chondrocytes [31–33]. In the hopes of treating osteoarthritis, not only has the transduction of chondrocytes been investigated but also other important cells, including osteocytes, meniscal fibrochondrocytes, tendon/ligament cells, muscle cells, cells of the synovial lining and progenitor cells that may differentiate to form joint tissues [6]. The gene therapy approach is aimed at targeting a variety of mechanisms involved in the development of osteoarthritis, including cell proliferation and survival, the stimulation of anabolic pathways, the inhibition of inflammatory or catabolic pathways, and finally a combination of these strategies.

Approaches looking at stimulating growth and regeneration focus primarily on expressing known growth and cell survival factors, such as fibroblast growth factor-2, bone morphogenetic proteins (BMPs), telomerase, and antiapoptotic molecules like Bcl-2. Stimulating anabolic pathways involves building new molecules out of the products of catabolism. It is thought to aid in restoring function/production to the extracellular matrix (ECM), using growth and transcription factors or signaling molecules, for example, insulin-like growth factor I (IGF-I), parathyroid hormone-related peptide, Indian Hedgehog, SOX factors, etc. Whereas the inhibition of catabolic pathways uses inhibitors of matrix-degrading enzymes, inflammatory cytokines, as well as that of chondroprotective cytokines like IL-4 and IL-10.

Caution needs to be taken when trying to implement the use of rAAV vectors in humans as a large proportion of the population have antibodies against AAV, which would greatly hinder its therapeutic efficacy. However, most of these antibodies are against the serotype AAV2 [34]. With several different serotypes, often therapeutic strategies aim to engineer variants to generate vectors with improved tissue specificity and transduction efficiency, while also avoiding the effects of preexisting neutralizing antibodies [35].

2.1.1. Using rAAV to treat bone regeneration

Bone loss occurs in a wide spectrum of inflammatory diseases including rheumatoid arthritis (RA), coeliac disease, Crohn's disease, asthma, psoriatic arthritis, nephritis and myositis [36, 37]. Bone loss and associated sequelae greatly reduce the quality of life of many patients. Bone remodeling/regeneration is a dynamic and highly complex process involving a delicate interplay between osteoclasts and osteoblasts. Each year, our bodies regenerate about a quarter of trabecular and 3% of cortical bone [38].

Several studies have shown the ability of rAAV vectors to efficiently express bone morphogenic proteins into myoblast C2C12. Skeletal myoblasts, fibroblasts, and bone marrow-derived cells are pluripotent and can be stimulated with various BMPs (or other factors) to become osteoblast lineage cells [39–41]. These studies even showed relatively good success *in vivo*, where new bone formation was detected in rats between 3 and 8 weeks post injection [41]. More recently, rAAV was also examined to repair bone in a cranioplasty model [42]. Calvarial autografts and allografts were coated with 10^9 particles/mm² of rAAV2 vector expressing BMP-2 and transplanted into osteocalcin/luciferase (Oc/Luc) transgenic female mice. Microcomputed tomography (μ CT) was used to measure the extent of bone formation, and findings showed that rAAV allografts resulted in significantly better bone repair. Furthermore, histological analysis also showed a variety of bone cells, as well as revitalization factors present in the grafts strengthening the conclusions of significant bone growth. However, the mechanisms involved in this AAV bone repair system still need to be elucidated.

Other studies have focused on expressing vascular endothelial growth factor (VEGF), receptor activator of nuclear factor κ B ligand (RANKL), and constitutively active form of the activin receptor-like kinase-2 (caALK2) in rAAV vectors. Koefoed et al. also used AAV-coated allografts in a murine femur model [43]. This model is fairly popular where a mid-diaphyseal femoral segment is removed and replaced by an autograft, isograft, or allograft, which is secured by an intramedullary pin. In this report, authors used a frozen allograft that was coated on the

cortical surface with 5×10^7 particles of rAAV, expressing caALK2. caALK2 can potently induce mesenchymal cell differentiation *in vitro* and *in vivo*, and its signals cannot be blocked by noggin or chordin, endogenous BMP antagonists. The results showed endochondral bone formation on the allograft. Interestingly, this procedure also prevented the formation of fibrotic tissue around the allograft, promoted blood vessel ingrowth, live bone marrow within the allograft, and stimulated osteoclastogenesis.

The group that opted to use rAAV expressing VEGF and RANKL did so because studies have shown that these factors significantly decrease during allograft healing [44]. Structural musculoskeletal grafts (i.e., bone, ligament), unlike other grafts, are often derived from allogenic cadavers. However, a significant drawback is that these transplants lack viability due to the absence of vascularization. This study aimed to examine that this rAAV could stimulate allograft vascularization and remodeling. The overarching hypothesis is that resorption of the graft through angiogenesis and osteoclast formation/activation leading to bone remodeling is a superior method to improve graft incorporation. VEGF/RANKL is known to regulate angiogenesis [45] and bone resorption [46] during skeletal repair. VEGF is secreted by hypertrophic chondrocytes and the perichondrium thereby recruiting endothelial cells and favor vascularization [47]. The data showed that if you block RANKL and VEGF signaling, there is indeed diminished bone formation on the autograft. A gain-of-function assay was also performed. RANKL and VEGF are sufficient to significantly improve healing by leading to a live, vascularized, remodeling.

Despite these positive results, more work is needed before this method can be used in humans. The connectivity between new and old bone needs to be ameliorated. In addition, technology allowing large animal, *in vivo*, 3D imaging of new bone formation and vascular ingrowth of allografts needs to be developed and biomechanical properties of rAAV-coated allografts must be determined and correlated with micro-CT parameters.

2.1.2. Using rAAV for cartilage repair

Cartilage is formed of connective tissue and found in many parts of the body, including joints. It is composed of chondrocytes surrounded by extracellular matrix, which contains glycoproteins, glycoaminoglycans, and structural and functional proteins. Articular cartilage is strong and flexible and protects the bones where they articulate to insure smooth movement and also absorbs shocks during weight-bearing activities. People with cartilage damage suffer from stiffness, pain, and swelling. Strategies for cartilage regeneration aim at modifying a variety of target cells including chondrocytes, synovial lining, osteocytes, meniscal fibrochondrocytes, tendon/ligament cells, muscle cells, and progenitor cells that may differentiate to form joint tissues [6]. Many rAAVs have been designed to target these cells.

Several papers have reported the ability to modulate cartilage both *in vitro* and *in vivo*. These studies aimed at over-expressing a variety of molecules like insulin-like growth factor-I (IGF-I), transforming growth factor- β (TGF β), SOX-9, fibroblast growth factor-2 (FGF-2), antioxidant protein heme oxygenase-1 (HO-1), CTLA4-FasL fusion gene, bone morphogenetic protein-7 (BMP-7), dominant negative to Ikappa β kinase β (IKK β dn), interleukin 38 (IL-38), interleukin-1-receptor antagonist (IL-1Ra), and osteoprotegerin (OPG) [32, 48–57]. These molecules can

act on a plethora of functions, including enhancing cartilage anabolism (IGF-1, FGF-2, TGF β , BMP-7), stimulating cartilage formation (SOX-9), exhibiting anti-inflammatory properties (CTLA4-FasL, HO-1, IKK β dn, IL-38, IL-1Ra), reducing oxidative stresses shown to exist in certain forms of arthritis (HO-1), and by blocking osteoclastogenesis (OPG). One paper examined using cystatin C (cysC) to inhibit cathepsin activity in the synovium of rabbit model of osteoarthritis. Unfortunately, this approach was unsuccessful. Despite completely blocking cathepsin activity in the synovium, synovitis, bone sclerosis and cartilage degradation remained [58].

Due to the large scope of these studies, for this review, we will summarize some of the main findings of rAAV and chondrocytes. The first attempts to transduce chondrocytes were in 2000. One group transduced primary human chondrocytes as well as human cartilage organ cultures with a rAAV-GFP. Their results were encouraging with GFP expression seen in more than 90% of monocultures after 7 days and over 45% of the cells in the organ cultures fluoresced for up to 28 days [59]. Around that same time, another group was looking at the ability of rAAV to be used *in vivo*. They used a rAAV-expressing bacterial beta-galactosidase (beta-gal) gene in an arthritis mouse model overexpressing tumor necrosis factor-alpha (hTNFalpha-Tg).

Another group also looking at transduction of a variety of primary human cells including tissues of mesenchymal, endodermal, neuroectodermal origin, and cartilage showed very different results. Chondrocytes appeared to have the lowest transduction rates along with dermal papilla follicles, epithelial cells, and fibroblasts. Transduction levels between 4.3 and 19.5% were seen [60]. Only melanocytes, G-CSF mobilized CD34+ and CD19+ cells fared worse, with no visible transduction seen.

Using genes, which are responsible for producing growth factors or molecules involved in cartilage repair, is a preferred method for viral therapy. Fibroblast growth factor-2 (FGF-2) is a member of the multifunctional fibroblast growth factor family and has broad mitogenic and angiogenic activities. One study examined whether rAAV is capable of delivering a functional FGF-2 gene cassette to isolated articular chondrocytes and to sites of articular cartilage damage *in vitro* and *in vivo* [61]. After encouraging results *in vitro*, the authors applied rAAV-hFGF-2 to osteochondral defects created in the patellar groove of knee joints in rabbits. Repair was seen at day 10 post infection and by day 20, the initial repair had progressed further, and integration into surrounding cartilage was seen. A follow-up at 4 months showed that the "new" cartilage now closely resembled the original tissue, but margins of new cartilage were barely visible. Results were even more encouraging as there were no apparent secondary effects such as synovitis or adverse reactions. Further histological analysis showed the absence of infiltrating cells at all time points observed. Earlier studies showed that rAAV could also transduce bone marrow-derived mesenchymal stem cells that migrate to injury sites [62]. Therefore, the mechanism of action is thought to be on two fronts: (1) rAAV can stimulate long-term FGF-2 transduction in damaged areas of cartilage, as well as in chondrocytes found in surrounding healthy areas and (2) rAAV also transduces the mesenchymal stem cells that will be recruited to damage areas and commence tissue repair. In 2008, *in vitro* studies using a combination of FGF-2 and SOX-9, a transcription factor that activates the expression of major cartilage matrix components, were also undertaken [53]. The premise behind this is that due to the complex nature of osteoarthritis and the plethora of processes involved in this pathology, efficient cartilage

repair may require expression of several therapeutic factors. Toward this, 3D cultures and cartilage explants were used. rAAV-FGF-2 showed greater transduction efficiency and effective expression of FGF-2. While the addition of SOX-9 was equally efficient, it did not add to the overall effectiveness of the expression. The authors did not test but did suggest that repeated administration of the combination might improve the outcomes of cartilage repair over time.

The most recent report in the literature examined the use of polymer micelles in aiding rAAV as gene therapy. The polymer micelles enhanced the stability and bioactivity of rAAV, leading to higher levels of transgene expression in human OA chondrocytes *in vitro*. It was also found to aid in human osteochondral defect cultures to mimic a more natural environment. In addition, the micelles protected the viral vector against neutralization of the viral capsid. No detrimental effect on cell viability was observed when delivering rAAV/micelles to the cells at any time point of the analysis.

An investigation looking at the use of adjuvants for *in vivo* rAAV articular cartilage gene therapy has also been done. One group showed that light-activated gene transduction (LAGT) could be one such method. UV light accelerates the formation of the double-stranded transducing rAAV vector episome by activation of a host DNA polymerase. The use of UV exposure at doses of up to 200 J/m² actively increases transduction efficiency and expression of the transduced gene eGFP in cultures of immortalized and primary human articular chondrocytes, as well as articular cartilage explants. Importantly, this amount of light was noted as insufficient to cause harm to cells [63]. A follow-up study looking at the ability of UV light-activated gene transduction (LAGT) in chondrocytes *in vivo* showed that in rabbit chondrocyte cultures, as well as in intra-articular transduction of rabbit knees, LAGT treatment resulted in higher efficiencies compared to nonirradiated samples [64]. However, after 3 weeks, the mean fluorescence intensity of positive cells of the non-LAGT group had increased to the same level as the LAGT group, despite the proportion of transgene-expressing chondrocytes were still higher in the LAGT group. Overall results showed that LAGT probably does not benefit healthy cartilage. However, in diseased tissue, more chondrocytes were transduced in general and especially those close to the irradiated surface respond to the treatment. Importantly, further investigation needs to be done to assess if the biological effect is sufficient to provide a desired metabolic response toward repair.

Despite being a promising avenue for gene therapy, consistency among findings and systems using rAAV appears to be difficult and unpredictable. Further experimentation and stringent conditions will need to be done to establish if this treatment strategy is a viable and promising avenue to promote cartilage restoration.

2.2. Recombinant adenovirus vectors (rAdV)

Adenoviruses (AdV) are medium-sized nonenveloped viruses, composed of a nucleocapsid and a double-stranded, linear DNA genome of approximately 36 kb. Over 50 different human serotypes can be found and they cause 5–10% of all childhood upper respiratory infections. Adults can also suffer from illness caused by adenoviruses, but disease is generally mild and resembles that of a common cold.

AdV are interesting because they can infect a broad range of human cells and tend to yield high levels of gene transfer compared to levels achieved with other currently available vectors. This also includes high *in vitro* gene transfer efficiencies in chondrocytes and mesenchymal stem cells [65, 66]. These viruses can accommodate large genomic insertions up to 14 kb and have the ability to transduce these genes in both proliferating and quiescent cells. At least three regions of the viral genome can accept insertions or substitutions of DNA to generate therapeutic vectors. Also, the viral genome is relatively stable and undergoes limited rearrangements and inserted foreign genes are very well maintained through successive rounds of viral replication. Genetic manipulation of these vectors is easy by using standard recombinant DNA techniques, and they are easily grown, reaching titers of up to high up to 10^{13} particles/ml. Taken together, these factors make adenoviruses excellent candidates for viral gene therapy.

Human serotypes AdV2 and AdV5 from group C are the classic adenoviruses used as therapeutic vectors. Early versions of adenovirus vectors were unsuccessful due to the deletion of E1 region to accommodate the therapeutic transgene and to prohibit viral replication [67]. This deletion led to a strong innate immune response followed by adaptive responses, which destroyed the transduced cells, thereby defeating the purpose of gene therapy. Second generation vectors were generated by deleting several areas of the genome and allowing a larger amount of DNA to be inserted. Unfortunately, these vectors still triggered immunogenicity and led to cell death. Third generation vectors were known as “guttled” vectors. All viral coding regions were removed to prevent an immunological trigger. However, they need a helper vector that codes for the viral genome to allow for replication. These third-generation vectors facilitate insertion of up to 35 kb of genetic material and are therefore deemed high capacity. Gutless AdV have been delivered to different tissues in rodents, dogs, and nonhuman primates. These third-generation vectors have been shown to be nonimmunogenic for the life of a mouse, whereas the first generation induced a response within 3 months [68].

Along with rAAV, AdV is a very popular choice for gene therapy delivery. Much work has been done in a variety of fields including cancer, metabolic diseases, motoneuronal injuries/diseases, and cerebrovascular diseases. One of the first reports in the early 1990s examined the ability of AdV vectors to be useful tools in overexpressing anti-inflammatory molecules in rabbit synoviocytes to alleviate rheumatoid arthritis. Synoviocytes were chosen due to the ease of access via the intra-articular space and their longevity (type A, macrophage-like synoviocytes are estimated to live for 3–6 months) making them ideal candidates for viral transduction [69]. This study showed the ability of rAdV vectors to express lacZ via different techniques including *in situ* staining, immunohistochemistry, and transmission electron microscopy. The transduction remained detectable for over 8 weeks; however, efficiency did wane over time. Clinically, the rabbits fared well with no signs of arthritis, synovitis, or adverse effects for up to 8 weeks post-transduction, despite having preexisting antibodies to either human or rabbit adenoviruses. The authors were unable to identify exactly to which one the animals were previously exposed to, human or rabbit adenoviruses, since antibodies against rAdV are crossreactive against many species including humans, rabbits, and cattle.

A follow-up study by the same group looked at replacing lacZ expression with that of human interleukin-1 receptor antagonist protein (IL-1ra) [70]. IL-1 is an important mediator of

inflammation and plays an important role in the pathogenesis of rheumatoid arthritis. IL-1ra is a natural receptor antagonist that competes with IL-1 for binding to type I IL-1 receptors and as a result blocks the effects of IL-1 [71]. Again, authors used New Zealand white rabbits as an *in vivo* model. After verifying *in vitro* that the expression of IL-1ra is biologically active, they found that direct intra-articular injection of rAdV into the synovium of rabbits led to the expression of high levels of IL-1ra within 1 week, as determined by Southern blot. However, like in their previous work, within 4 weeks, the levels of IL-1ra expression within synovio-cytes decreased a major limitation to the approach. However, it is noteworthy to mention that these studies were undertaken using first generation vectors, which as mentioned above, are associated with major drawbacks.

Since the first studies in the 1990s, a plethora of publications regarding AdV have been published. Similar to rAAV, the focus of these studies seems to be targeted mainly on either bone or cartilage repair. Many studies have been interested in using rAdV to transduce bone morphogenic proteins including BMP-2, BMP-4, BMP-7, and BMP-13. In addition, soluble growth factors like PDGF, FGF and IGF, anabolic factors like growth factor and PTH, systemic angiogenic factors like VEGF as well as transcription factors associated with bone- and cartilage-related gene expression like Runx2, SOX9, osterix, and extracellular matrix molecules associated with induction or repression of mineralization like Gla protein, osteopontin, and bone sialoprotein.

2.2.1. Using rAdV to treat bone regeneration

Most of the studies using rAdV focus on the transduction of the various BMPs. One study by an American group based in Chicago investigated the feasibility of using a recombinant AdV to express 14 different bone morphogenic proteins (BMPs) [72]. It is known that bone demineralization can induce *de novo* synthesis of bone formation [73]. BMPs have been demonstrated to be the factors involved in bone regeneration. They belong to the TGF β superfamily and are important in embryogenesis as well as in bone modeling. There are at least 15 different BMPs in humans, and this study attempted to establish which BMPs were the most effective at bone regeneration. The authors first examined the ability of the rAdVs to express ALP (an osteogenic marker) in the C2C12 cell line that is a precursor of osteoblasts. Four days after transduction, five BMPs were able to express ALP. These were BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9. Findings were similar when looking *in vivo* at athymic nude mice. AdV were used to transduce C2C12 *in vitro*, and cells were then injected into the quadriceps muscle. Ossification was seen in animals that received AdBMP-2, 6, 7, and 9. However, BMP-7 was less robust than the other BMPs, and interestingly, BMP-6 and 9 were the most efficient. Since this study, numerous others have investigated the use of these bone-regenerating BMPs for *in vitro*, *in vivo*, and clinical studies [74].

Two studies of interest showed the ability of AdBMP-7 and AdBMP-2 to form bone intramuscularly and subdermally in immunocompetent rodents. A key factor in the success of these studies was to reduce immune responses to the adenoviral vector. Strong immune responses can decrease or inhibit therapeutic transgene expression. It was found that when the vector is delivered in conjunction with a collagen carrier, the vector becomes more effective in decreasing

immunogenicity [75, 76]. Another method of prolonging transgene expression is by administering anti-T cell receptor monoclonal antibody following adenovirus-mediated *in vivo* gene transfer [77].

One of the most recent publications examined the effect of AdBMP-2 on the osteogenic ability of human mesenchymal stem cells (hMSCs) [78]. MSCs are multipotent somatic stem cells that are able to differentiate into a variety of cell types, including chondrocytes, myocytes, osteoblasts, and adipocytes. Targeting these cells with BMP-2 could potentially lead to their osteogenic differentiation and promote bone healing. *In vitro* experiments showed that when treated with AdBMP-2, hMSCs change phenotypes and resemble osteoblast-like cells. Further analysis showed that these changed cells also expressed ALP, an enzyme present in osteoblasts and critical for bone mineralization and calcification. Immunohistochemistry using a von Kossa stain (used for the quantification of mineralization in cell culture and tissue sections) showed increased positive staining at d14 post-treatment. Taken together, this study showed the potential of AdBMP-2 to skew the differentiation of hMSCs toward osteoblast-like cells, thereby potentially becoming a novel treatment for delayed or nonunion fractures.

In addition to BMPs, several other factors have been investigated to determine if their expression via an adenoviral vector leads to bone healing. Nell-1 is a novel direct transcriptional target of runt homology domain transcription factor-2 (Runx2). Nel-like molecule-1 (Nell-1) is osteoinductive on cells of the osteochondral lineage. Adenovirus vectors containing Nell-1 was shown to promote osteoblastic differentiation in calvarial cells (from the skull cap) [79]. An *in vivo* study demonstrated that Null-1 could be as efficient as BMP-2, one of the most potent BMPs, to induce rat calvarial bone formation [80]. VEGF, Sox9, Core binding factor alpha 1 (Cbfa1), Runx2, and noggin have all been investigated with varying degrees of success.

2.2.2. Using rAdV to treat cartilage regeneration

A recent publication showed that AdBMP-2 stimulates chondrogenesis of equine synovial membrane-derived progenitor cells. Chondrogenesis was determined by the up-regulation of collagen II, X and aggrecan, as well as the secretion of sulfated glycosaminoglycans and production of alkaline phosphatase [81]. Two other growth factors, Insulin-like growth factor-I (IGF1) and human growth and differentiation factor-5 (GDF-5), have also been examined for cartilage regeneration using rAdVs. IGF-1 is the major anabolic mediator for articular cartilage and plays an important role in maintaining cartilage homeostasis. IGF-1 enhances cartilage matrix metabolism by increasing the production of aggrecan, hyaluronan, and proteoglycan link protein-1 and by preventing degradation of proteoglycans. It also protects cartilage from the harmful effects of interleukin-1 or TNF following assault or injury. In one study, the ability of adenovirus vector encoding equine IGF-1 (AdIGF-1) to heal cartilage in an equine femoropatellar joint model was examined [82]. Then, 2×10^7 AdIGF-1-modified chondrocytes were injected into the joint and the animals were monitored for repair over the course of 8 months. The results showed that the AdIGF-1-modified chondrocytes were able to induce high levels of IGF-1, which persists for up to 9 weeks post-transplant. The increase in IGF-1 also led to an increase in collagen II expression. Histological analysis of tissue repair showed significant amelioration over control joints. Furthermore, no difference in inflammation was

seen between naive chondrocyte-implanted or AdIGF-1-transduced repair tissues. These data were determined by examining inflammatory markers (including MMP-1, MMP-3, MMP-13, and aggrecanase-1) by qPCR. In addition, it was shown that IGF-1-enhanced repair also involved an increase in tissue thickness. It appears that there was a greater defect filling, and upon examination, these cells morphologically resembled chondrocytes rather than a fibrocartilaginous-like phenotype seen within the control tissues. Another study in humans looked at the effects of AdV gene transduction FGF-2, FGF-2 combined with interleukin-1 receptor antagonist protein (IL-1Ra), and/or insulin-like growth factor-1 (IGF-1). This was determined in both human osteoarthritis (OA) chondrocytes as well as in a leporine OA model [83]. FGF-2 expression protected human OA chondrocytes and decreased cartilage degradation *in vivo* (rabbit model). *In vitro*, FGF-2 induced collagen type II and an increased production of GAG. Furthermore, combining all three factors FGF-2, IL-1Ra, and IGF-1 leads to significantly lower levels of ADAMTS-5, MMP-13, and MMP-3, and increased amounts of TIMP-1. This was also true as seen in the rabbit model. The combined therapy seems to have a synergistic effect to achieve optimal results. The trigene expression system appears to promote GAG synthesis of chondrocyte, increases TIMP-1 expression, and reduces ADAMTS-5, MMP-13 and aggrecanase expression. Haupt et al. also found that an adenovirus-mediated gene therapy combining several factors was more efficient. In this study, IGF-1 and IL-1Ra were shown to promote the healing of cartilage injury in degenerative joint diseases, suggesting combination therapy could be beneficial for cartilage repair in degenerative joint diseases [84].

GDF-5 has been shown to be essential for normal appendicular skeletal and joint development in humans and mice. It positively regulates differentiation of chondrogenic tissue through its binding with bone morphogenetic protein receptor type 1 A and B (BMPRI1A and BMPRI1B). It also negatively regulates chondrogenic differentiation through its interaction with noggin (NOG). One study conducted by Luo et al. investigated the effects of adenovirus-mediated GDF-5 (AdGDF-5) on ECM expression in human degenerative disc nucleus pulposus (NP) cells in order to determine if AdGDF-5 is a viable therapy to treat intervertebral disc degeneration (IDD) [85]. Like many other studies, they began by determining the expression of GDF-5 *in vitro* after treating HEK293 cells with AdGDF-5 and then determined the optimal amount of viral vector needed for efficient transduction of NP cells. Following this, they investigated the effects of expression of GDF-5 had on the ECM. It was noted that GDF-5 promotes the synthesis of sulfated glycoaminoglycans and hydroxyproline, two major structures forming the ECM network. In addition, immunohistochemistry showed an increase in proteoglycans in the AdGDF-5-treated NP cells, stimulated NP proliferation, and increased the expression of collagen II and aggrecan genes. The outcome of this study indicates that NP cells within degraded discs would be ideal targets for the transduction of transgenic proteins and that AdV therapy could be a promising new avenue for the treatment of disc degeneration.

As like for rAAV, the effects of Sox9 on MSCs have been examined as a novel treatment of cartilage repair. This is of no surprise, considering that Sox9 is considered a master regulator of chondrocyte phenotype [86]. Like that of rAAV, Sox9 has been shown to be able to modulate cartilage both *in vitro* and *in vivo*. In the study led by Cao et al., Sox9 expression successfully promoted a chondrocyte morphology after AdV transduction of rabbit bone marrow mesenchymal stem cells (BMSCs) [87]. Overexpression of Sox9 resulted in the upregulation

of collagen II and aggrecan, while inhibiting osteogenic differentiation. The latter was shown by a decrease in ALP staining and reduced expression of Runx2, Col I, and osteopontin. In rabbits, the AdVSox9 group had a better outcome regarding cartilage repair. This was seen by integration of *de novo* cartilage tissue repair, cells in the repaired tissue had distinctive morphology resembling chondrocytes that were surrounded by matrix that stained positive for safranin O and type II collagen. Finally, overexpression of Sox9 led to suppressing makers of hypertrophic chondrocytes (ColX and osteocalcin), thereby avoiding cartilage calcification.

In summary, like for rAAVs, rAdVs show a promising future for gene therapy to treat, or limit, joint damage. They have the advantage of growing to high titers, allowing high transduction efficiencies in a variety of cells and have shown promise in animal experiments as well as in explants. However, the main drawbacks for AdVs remain a long-term efficiency and overall safety. Prior exposure to various strains results in robust host immune responses against the vectors, greatly hindering long-term transgene expression in targeted patients. Moreover, the first patient death associated with gene therapy trials was that of an 18-year-old boy receiving a rAdV [88]. This vector contained ornithine transcarbamoylase (OTC), an enzyme needed to eliminate ammonia, and essential to treat the patient's partial OTC deficiency, which was present since birth. Unfortunately, the boy died 4 days after receiving the infusion and this adverse effect sparked controversy and ended in a lawsuit and formal investigation. Despite being the only death in nearly 4000 gene-therapy patients (over 400 trials), this hindered progress and saw extra measures for monitoring, reporting, and obtaining informed consent. The FDA and participants will probably still err on the side of caution when it comes to these types of clinical trials.

2.3. Retroviruses and lentiviral vectors

Lentiviral vectors are members of the *Retroviridae* family. These vectors can deliver a substantial amount of genetic information by spontaneously penetrating the intact nuclear membrane and inserting the "carried" DNA into the host's DNA. Due to this unique property, they are among the most efficient methods for gene delivery. Furthermore, they can integrate into either actively replicating or quiescent cells. For these reasons, they are commonly used for *in vivo* delivery of genome editing therapies. However, this ability to integrate into the host's DNA also raises a number of safety and ethical concerns. Another drawback of this class of vector is the possibility to activate tumor genes and to provoke insertional mutagenesis events upon integration. Examples of most frequently used lentiviruses include human, simian, and feline immunodeficiency viruses (HIV, SIV, and FIV).

Compared to other forms of viral gene therapy, the main advantages of using lentiviruses include low or absence of preexisting immunity, ability to transport one or more transgenes, delivery of genetic material to replicating and nonreplicating cells, as well as prolonged transgene expression (upward of 6 months). In order to make a lentivirus vector, a split component system is needed, where each part is in itself nonpathogenic and only the sum of its parts can actively infect cells. Target cells are usually transfected with the viral vector, which is flanked with long terminal repeats (LTRs). It is this feature that allows the carried transgene to integrate into the genome of the target cell. The vector could also contain the Rev-responsive element (RRE) for most efficient vector production and, of course, the gene of interest. In parallel, a

plasmid containing gag and pol structural genes are needed to supply reverse transcriptase and integration functions for the therapeutic vector particles. Finally, the last part is composed of plasmids encoding envelope proteins for the therapeutic viral particles and perhaps Rev. protein. Typically, envelope gene used is that of the glycoprotein G from vesicular stomatitis virus (VSV-G). The addition of this foreign viral envelope is called pseudotyping, and it alters the viral tropism to specifically target certain cell types.

Retroviruses and lentiviruses have been used to transfer genetic material since the 1980s. In the early 1990s, γ -retrovirus gene transfer was shown to be possible in hematopoietic stem cells [89]. This era also saw the first clinical trial that aimed at treating severe combined immunodeficiency (SCID) [90]. A major accomplishment in this field happened in the early 2000s, when 11 children were successfully treated for X-SCID by introducing the common interleukin receptor γ -chain in bone marrow using a retrovirus vector based on mouse leukemia virus (MLV) [91].

2.3.1. Using lentiviruses for joint repair

One of the first reports of using a lentivirus for the treatment of joints occurred in 2008. Ricchetti et al. overexpressed IL-10 in the patellar tendons of mice. IL-10 is known for its potent anti-inflammatory properties that limit host response to pathogens, but also can inhibit scar formation in fetal wound healing. In this study, a murine model of patellar tendon injury was used to investigate the effect of IL-10 overexpression on the properties of adult healing tendon. Findings showed successful transfer of IL-10 into patellar tendons with more than six times greater expression in comparison with endogenous IL-10 levels. IL-10 expression peaked at 10 days after injury. Furthermore, treated tendons showed improved maximum stress and percent relaxation was increased in the treated group. However, there were significant limitations regarding the study. The empty vector control also showed improved tendon properties compared to the sham control group, which could indicate that injection of the vector itself, rather than IL-10, as a beneficial effect. The authors hypothesize that injection of the viral vector may actually lead to more robust immune responses that subsequently drive better scar formation and wound healing.

2.3.2. Lentiviruses toward cartilage regeneration

Many attempts have been made to use retroviruses and lentiviruses for a long-term transgene expression in chondrocytes. Toward this, many different animal cells have been used, including human, rat, rabbit, goat, and cattle [92–95]. One group showed that transduction of chondrocytes with GFP was associated with an approximate 60% success rate [92]. After 6 weeks, only 21% of the cells remained GFP positive, whereas other studies showed greater efficiency rates with up to 85% of osteoarthritic chondrocytes being transduced [94]. Human articular chondrocytes have been shown to be highly susceptible to lentiviral infection, with 74% being GFP positive and expression was maintained *in vitro* for up to 22 weeks [93].

Like for the other viral vectors described in this chapter, studies have focused on inserting factors, which could help cartilage or bone repair, either by incorporating molecules stimulating the ECM, chondrogenesis, or immunomodulatory molecules. One such study examined the

possibility of expressing a member of the nuclear factor of activated T-cells (NFAT) as a means to treat osteoarthritis [96]. NFAT was initially identified as a regulator of gene transcription in response to T-cell receptor-mediated signals in lymphocytes. However, it is also involved in regulating bone formation and osteoclastic bone resorption [97, 98]. Interestingly, NFAT knockout mice have normal skeletal development, but with age, display loss of type II collagen, and aggrecan. They also show overexpression of specific matrix-degrading proteinases including MMPs and ADAMTS in addition to proinflammatory cytokines. The authors then used a lentiviral vector to express NFAT1 in cultured primary *Nfat1*^{-/-} articular chondrocytes. This rescue of NFAT partially or completely rescued the abnormal catabolic and anabolic activities of *Nfat1*^{-/-} articular chondrocytes.

Another study looked at using the lentivirus vector to knock down aggrecanase activity [99]. RNAi was used to specifically target both aggrecanase-1 and -2 in primary rat chondrocytes. This approach was relatively successful *in vitro* with increased amounts of glycosaminoglycans and total collagen being produced as well as an increase in chondrocyte proliferation. This data provided the proof-of-principle that it is feasible to use this vector system to modulate chondrocyte phenotype and may be useful for future studies.

Several reports examined the ability of lentivirus vectors to be used to target MSCs in order to ameliorate the ECM surrounding the joints. One interesting example is the use of these vectors to help create a bioactive scaffold where sustained transgene expression and ECM formation are accomplished by human MSCs (hMSCs) [100]. The lentivirus vectors were used to express transforming growth factor β 3 (TGF- β 3) under the control of a constitutive EF-1 α promoter. TGF- β 3 was chosen as it was previously shown to be the most potent driver for chondrogenesis in hMSCs. After transduction, hMSCs developed a spherical shape comparable to chondrocyte-like morphology. Also, there was a substantial increase in col. II and glycosaminoglycan. Bioactive scaffolds with immobilized TGF- β 3 expressed in lentivirus vectors showed a production of 17 ng/mL TGF- β 3 and 12.87 μ g sGAG/ μ g DNA at 1–3 weeks after seeding scaffolds. The results of this study indicate that the scaffold-mediated transduction technique could eventually be used *in vivo* to direct cell lineage commitment and ECM development in a controlled and persistent manner. The field of bioengineering is rapidly growing and the possibility of creating alternative methods for tissue replacement is not so far away.

One of the most recent publications examining the use of lentiviruses for cartilage repair used ovine perivascular stem cells (oPSCs). These cells are said to be natural ancestors of mesenchymal stem cells. The goal of this study was to develop an autologous large animal model for PSC transplantation and determine if implanted cells are retained in articular cartilage defects. oPSCs could be sourced from various locations including bone marrow, subcutaneous fat, and the infrapatellar fat pad. The lentivirus was used to transduce the cells with eGFP to allow tracking when implanted into the animals. The transduced cells were implanted into articular cartilage defects on the medial femoral condyle using hydrogel and collagen membranes. Results showed that GFP-emitting cells could be found at the base of the articular cartilage defect up to 4 weeks after transplantation. However, no repair tissue was seen by immunohistochemistry. Overall, more work needs to be done for this model to be a robust example of cartilage repair, but it could be an alternative replacement to the current canine model.

Despite some promising results, the use of lentiviruses will probably always raise concerns about safety due to the ability to integrate into the host genome. Clinical trials will be challenging due to the unknown risks associated with their administration. Thorough justification for their use will be warranted especially with so many other types of viral vectors currently available, although it is possible to see successful joint repair using such a system.

Overall, this chapter examined some of the most recent literature surrounding the use of viral vectors for bone and cartilage repair. This is a vast field with many exciting studies and promising developments. There has been a huge amount of progress since the early development of viral gene therapy, and it is only a matter of time before joint disorders and injuries will be treated using these approaches.

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