REVIEW ARTICLE

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The use of recombinant adeno-associated virus for skeletal gene therapy

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Dates:
Accepted 24 December 2006

To cite this article:
Dai J, Rabie ABM:
The use of recombinant adeno-associated virus for skeletal gene therapy
Orthod Craniofacial Res 10, 2007; 1–14

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Structured Abstract

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Objectives – To provide a comprehensive literature review describing recent developments of the recombinant adeno-associated virus (rAAV) vector and exploring the therapeutic application of rAAV for bone defects, cartilage lesions and rheumatoid arthritis.

Design – Narrative review.

Result – The review outlines the serotypes and genome of AAV, integration and life cycle of the rAAV vectors, the immune response and regulating system for AAV gene therapy. Furthermore, the advancements of rAAV gene therapy for bone growth together with cartilage repair are summarized.

Conclusion – Recombinant adeno-associated virus vector is perceived to be one of the most promising vector systems for bone and cartilage gene therapy approaches and further investigations need to be carried out for craniofacial research.

Key words: adeno-associated virus; cartilage; gene therapy; mandible; rheumatoid arthritis; temporomandibular joint

Abbreviations: bGHpA, bovine growth hormone polyadenylation signal sequence; BMP2, bone morphogenetic protein; FGFR1, fibroblast growth factor receptor-1; HSPG, heparan sulfate proteoglycan; MPCs, mesenchymal progenitor cells; PDGFR-α, platelet-derived growth factor receptor; RA, rheumatoid arthritis; rAAV, recombinant adeno-associated virus; TGFβ1, transforming growth factor; TMJ, temporomandibular joint; WPRE, woodchuck hepatitis B virus post-transcriptional regulatory element.

Introduction

Gene therapy is a technique to deliver small DNA or RNA sequences to cells or tissues to correct a genetic defect or treat a disease. Currently, the majority of gene therapies focus on adding genetic information to cells rather than alteration of the genome (1). Gene transfer can be completed *in vivo* or *ex vivo*, and both approaches have been examined for bone regeneration. During *in vivo* gene transfer, a vector carrying the therapeutic DNA is directly implanted or injected into the patient. During *ex vivo* gene transfer, a patient's cells are harvested, expanded using cell culture techniques, and transfected *in vitro* prior to implantation or injection of the cells into the patient. During the past decade, gene therapy has attracted increasing interest as a novel strategy for inducing craniofacial bone formation (2).

An ideal gene transfer vector should meet a number of requirements: efficiency, safety, economy and convenience. It must be safe, i.e. it must not induce cellular toxicity or provoke an immune reaction. It must be capable of being delivered by injection, efficiently injecting target cells, and expressing the transgene product at a therapeutic level and under tight regulation for the required amount of time (3). A number of vector systems have been developed recently, both viral and non-viral, to meet these requirements in delivering therapeutic genes to bone tissues. The non-viral vector based systems involve either the physical or chemical transfer of genetic material, and are dependent on cellular transport mechanisms for uptake and expression in the host cell. They include naked DNA alone (4, 5), or DNA associated with carrier molecules (such as liposomes or a polymer matrix) (6). Non-viral vector are easy to manufacture, accept different sizes of inserted DNA on the size of the inserted DNA, and display fewer immunological and safety problems. However, in Ohashi's research (7), a naked DNA injection alone (25–50 μg) resulted in a very faint expression of transgene. This is consistent with Yavandich's findings (8) that direct intra-articular administration of 100 μg naked DNA induced a very low level of marker gene (Lac z) expression in both the rat and rabbit synovium. Therefore, the poor transduction efficiencies, transient transgene expression and non-selective cell targeting prevent them from wide clinical usages (7, 9, 10). In contrast, viral vectors, e.g. retrovirus, lentivirus, adenovirus and adeno-associated virus (AAV) are considered to be the appealing delivery vehicles as they are quite efficient, associated with higher infection efficiency, and generally provide more preclinical and clinical utility than non-viral vectors (11). They can achieve prolonged expression, and their transfection efficiency approaches 100%, dramatically exceeding the level reached by most non-viral methods (2). However, retrovirus are incapable of infecting non-dividing cells such as muscle cells and neurons, and may give rise to insertional mutagenesis (the activation of a cell proto-oncogene or the disruption of a tumor suppressor gene) (12, 13). Lentivirus, a member of retrovirus, can transduce dividing as well as non-dividing cells with a risk of random integration into host genome giving rise to insertion mutagenesis, a fact that limits its clinical application (13). The great shortcoming of adenoviral vectors is the stimulation of a significant host immune response. A T cell-mediated immune response against capsid proteins of adenovirus results in a local inflammatory reaction leading to transduced cells lysis and a shorter duration of transgene expression in immunocompetent animals and humans (14).

Since 1984, when Hermonat and Muzyczka demonstrated the AAV as a vector for the transduction of a foreign gene into a host chromosome (15), the cloning of rAAV has become routine for gene therapy study. And the improvement of *in vitro* packaging systems has been developed by constructing the AAV helper plasmid, which makes it possible to produce high-titer AAV vectors in the absence of infectious helper adenovirus (16). AAV is a favorable choice as it has several major advantages. It can efficiently infect dividing as well as non-dividing cells with a broad host range including human and murine embryonic stem cells (17), hematopoietic progenitor cells (18, 19), mesenchymal stem cells (20–23), chondrocytes (24–27), osteoblasts (28), myoblasts (13), brain cells (29), hepatic stellate cells (30), and epithelial cells (31). Delivery by rAAV vectors results in long-term expression of therapeutic genes as it persists mostly in episomal or concatameric form but not integration into host chromosomal DNA, does not result in destructive cellular immune responses against infected target cells, and has not been associated with any human disease (32–40). The positive results from proof-of-concept studies in cell culture and animal models and the accumulation of pre-clinical safety data has led to the initiation of phases I and II clinical trials of rAAV2 mediated gene therapy. The primary drawback of this vector system is the limited packaging size with a maximum capacity of 5.2 kb, which has always been thought to preclude its application for delivering larger size of transgene (41). Recently, several strategies have been developed to overcome this size limitation by exploiting the unique heterodimerization ability of AAV DNA (42). The split-gene or trans-splicing strategy has effectively increased the packaging size of rAAV vectors up to 10 kb and has been applied to factor VIII cDNA (7 kb) (43, 44). Hence, based on the characteristics of the vectors and the nature of the target tissues, rAAV-mediated gene transfer is under investigation to treat a large number of bone and cartilage diseases and is thought to be the most promising approach (45).

In this review, we will focus our discussion on the ongoing efforts made in (1) understanding the serotypes of AAV and the structure of the AAV2 genome; (2) exploring the integration and life cycle of AAV; (3) investigating the immune response and regulating system for AAV gene therapy; (4) summarizing the advancements of AAV application for bone growth, growth modification of temporomandibular joint (TMJ) together with rheumatoid arthritis (RA) treatment.

Serotypes and structure of AAV

Serotype of AAV

Adeno-associated virus is a small, non-enveloped, single-stranded linear DNA parvovirus and is the smallest known virus (46). It belongs to the dependovirus genus of the parvovirus family. The virion is icosahedral in shape and measures 20–25 nm in diameter (47). Up to now, 11 serotypes were identified and they have different intrinsic properties. The sequence homology among the different serotypes is high. Sequence comparison revealed that the greatest divergence lies in the capsid proteins (48–50) leading to differences in both tropism and serological neutralization (51). The natural host of AAV-1 is not clear at present. AAV-2, 3 and 5 were later isolated from human clinical specimens. AAV-4 was isolated from a culture of a rhesus monkey kidney cells. AAV-6 appears to be have arisen from homologous recombination between AAV-1 and 2 (52). Recently, five novel serotypes were isolated from non-human primates. AAV-7, 8 and 9 were found in rhesus monkeys (53, 54) and AAV-10 and 11 have been isolated from cynomolgus monkey (55).

A tremendous amount of study has been performed on understanding the biology of AAV2. Therefore, it has been the most widely utilized serotype and is the best characterized among all naturally discovered serotypes (56). AAV2 uses heparan sulfate proteoglycan (HSPG), a widely-expressed cell surface receptor, as a primary receptor for cell attachment (57), and it also utilizes co-receptors to assist its internalization, including the fibroblast growth factor receptor-1 (FGFR1) (58), integrin $\alpha_v\beta_5$ (59), and hepatocyte growth factor receptor (HGFR) (60). It has been demonstrated that AAV4 binds to O-linked α2-3 linked sialic acid, while AAV5 binds to N-linked α2-3 or 2-6 sialic acid (61). A co-receptor, platelet-derived growth factor receptor (PDGFR-α), has also been identified for AAV5 (62). While the cellular receptors for some AAV serotypes are still unknown. Moreover, recombinant cross-packaging of AAV genome of one serotype into other AAV serotypes has opened the possibility to optimize tissue-specific gene transduction and expression. Interest in these alternative serotypes has been driven by the fact that they exhibit different cellular tropisms and are often more efficient than AAV2 *in vivo*. For example, by injection of different serotypes via tail vein, serotype 2 preferentially transduces liver and spleen while serotype 5 is likely to infect spleen and lung in mice (63, 64). The transduction efficiencies of differentiation status of host cells also vary among various serotypes. Whereas AAV-2 transduced undifferentiated C2C12 mouse myoblasts more efficiently than differentiated ones, AAV2/10 and AAV2/11 transduced the undifferentiated myoblasts less efficiently than differentiated ones (55). These hybrid serotypes not only could achieve high efficiency of gene delivery to a specific targeted cell type, but also serve as a tool for studying AAV biology such as receptor binding, trafficking and genome delivery into the nucleus.

Structure of AAV

The AAV-2 is the most extensively studied serotype. The AAV-2 virion has a genome of 4675 bases (65). The genome of AAV contains two large open reading frames (ORFs): the 5′ (or left) ORF (*rep*) encodes the non-structural Rep proteins for viral replication and the 3′ (or right) ORF (*cap*) encodes the structural capsid proteins (66). The Rep proteins are required in all phases of the viral life cycle, including transcription, replication, encapsidation, integration, and rescue from the latent state (66). The *rep* contains four overlapping ORFs which encode four proteins of 78, 68, 52 and 40 kDa. Rep 78 and Rep 68 are involved in the DNA replication process through their interactions with the Rep-binding elements and the terminal resolution sites which are located in the ITRs. Rep 52 and Rep 40 participate in the generation and accumulation of single-stranded viral genome from the double-stranded replicative intermediates (67). The capsid proteins are crucial for rescue, replication, packaging, and integration of AAV. The *cap* encodes three capsid proteins, VP1 (90 kDa), VP2 (72 kDa) and VP3 (60 kDa), under the control of the p40 promoter (31). At each end of the

genome there are a 145-base inverted terminal repeats (ITRs) (68).

Integration and life cycle of AAV

Integrative systems

The defective replication and non-pathogenic nature of wild-type AAV triggered the rapid development of rAAV derived from AAV2. The construction of recombinant AAV vectors is based on transient triple transfection protocols of target/producer cells (such as human embryonic kidney cell line (HEK-293), which requires the following genetic elements (Fig. 1) (69):

- The plasmid with the sequence of the rAAV genome, which is derived by deleting structural genes (*cap*) and genes essential for virus replication (*rep*) and replacing them with sequences carrying a therapeutic gene. It consists of the expression cassette of the therapeutic gene (flanked by ITRs), along with an appropriate promoter, woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE), and bovine growth hormone polyadenylation signal sequence (bGHpA). The insert gene cannot exceed 4.5 kb.
- The plasmid with the sequence encoding the two AAV ORFs of *rep* and *cap*, which called pHelper (helper plasmid) for complementing the missing rAAV functions.
- The plasmid with the required helper functions encoded by the natural auxiliary virus, usually Adenovirus (E1A, E1B, E4, E2A and VA1 being provided by the HEK-293 genome) (16).

The vector plasmids and helper plasmids were co-transfected into permissive cells (usually HEK-293 cells), then packaged rAAV2 virions containing only the therapeutic vector genomes. Recombinant vector particles, thus produced and purified. The most classical and easy way remain the ultracentrifugation on a CsCl or iodixanol gradient (70). More complex techniques based on chromatography especially for rAAV2 (71, 72) are now well developed for the generation of high purity grade and up-scaled production suitable for human clinical application.

Fig. 1. The three plasmids-packaging system for rAAV vectors (see text for details).

In absence of helper virus, AAV in infected cells cannot produce any progeny virus and enters a latent state. Latent AAV genomes exist in a number of forms including site-specific integrated forms, episomal forms, and randomly integrated forms (Fig. 2). The ITRs and either *Rep78* or *Rep68* were sufficient for the replication of the AAV genome and its integration into a specific site, referred to as AAVS1, which is found at chromosome 19 (19q13.3qter) and is mapped to the first exon of myosin binding subunit 85 of protein phosphatase 1 (18, 73, 74). The rAAV vectors were deleted all the viral genes of *rep* and could not integrate in the AAVS1 locus and most of them would persist in an episomal state, which ensures that the treatment is innocuous, and that there is no risk of insertional mutations (75, 76). Although rAAV are devoid of viral genes, various approaches aiming at providing the necessary Rep function along with the rAAV have been envisioned. If Rep protein is provided trans, site-specific integration can occur (73). When a latently infected cell encounters superinfection by any of the helper viruses, the integrated AAV genome undergoes a productive lytic cycle (77). Random integration has been demonstrated in established cell lines, but it is at low frequency in primary cultures together with *in vivo* investigation (78). The major advantage of AAVS1-directed integration system lies in the theoretical reduction of random integration, with its possible adverse insertional mutagenesis. Several preliminary attempts have relied on different systems ranging from

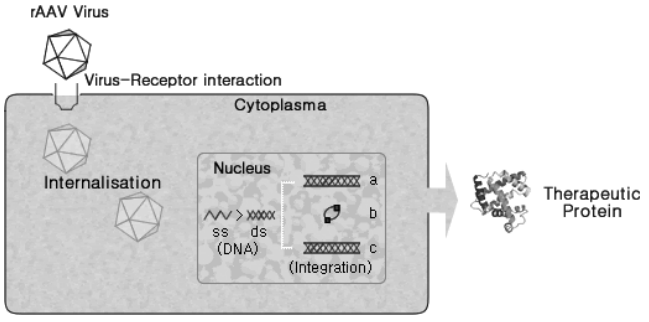

Fig. 2. Life cycle of rAAV virus. The recombinant virus first attaches to the cell surface by binding to the receptors and coreceptors, followed by internalization and intracellular trafficking. The virus then penetrates the nuclear membrane and release the vector genome from single strand DNA (ss) conversed to double strand (ds) DNA. The rAAV genomes exist in three forms in the nucleus including site-specific integration (a), episomes (b), and randomly integration (c). Finally, the infected cells secreted the therapeutic protein.

plasmid transfection to viral infection by multiple AAVs (one being the recombinant and one bearing the rep gene) or of hybrid viral systems based on chimeric Ad/AAV vector. These approaches are still in their infancy and have been essentially assessed *in vitro* (79–81). However, if such a specific integrative system is to be further developed for human application, the following safety and regulatory constraints should be considered. First, the Rep proteins should be temporarily supplied, to allow only the integration of the rAAV but not a later excision/rescue event from the integrated provirus. Second, a low level of Rep would presumably be preferable, this allows the integration pathway rather than the replication pathway to be utilized, but also because Rep proteins are cytotoxic at high level. Finally, AAVS1 is closely correlated with the slow skeletal troponin T gene (82). Although natural integration seems to have no adverse effect in humans, integration of rAAV close to this gene needs to be shown to be innocuous.

Life cycle of AAV

The life cycle of AAV has been studied in detail. At the cellular level, AAV undergoes five major steps before achieving gene expression (67) (Fig. 2): 1) binding to cellular surface receptors and then be internalized through interactions with coreceptors; 2) clathrin-mediated endocytosis, a dynamin-dependent process; 3) trafficking through the cytoplasm to the nucleus; 4) uncoating of the virus to release the vector genome; and 5) conversion of the genome from single-stranded to double-stranded DNA as a template for transcription in the nucleus. The viral genome of AAV is transported to the nucleus within minutes after infection, *in vivo* transduction takes days to weeks as a consequence of lagging second-strand synthesis (83). The cumulative efficiency with which rAAV can successfully execute each individual step, determined the overall transduction efficiency. Rate limiting steps in rAAV transduction include the absence or low abundance of required cellular surface receptors for viral attachment and internalization, inefficient endosomal escape leading to lysosomal degradation, and slow conversion of single-stranded to double-stranded DNA template (84). The circular concatamers (head-to-head and head-to-tail) are formed by means of intra- and inter-molecular recombination. Hence, rAAV vectors are mostly persist in an episomal state with low frequency of integration (85, 86).

Immune response to AAV and regulated viral expression system

Immune response

The immunologic profile of the AAV is of great importance in the evaluation of the vector system for the use in skeletal field. The host immune responses elicited by the vector may severely impair the success of a gene transfer protocol in clinical trials. Moreover, recent studies have shown that one of the disadvantages of AAV vectors is that they can induce both cellular and humoral immune responses against the transgene product (87). To understand the scope of this challenge, studies have determined the prevalence of serum antibodies in the population. These studies have defined the subset of the population with seropositive antibodies against AAV type 2 over age 50–96%, teenagers to 30% being neutralizing (88, 89). In experimental animals, neutralizing antibodies have been shown to eliminate (90) or greatly reduce (91) the levels of transgene expression of the readministered vector. This host humoral immune response directed against the capsid proteins, but not the transgene product, is responsible for the failure of successful rAAV2 readministration. Recently, Cottard *et al.* collected synovial fluid (SF) from patients with joint disease and tested the influence of SF on AAV_2 mediated gene transfer to chondrocytes. The results suggested that anti-AAV_2 IgG were identified in SF from 13 of 18 patients. Moreover, anti-AAV IgG level in SF was highly linked to the neutralizing activity. Therefore, a more efficient way to increase AAV-mediated gene transfer and bypass the immunity to AAV2-derived vectors has emerged with the development of other alternative AAV serotypes to avoid a pre-existing immune response or manipulating the virion to alter antigenic determinants.

Regulated viral expression system

A recent advancement in gene therapies for controlling and monitoring bone and cartilage regeneration is the incorporation of regulators in the gene construct. By controlling the timing, duration, and level of expression, the transgene function could be controlled *in vivo*. The regulatory system based on the use of small molecules, such as tetracycline, doxycycline or rapamycin, is the most widely used and represents a versatile system for gene therapy applications (92, 93).

This approach allows for transient expression of the therapeutic protein even though the transgene has been incorporated into the host chromosome. Gafni *et al.* constructed a dual-construct vector using rAAV-BMP2 (bone morphogenetic protein) mediated gene delivery, which was regulated by the tetracycline sensitive promoter tetON (28). Post-administration, doxycycline binds to the transactivator and the newly formed complex acquires a high affinity for the Tet-responsive element, which is located upstream of the cytomegalovirus (CMV) minimal promoter, and thus activates the transgene's promoter and upregulates BMP-2 transcription. In the absence of doxycycline, the transactivator cannot bind to the tet-responsive element and transcription of the BMP-2 gene is suppressed. Therefore, controlled bone formation can be achieved by regulating BMP-2 expression via tetracycline by using the TetON-regulated promoter.

Therapeutic application of AAV

Table 1 gives an overview of rAAV-mediated target genes on bone growth and cartilage repair. These will be discussed in the following sections.

AAV gene therapy for bone regeneration

Adeno-associated virus mediated BMP2, 4, 7 could infect and efficiently convert C2C12 cells from myoblasts into osteoblast lineage cells *in vitro* (13, 94, 95). *In vivo*, AAV-BMP2 vectors were directly injected into the hindlimb muscle of Sprague–Dawley rats. Significant heterotopic new bone formation was detected as early as 3 weeks post-injection, and BMP-2 expression could persist for at least 8 weeks in some immature regions (94). Using the same animal model, the further work demonstrated a greater osteogenic response was achieved when a lower dosage of rAAV-BMP-2 was combined with an Ad-BMP-2 vector, at a dosage low enough to avoid triggering an immune response (96). AAV-BMP-4 delivery could also successfully induce endochondral bone formation at the immediate site of the vector injection in SD rats (13). More recently, Li and colleagues compared the osteogenic potential of AAV5hBMP6 and ADhBMP6 in immunodeficient and immunocompetent rats. The obvious ectopic bone induced by AAV5-BMP6 appeared later than by AD-BMP6. In immunodeficient rats (athymic nude rats), the amounts of ectopic bone induced by AD-BMP6 (injection of 5×10^7 PFU) were significantly larger than by AAV5-BMP6 (injection of 2.3×10^{12} particles). However, the amount of bone in immunocompetent rats (SD rats) induced by AAV5-BMP6 was significantly greater than that induced by AD-BMP6 (97).

Previous study demonstrated that AAV-osteoprotegerin (OPG) gene therapy effectively reversed established osteopenia in ovariectomized mice (98). The recent study demonstrated that a single intramuscular injection of AAV-*h*OPG resulted in vividly increased serum concentrations of *h*OPG within a few days after transduction followed by consistently elevated levels of *h*OPG till 8 weeks. The increased level of OPG could significantly reduce the osteoclast number and activity in the fracture callus. AAV-OPG decreases the fracture remodelling but did not impair the enhancement in structural strength or ultimate stress of the fractures (99). It has been shown that AAV vector-mediated gene delivery of OPG to mice with micro-particle-induced osteolysis reduces both osteoclasteogenesis and bone resorption (100) and (101). A recent work by Kostenuik *et al.* (98) investigating systemic vector-related toxicity revealed that AAV-OPG has an acceptable safety profile and safety margin. It has recently been shown that ultraviolet (UV) light irradiation combined with rAAV markedly improved the protein expression of transforming growth factor-β1 (TGFβ1) from human bone marrow derived-mesenchymal stem cells (HuMSCs) (23). Kumar *et al.* (20) established the ovariectomized mouse model and demonstrated the *ex vivo* osteoporosis gene therapy with rAAV-BMP2 transduced MSCs could promote new bone formation. More recently, Ito and colleagues established an unconventional means to evaluate cortical bone healing with femoral allografts coated with freeze-dried rAAV encoding receptor activator of nuclear factor kappaB ligand (RANKL) and vascular endothelial growth factor (VEGF) (102) or receptor of activin receptor-like kinase-2 (caAlk2) (103) and identified a significant increase in bone formation. The results convinced that cell-free, rAAV-coated allografts have the potential to revitalize *in vivo* following transplantation. Therefore, AAV vector is an efficient and safe procedure to facilitate gene induction in the skeletal system, and has advantages over all the viral and non-viral vectors. However, its ability to transduce

Table 1. **Effects of rAAV-mediated target genes on bone growth and cartilage repair**

Growth factor	Animal model	Route of gene delivery	Target cell/tissue	Effects	References
BMP2	SD rats	*In vivo:* Local intramuscular injection	Myoblasts	Significant new bone could be detected as early as 3 weeks post-injection	(94)
BMP4	SD rats	*In vivo:* Local intramuscular injection	Myoblasts	Bone formation was induced	(13)
BMP-6	SD rats athymic nude rats	*In vivo:* Local intramuscular injection	–	The bone was visible earlier at day 30 in 2-month-old SD rats and 60 days in 18-month-old SD rats	(97)
OPG	Rats with tibia fractures	*In vivo:* Systemic intramuscular injection	–	The fracture remodelling was decreased, but this does not influence the structural strength of healing fractures	(99)
OPG	Ovariectomized OVX) mice	*In vivo:* Systemic injection via the portal vein	–	The treatment reversed established osteopenia in OVX mice	(98)
OPG	Murine air pouch model	*In vivo:* Local injection in the air pouch	–	It effectively protected particulate polyethylene-induced bone resorption	(101)
RANKL VEGF	C57BL/6 mice for femoral grafting	*In vivo:* the AAV vectors are freeze-dried and coated to cortical surface of allografts bone	Fibroblasts osteoblasts	The coated allografts shows marked vascularization and new bone formation around	(102)
caAlk2	C57BL/6 mice for femoral grafting	*In vivo:* the AAV vectors are immobilized to the cortical surface of the bone allografts by freeze-drying	Osteoblasts	It demonstrated a significant increase in new bone formation	(103)
FGF-2	Rabbit cartilage defect	*In vivo:* Local injection into the knee joints	Chondrocytes	It significantly improved the overall repair, filling, architecture, and cell morphology of osteochondral defects in rabbit knee joints.	(107)
bFGF	Rabbit	*In vivo:* Local injection into the knee joints	Synovial cells	Achieved a high infection efficiency with a complementary enhanced articular cartilage repair	(106)
bFGF	Rabbit cartilage defect	*Ex vivo:* The infected chondrocytes were embedded in collagen gel and transplanted into the articular cartilage defect	Chondrocytes	The infected cells were found at the transplant sites for at least 4 weeks and the repair of articular cartilage defects was improved	(115)
TNFR1	TNF-*a* induced transgenic mice	*In vivo:* Local injection into the knee joints	Synovial cells muscle cells	It effectively suppressed the severity of arthritis	(118)
TNF:Fc	Streptococcal cell wall (SCW)-induced arthritis rat	*In vivo:* Systemic intramuscular injection. Local injection into the knee joints	–	It significantly suppressed the arthritis by decreased inflammatory cell infiltration, pannus formation, cartilage and bone destruction	(121)

Table 1. Continued

Growth factor	Animal model	Route of gene delivery	Target cell/tissue	Effects	References
IL-4	Collagen-induced arthritis (CIA) mice	*In vivo*: Local injection in the tarsus area	Muscle cells	It showed a significant reduction in paw swelling and attenuated histological synovitis	(119)
IL-4	CIA mouse	*In vivo*: Local injection into the knee joints	Synovial cells	It reversed the loss of proteoglycan synthesis in patellar chondrocytes, but had no effect on synovial inflammation	(111)
IL-10	CIA mouse	*In vivo*: Systemic intramuscular injection	Synovial cells	It significantly decreased the frequency of arthritis, delaying the onset and reducing the severity of arthritic symptoms	(120)
IKKβ	Lewis rats with adjuvant arthritis	*In vivo*: Local injection into the knee joints	Synoviocytes macrophage	It reduced severity of inflammation in adjuvant arthritis *in vivo* and proinflammatory cytokine production in human RA synovial tissue	(124)
IL-1 antagonist	LPS induced rat	*In vivo*: Local injection into the knee joints	Synovium	Both the primary and recurrent arthritis were suppressed	(113)
Angiostatin	CIA mouse	*In vivo*: Local injection into the knee joints	Chondrocytes synovial cells	It efficiently inhibited the development of collagen-induced arthritis in the treated joint	(122)

craniofacial bone *in vivo* is still unknown, and this requires further investigation.

AAV mediated gene delivery to joint for growth modification

Adeno-associated virus has been shown to be an effective vector for gene transfer to human chondrocytes, achieving transfection efficiency of nearly 50% for at least 28 days (25). Using ultraviolet (UV) light, it was found that the transduction efficiency and expression of the transduced gene were significantly enhanced in rAAV-infected primary chondrocytes and in the superficial zone of cultured intact articular cartilage without simultaneously causing cytotoxicity and DNA mutations (104, 105). More recently, the *in vivo* study demonstrated that pre-treatment with 6000 J/m^2 of long-wavelength UV leads to a 10-fold increase in the transduction of articular chondrocytes after 1 week (105). Furthermore, repair of full-thickness defects in rabbit articular cartilage could be enhanced by intra-articular administration of AAV2-fibroblast growth factor (FGF-2) and basic fibroblast growth factor (bFGF) (106, 107). However, most of the available gene delivery systems could hardly efficiently and persistently transduce the chondrocytes of articular cartilage embedding with the rich matrix *in vivo*. Kuboki *et al.* (108) firstly delivered reporter gene (LacZ) into the articular surface of the TMJ using the adenovirus vector. Using the Hartley guinea-pig model, they observed LacZ gene expression in the articular surfaces of the temporal tubercle, articular disc and synovium of the TMJ, which lasted 4 weeks after injection. However, a low transduction rate into the chondrogenic layer was observed. Recently, effective transfer of lentivirus mediated LacZ gene into the hypertrophic layer of condyle was reported by local injection into the TMJ space (109). However, Lentivirus, the member of retrovirus can be integrated into host genome randomly and may give rise to insertion mutagenesis, so as to limit its clinical application (12, 13). More recently, we were able to construct a rAAV2 mediated delivery vehicle where reporter gene of eGFP could be delivered in the deeper layers of the mandibular condyle, which provides a basis to select some target genes to regulate mandibular condylar growth (110). Moreover, the response of condyle by rAAV delivery is different from that of knee joint, the transgene expression was only observed in synoviocytes and chondrocytes in arthritic knee joints of transgenic

mice by rAAV delivery, but no or less expression of the delivered genes was detected in the normal articular cartilage (106, 111, 112). In the knee joint cartilage, the chondrocytes are embedded in a well-organized extracellular matrix which is different from the articular surfaces of the TMJs covered by a fibrous connective tissue. Hence, the pattern of expression for AAV after intraarticular injection is variable according to the different anatomical structure.

AAV gene therapy for arthritis

Among potential vector systems for gene therapy application in RA, rAAV serotype 2 has been frequently employed for gene transfer experiments in arthritis and has received increasing attention. Madry *et al.* (26) demonstrated that infection efficiency of AAV-LacZ exceeded 70% for isolated normal human adult articular chondrocytes and osteoarthritic human articular chondrocytes and the marker genes were stably expressed. Another *in vitro* study demonstrated that the AAV-TGFβ1 vector equally and efficiently transduced both osteoarthritic human chondrocytes and a human normal articular chondrocyte cell line (24). The feasibility of direct *in vivo* gene transfer to rat and mouse arthritic joints, using AAV, has been well investigated (111, 113). In most of the studies, reporter gene expression peaked on days 3–7 and returned to baseline levels 3–4 days after injection, whereas AAV2 genome persisted within joints for at least 100–200 days (52, 112, 113). Importantly, transduction efficiencies revealed a striking correlation with disease severity, maximal transgene expression being observed at the peak of joint inflammation (52). The pattern of expression after intraarticular injection of AAV2 has been variable according to studies, from periosteum-derived cells (114, 115), synovial lining cells (52), fibroblast-like synoviocytes (FLS) (116) to chondrocytes (112) and muscle cells (117, 118). The rAAV gene constructs encoding immunosuppressive cytokines such as interleukin 4 (IL-4) (111, 119), IL-10 (120), or IL-1 antagonist (113) tumor necrosis factor-α (TNF-α) inhibitors (118), TNF receptor-immunoglobulin Fc (TNF:Fc) (121) and anti-angiogenic factor such as angiostain (122) showed high therapeutic efficiency after both local and systemic administration. Moreover, vectors based on at least seven other AAV serotypes have also been developed and are being actively pursued as gene delivery vectors. A recent study reported that rAAV5 is the most effective rAAV serotype for local gene therapy in RA over other 4 serotypes of rAAV1-4 (123). Moreover, rAAV5-mediated gene therapy targeting the nuclear factor (NF)-κB-activating kinase IκB kinase (IKKβ) locally in the joint significantly reduced established arthritis *in vivo* (124). Recently, proteasome inhibitors have been demonstrated to enhance rAAV-mediated transgene expression in human RA FLSs and suggested a possible approach to regulate synovial transgene expression *in vivo* (116).

Stem cell gene therapy with AAV

Mesenchymal stem cells (MSCs) from bone marrow possess the capacity for self-renewal and multilineage potential to differentiate into osteocytes, chondrocytes, myocytes, tenocytes, adipocytes, and neural cells *in vitro* (125, 126). These mesenchymal progenitor cells (MPCs) are attractive candidates as cellular vehicles for skeletal tissue regeneration. Earlier reports demonstrated the difficulties and problems in transduction efficiency of rAAV into MSCs. The expression of exogenous gene was partly limited by lack of low-affinity membrane-associated HSPG, and at least one of two high-affinity co-receptors (the FGFR and the αVβ5 integrin) (58, 59). Ju (22) reported that hydroxyurea and etoposide can increase the transduction of hMSCs by rAAV. With UV irradiation, the secretion of TGFβ1 protein from infected hMSCs with AAV-TGFβ1 can be significantly increased (23). Recently, Chamberlain and colleagues used AAV to disrupt the exon 1 of the chromosomal COL1A1 collagen gene in MSCs which were isolated from the patients with osteogenesis imperfecta, demonstrating successful gene targeting in adult human stem cells (127).

Conclusion

Local delivery of therapeutic genes by rAAV is a promising approach to treat bone and cartilage disorders with several potential advantages over systemic forms of targeted therapy. In the field of dentistry, congenital and acquired defects include hemifacial microsomia, micrognathia, segmental craniofacial bone defects, cartilage damage, TMJ arthritis and periodontal bone loss. The challenge now is to precisely define optimal cellular targets, therapeutic genes, and to develop safe, efficient and

controllable ways to deliver such therapeutic genes to target cells. Advances in determining the functional mechanism of many of the new gene candidates for craniofacial bone formation continue to be made (128). Likely other potential therapeutic genes will soon become available to facilitate the localized regeneration of bone and cartilage repair. In the light of advances in the recombinant molecular technology and viral biology, rAAV production facilitated its use in human clinical trials (129). Recombinant cross-packaging of AAV genome of one serotype into other AAV serotypes has opened the possibility to optimize tissue-specific gene transduction and expression. Hence, different rAAV serotypes need to be compared and select the efficient one for different target cells. After studies in rodents, vectors should be tested in large animals to ensure that they mediate safe and long-term gene expression. In addition, studies on appropriate regulation of therapeutic gene expression will be important. In the near future, this novel technology of *in vivo* rAAV-mediated gene therapy allowing cell-specific, long-term and regulable therapeutic gene expression could be applied in the field of dentofacial-orthopedic research to treat patients with serious bone and cartilage problems. Further proof-of-concept advances are needed to bring this approach to fruition, and to perfect a method that is low-risk, effective, and requires only a short-treatment period.

Acknowledgement: Work from the author's laboratory cited in this article was supported by a grant to The University Strategic Research Theme: Genomics, Proteomics and Bioinformatics, The University of Hong Kong, grant no. 10206152.11222.21700.302.01. And the CERG grant awarded to Professor Rabie, no. 10206968.22311.08003.324.01. We thank Ting Tung Yuen for illustration processing.

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