[Frontiers in Bioscience 12, 2212-2220, January 1, 2007]

Direct AAV-mediated gene delivery to the temporomandibular joint

Juan Dai, A. Bakr M. Rabie

The Biomedical and Tissue Engineering Group, Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong, SAR

TABLE OF CONTENTS

1. ABSTRACT

Successful intra-temporomandibular joint gene transfer is a prerequisite for gene therapy to induce mandibular condylar growth. This study was carried out to investigate the expression of recombinant adeno-associated virus (rAAV) based vector-mediated enhanced green fluorescence protein (eGFP) in the mandibular condyle. The transduction efficiencies for primary chondrocytes using different hybrids of rAAV vectors were identified by fluorescence microscopy and FACS. Sprague-Dawley rats were injected with either $rAAV_{2/2}$-eGFP construct or saline into both mandibular condyles. The spatial and temporal transgene expression was detected by *in situ* hybridization, RT-PCR and real-time PCR. Our result showed that rAAVs were capable of infecting rat chondrocytes. $rAAV_{2/2}$ was the best serotype to infect chondrocytes *in vitro*. By *in situ* hybridization, eGFP expression was clearly detected in the deeper layer of mandibular condyle as early as 7 days after injection. By real-time PCR, the transgene expression reached a peak at 21 days. This study was the first report to explore that rAAV-mediated genes could be transferred to mandibular condyle *in vivo*. Our results strongly suggest that $rAAV_{2/2}$ mediated gene delivery is a promising approach to deliver candidate genes for future regulating mandibular condylar growth.

2. INTRODUCTION

Congenital and acquired craniofacial malformations such as micrognathia, hemifacial microsomia resulting from impaired growth of the mandibles are commonly found and require a dental, speech, medical, behavioral and extraordinary surgical interventions (1). Because of the tremendous impact on patients' lives, researchers have struggled to explore improved methods to treat those craniofacial deformities.

The rapid development of recombinant DNA technology has also led to the development of factor-based approaches. Protein treatment has been used for some time, but it has some disadvantages of denature during the protein purification process, frequently delivering, low yield rates and short biological half-life (2). On the basis of recent insights into the development, growth, and adaptation of bone, together with the significant advances in molecular biology, gene therapy is increasingly becoming recognized as the most promising way for novel approaches to treat both genetic and acquired bone diseases by the introduction of new genetic material into the appropriate cells in the body (1, 3). In 1995, Baum and O'Connell firstly described the potential impact of gene therapy on dentistry (4). Over the past decade, gene therapy

has forged its own position to be a novel strategy to induce bone formation. Although non-viral vector systems such as naked DNA and plasmids are utilized by many research groups for gene delivery with a long history and a lot of improvements have been achieved, low transduction efficiency prevents them from wide usages. In contrast, viral vectors are more efficient for cell entry. Hence, the critical point is the identification of high-efficiency viral vectors suitable for direct gene delivery in the craniofacial bone (1). To date, candidates including retrovirus, adenovirus, lentivirus, and adeno-associated virus (AAV) have been used to target gene expression *in vivo* (5, 6). Moreover, most of the available gene delivery systems could hardly efficiently and persistently transduce the chondrocytes of articular cartilage embedding with the rich matrix. Kuboki *et al* (7) firstly delivered reporter gene (LacZ) into the articular surface of the temporomandibular joint using the adenovirus vector. Using the Hartley guinea-pig model, their observation showed that LacZ gene expression was found in the articular surfaces of the temporal tubercle, articular disc and synovium of the tempormandibular joint (TMJ) and lasted 4 weeks after injection. However, a low transduction rate into the chondrogenic layer was observed. Recently, it was reported effective transfer of lentivirus mediated LacZ gene into the hypertrophic layer of condyle by local injection into the TMJ space (8). However, Lentivirus, the member of retrovirus can be integrated into host genome nonrandomly and may give rise to insertion mutagenesis, so as to limit its clinical application (9, 10).

More recently, vectors based on the AAV have convincingly mediated transduction of reporter genes to human articular chondrocytes with a transduction efficiency of nearly 20% on day 1, 95% on day 7 and 50% for at least 28 days *in vitro*. In cartilage organ culture, GFP expression was observed in cells located in both superficial layer and deep layer (11). These studies have identified that the AAV vector is capable of efficient gene transfer and sustained gene expression in both normal primary articular chondrocyte cultures, and articular cartilage explants, and direct *in vivo* gene therapy (12). Overall, among potential vector systems for gene therapy application in TMJ, AAV, which is a small and single stranded DNA virus, is a favorable choice as it has several major advantages such as low immunogenicity, persistent expression, the ability to infect both dividing and non-dividing cells and a broad host range (13-15). Up to now, 11 serotypes were identified and they have different intrinsic properties, the serotype 2 of AAV is the first one used as recombinant vector (16) and is the most commonly used. Recent improvements in the technology of rAAV production facilitate its use in human clinical trials (13, 17). Recently, the potential for gene delivery to treat joint disorders with rAAVs has received much attention. What's more, mandibular condylar cartilage has been of much interest in many studies, due to its integral role in mandibular growth (18-20). However, so far, none of the rAAV vectors-mediated gene delivery systems was tested in mandibular condyle.

Therefore, it would be of great interest to determine if rAAV mediated gene therapy will present an alternative choice for delivering some candidate gene to control the growth of the mandibular condyle in the near future.

3. MATERIALS AND METHODS

3.1. Generation of hybrids of rAAV- eGFP Particles

Recombinant AAV vector encoding eGFP (rAAV-eGFP) was constructed with restriction enzyme site of *EcoR* I and *Xho* I. The gene expression was under the control of CAG (cytomegalovirus enhancer plus chicken β-actin promoter). Woodchuck post-regulatory elements (WPRE) and bovine growth hormone polyA sequences were utilized to boost gene expression, as previously described (21). The integrity of the cDNA constructs and the vector was confirmed by DNA sequence analysis. Different pseudotypes of rAAV were generated by standard production and purification protocols (22). Recombinant $AAV_{2/2}$ ($rAAV_{2/2}$) and pseudotyped $rAAV_{2/1}$ and $rAAV_{2/5}$ were generated by packaging identical rAAV2-inverted terminal repeats (ITR) recombinant genomes in AAV_2, AAV_1, and AAV_5 capsids, respectively. They were generated by using a three-plasmid transfection protocol as described elsewhere (23), with minor modifications. Briefly, HEK293 cells were tritransfected by calcium phosphate precipitation with an adenovirus helper plasmid pFd6 (24), a AAV packaging helper plasmid expressing the *rep* and *cap* genes, and a plasmid bearing the recombinant pAAV-eGFP. The packaging plasmids were pH22 for $rAAV_{2/2}$, pH21 for $rAAV_{2/1,}$ pH25 for $rAAV_{2/5}$ (25). The transfected cells were harvested 70 hours after transfection. All the recombinant vectors were purified by an OptiPrep-based gradient ultracentrifugation method followed by dilution with sterile phosphate buffered saline (PBS) and subsequent reconcentration (26). After DNase and proteinase K treatment, the aliquot of virus was titered by quantitative real time PCR (7700, Applied Biosystems) using SYBR Green to detect linear amplification of a region within the common WPRE sequence (27). The viral vector was stored at -80^{o}C prior to *in vitro* and *in vivo* studies.

3.2. Isolation of rat chondrocytes and *in vitro* Expression of eGFP

Primary rat chondrocytes were obtained from cartilage removed under sterile conditions from mandibular condyles. To remove adherent fibrous tissues, the cartilage was incubated in DMEM medium containing 0.05% trypsin for 1/2h at 37°C. The medium was discarded and the tissue fragments were minced and digested overnight at 37°C in DMEM containing 2mg/ml collagenase II, 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were collected by centrifugation at 250g for 5min and washed four times with complete medium (28). The chondrocytes used in this study were maintained as monolayer cultures for no more than two passages, to maintain the differentiated chondrocyte phenotype. The chondrocytes were allowed to adhere for at least 24 h before addition of AAV. The cells were washed once with 1×PBS, and then the virions were added at the indicated number of multiplicity of infection (MOI) either 5×10^3, 1×10^4 or 5×10^4 in DMEM medium. After 5 h, cells

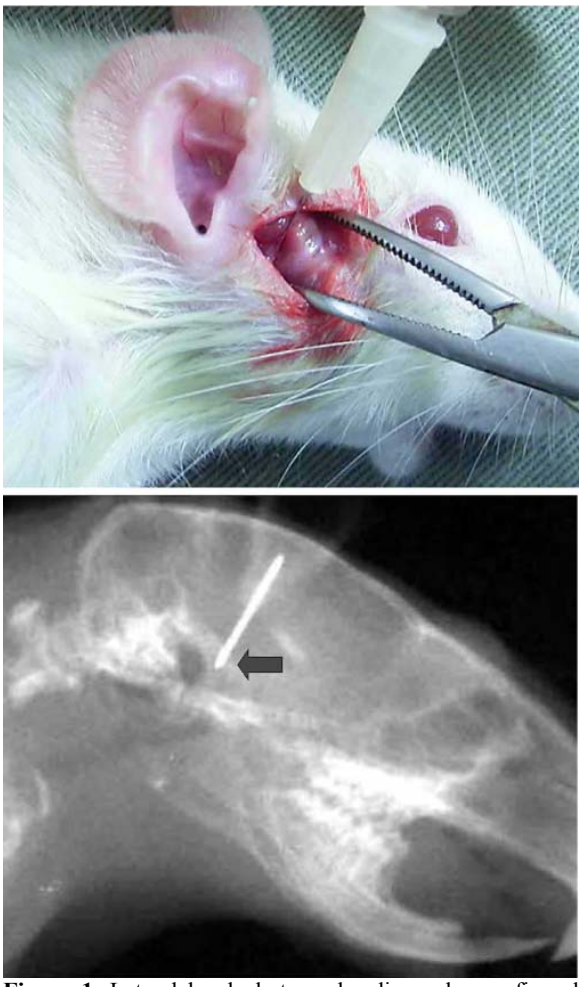

Figure 1. Lateral head photo and radiography confirmed the injection site just in the posterior attachment of mandibular condyle.

were incubated in DMEM medium with 10% FBS and antibiotics. At days 3 after transduction, the capacity of the rAAV-eGFP infection on primary chondrocytes was analyzed by fluorescence microscopy and the transduction efficiency was performed by a FACSCalibur (Becton Dickinson). Then 5×10^4 of MOI was selected to test the infection efficiency on day 3, 5, 7, 9. As previously described (29), 1x $l0^4$ cells were counted per acquisition. The percentage of live eGFP-expressing cells in this population was evaluated. The data were further analyzed with CellQuest software (Becton Dickinson). A maximum level of 5% was set as the background autofluorescence in live, uninfected chondrocytes.

3.3. Local injection of $rAAV_{2/2}$-eGFP into rat condyle posterior attachment

35 days-old female Sprague-Dawley (SD) rats were obtained from the Animal Units of the University of Hong Kong. All surgical procedures and care administered to the animals were approved by the University Ethics Committee and performed according to institutional guidelines. Sixty SD rats were randomly allotted into 1 experimental group and 1 control group. The body weight of each rat was measured both at the beginning and at the end of the experiment. Injection procedure was performed as previously described (8). The 50µl volume of rAAV-eGFP (2×10^{11} genome copies) or PBS was slowly injected directly into the posterior attachment of rat condyle through a 30 gauge needle connected with a microsyringe with the direction of downbackward (Figure 1). The needle was allowed to remain in place for another 5 minutes before being slowly retracted at the end of each injection. One interrupted 4-0 silk suture was used to close the incision. Take care to avoid inject the blood vessels around the condyle. Six rats from each group were sacrificed at day 7, 14, 21, 30 & 60 postinjection by an intraperitoneal injection of Dorminal. The heads of those SD rats were skinned, dissected into two halves. Left halves were immediately fixed at 4% paraformaldehyde and later for *in situ* hybridization. The right halves of mandible were cleaned off the adherent connective tissues and snap stored in liquid nitrogen for further RT-PCR analysis.

3.4. *In situ* hybridization

The procedure was carried out following the procedure by Rabie *et al* (19). Briefly, hybridization was performed with digoxigenin-11-UTP -labeled hybridizing (antisense) and non-hybridizing (sense) RNA probes transcribed from WPRE cDNA (600bp, GenBank accession number AY468486) subcloned in pBluescript (Stratagene) plasmid by T3 or T7 RNA polymerases as protocols of *in vitro* transcription using a DIG RNA labelling kit (Roche Diagnostics GmbH). Hybridization was carried out at 60 °C for 16 h in a humidified chamber. Samples were then incubated 2 h with 2.5 U/ml ALP−conjugated anti-digoxigenin Fab fragments (Roche Diagnostics GmbH) and developed with NBT/BCIP solution (Roche Diagnostics GmbH). Specimens were finally treated with 0.02% methylgreen as counterstaining. Rat condyle sections treated with PBS served as controls.

3.5. RT-PCR

Mandibular condylar cartilage, liver, kidney, spleen and heart were harvested. Specially, the condylar cartilage was homogenized with Mikro-dismembrator U (Braun Biotech International). Total RNA was isolated from each organ by means of RNeasy Fibrous Tissue Mini Kit (QIAGEN) and was reverse transcribed using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen Corp) followed the manufacturer's instructions. Primers used for eGFP mRNA were: forward: 5'- CCT ACG GCG TGC AGT GCT TCA GC-3' and reverse: 5'-CGG CGA GCT GCA CGC TGC GTC CTC -3' (320bp, GenBank accession number AY841887) and for GAPDH mRNA were forward: 5'-ACCACAGTCCATGCCATCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3' (136bp, GenBank accession number XM237330).

3.6. Real time PCR

The PCR amplifications were performed using real time PCR (7700, Applied Biosystems), according to the manufacturer's instructions. A total volume of 20 µl reaction mixture containing 2 µl of cDNA sample, 1×SYBR® Green master mix (Applied Biosystems), 20 pmol sense and antisense primers. Fluorescence signals

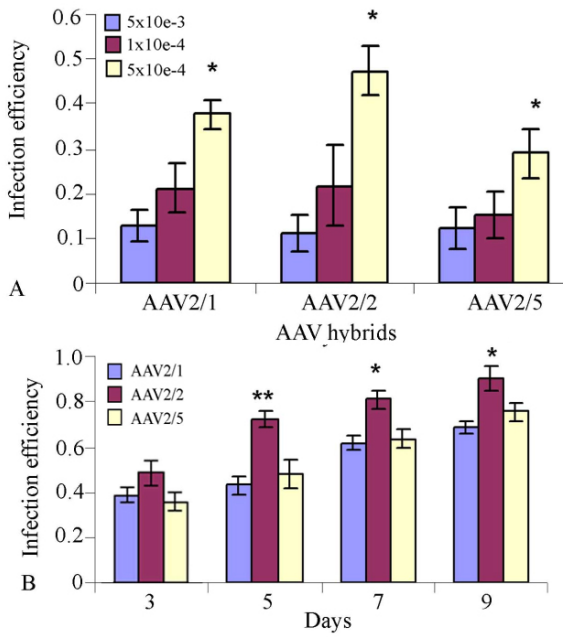

Figure 2. *In vitro* infection efficiency identified by FACS at different MOI (A) and incubation time (B).

were detected at the end of elongation phase for each amplification cycle. To confirm the specificity of the reaction, melting point analysis was carried out after the end of the last amplification cycle. Dissociation of the PCR product was carried out at 90ºC for 1 minute followed by 55ºC for 1 minute. Also, negative controls were tested with the same amplification procedures in order to eliminate any false positive result. Primers for WPRE were: sense 5'-TGGCGTGGTGTGCACTGT-3'; antisense, 5'-GTTCCGCCGTGGCAATAG-3' (110 bp, GenBank accession number AF410861). For GAPDH, the sequences used were: sense primer, 5'-catgttccagtatgactctaccc-3'; antisense primer, 5'-agcatcaccccatttgatgt-3' (136 bp, GenBank accession number XM237330). The quantification of gene expression was Real-Time PCR Detection System Software (Applied Biosystems). Absolute quantification was performed by comparing the target threshold cycles (Ct) directly with the absolute standard curve for each individual amplicon. The target gene copy numbers were normalized with the copy numbers of GAPDH.

3.7. Statistical Analysis

The data was processed with SPSS for Windows Release 11.0.0 (SPSS Inc.). All data acquisition and analyses were performed blindly. The in vitro infection efficiency was presented as mean percentage of positive cells and standard deviation. The in vivo infection efficiency was presented as the copy numbers of WPRE to GAPDH and standard deviation for each group. To compare the time-course change of rAAV gene expression level, one-way ANOVA with post hoc test of Bonferroni multiple comparisons test was performed for each time points. P values were considered to be statistically significant when less than 0.05.

4. RESULTS

4.1. *In vitro* detection of transgene expression

To evaluate the effects of rAAV hybrids-mediated transduction of chondrocytes *in vitro,* monolayer cultures of primary rat condylar chondrocytes were infected at various MOIs with rAAVs-eGFP immediately afterward. The relative transduction efficiency of eGFP gene expression in the primary condylar chondrocyte cultures with different MOIs and different time points after infection were determined by flow cytometric analysis (FACS) (Figure 2). At the MOI of $5x10^3$, on day 3, only about 10% chondrocytes can be tranduced with AAV-eGFP, when increase the MOI to $5x10^4$, significant higher proportion of chondrocytes can be tranduced. Moreover, the transduction efficiency increased with the incubation time with AAVs. On day 3, less than half of the chondrocytes were tranduced by the rAAVs and there were no significant differences among three serotypes; from day 5 to day 9, the transduction rate increased gradually and best efficiencies had been identified for $rAAV_{2/2}$; on day 9, the transduction rate of all the rAAV vectors increased to 70-90%.

4.2. *In vivo* detection of transgene distribution

AAV-eGFP injection into the TMJ posterior attachment resulted in transgene expression in cells located within the posterior attachment of condyle and condylar cartilage (Figure 3). Specially, chondroblast, hypertrophic chondrocyte and some osteoblasts in the erosive zone showed positive staining. This transgene expression was detected as early as day 7 and lasted for at least 60 days. However, there was a lack of transgene expression in the PBS-injected animals. These results suggest that rAAV successfully infected and stably expressed the exogenous gene to condylar cartilage of growing rats.

4.3. Expression of exogenous gene in the mandibular joint and remote organs

After injection of AAV-eGFP and PBS at different time points, total RNA was extracted from each organ, and RT- PCR was conducted. In the remote organ of heart, kidney, spleen and liver, of AAV-eGFP injected animals, mRNA expression of eGFP was not detected thereby reducing the prospects of systemic adverse effects (Figure 4). In the mandibular condyles, expression of eGFP mRNA was detected as early as day 7 and reaching the maximum at 21days, this expression lasted at least for 60 days, which was consistent with the result of *in situ* hybridization. The exogenous gene expression was not detected in the joints of PBS -injected animals.

4.4. Quantification of delivered AAV expression

To quantify the temporal transgene expression in the mandibular condyle, mRNA expression of transgene was assessed by real-time RT-PCR as shown in (Figure 5-6). The transgene was expressed constantly throughout the experiment. At day 21, there was a substantial increase in transgene expression, which was 18.42 fold more than that of day 7 and 7.18 and 4.63 fold increase when compared with that of day 14 and day 30 respectively. And this difference was statistically significant ($p<0.001$). Then

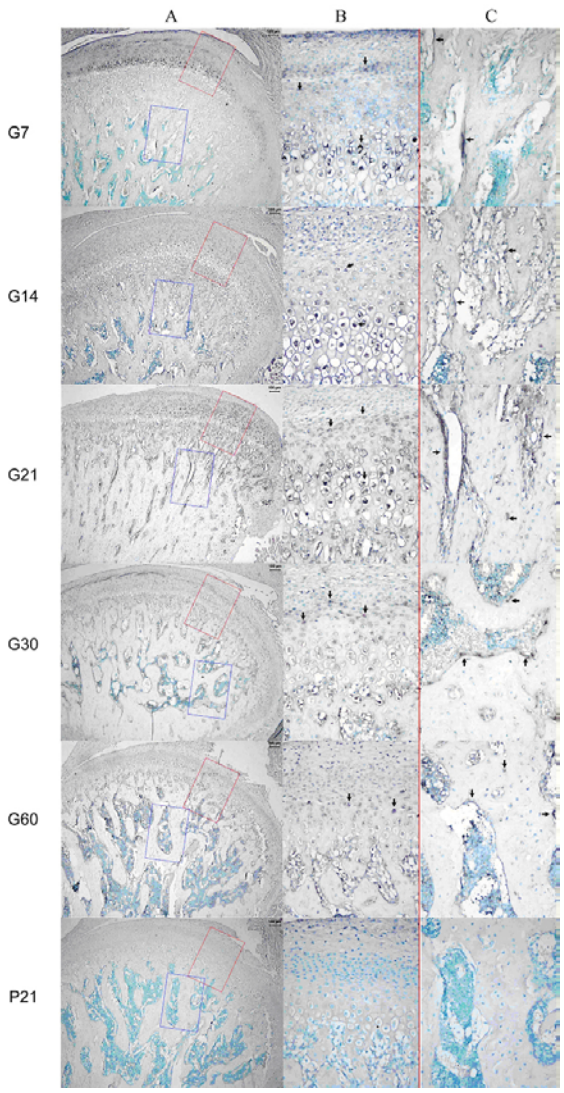

Figure 3. The transgene distribution in the mandibular condyle. It was identified by *in situ* hybridization with Dig-labeled WPRE probe (rAAV) demonstrating positive signal (*mulberry staining, arrow*) in the posterior attachment *(A)*, chondrocyte layers *(B)* and erosive layer *(C)* of rAAV-eGFP injected condyles at day 7(G7), 14(G14), 21(G21), 30(G30) and 60(G60). The corresponding PBS injected condyle at day 21(P21) showed no positive staining.

from day 60 on wards, the transgene expression returned to a level similar to day 7.

5. DISCUSSION

The rAAV vector generated in the present studies has provided as an efficient vehicle for the delivery and expression of eGFP in the primary chondrocyte, which was consistent with previous *in vitro* observation that rAAV vectors expressing various reporter genes could transduce normal and osteoarthritic human articular chondrocytes (30). Moreover, we found that all the selected rAAVs were capable to transduce rat chondrocytes *in vitro*. Up to now, 11 primate AAV serotypes have been identified. The sequence homology among the different serotypes is high. Sequence comparison revealed that the greatest divergence lies in the capsid proteins (31-33) leading to differences in both tropism and serological neutralisation (33, 34). Differential tissue tropism among various serotypes is a characteristic of AAV. Therefore, we compared three rAAV serotypes for their relative efficiencies to transduce primary chondrocytes isolated from mandibular condyle. The rAAV pseudotypes were generated by packaging identical rAAV2-ITR recombinant genomes in AAV_2, AAV_1, and AAV_5 capsids. This could avoid interference from *cis*-acting elements caused by the differences in the vector genome. Compared with two hybrids, $rAAV_{2/2}$ showed higher transduction rate *in vitro*. Moreover, the transduction efficiency (percentage of eGFP-positive cells) of different hybrids infection was dependent upon the MOI, and incubation time. The higher MOI and longer incubation time resulted in greater infection efficiency.

Our study was the first report to confirm that transgene can be transferred to the mandibular condyle *in vivo*. In the previous study, adenovirus delivered LacZ gene expression was found in the articular surfaces of the temporal tubercle, articular disc and synovium of the TMJ even four weeks after injection (7). However, no signal was detected in the chondrogenic layer. In the cartilage organ culture, rAAV delivered GFP expression was observed in cells located in both superficial layer and deep layer over a period of 7 days and for 28 days (11). In our study of SD rats, after local injection of rAAV-eGFP, the transgene expression was detected in the deep layer of condylar cartilage as early as day 7 and lasted for at least 60 days. It is easily speculated that AAV, 22–25 nm in diameter is quite smaller than adenovirus which is 70-100 nm and can be easier to diffuse into the deeper layer of condylar cartilage. On the other hand, the transgene expression was observed in synoviocytes and chondrocytes in arthritic knee joints of transgenic mice by AAV delivery, but no or less expression of the delivered genes was detected in the normal articular cartilage (5, 35). Hence, the pattern of expression for AAV after intraarticular injection is variable according to the different anatomical structure. In the knee joint cartilage, the chondrocytes are embedded in a well organized extracellular matrix. To deliver genes into knee chondrocytes *in vivo*, the virus has to pass through the surrounding extracellular matrix. In contrast, the articular surfaces of the temporomandibular joints are covered by a fibrous connective tissue. In our study, local gene delivery restricts transgene expression to defined anatomical locations and none in the remote organs suggesting an absence of widespread infection. Those infected cells were located around the posterior attachment, the different layers of cartilage and even the wall of bone marrow cavity. This observation might reveal that either the rAAV vector is able to diffuse from the injected joint space to the deeper layer to infect chondrocytes, or the cells infected within the posterior space migrated with the branch of blood vessels. Both hypotheses were considered because that during postnatal endochondral ossification, blood vessel invasion induces vascularization channel formation

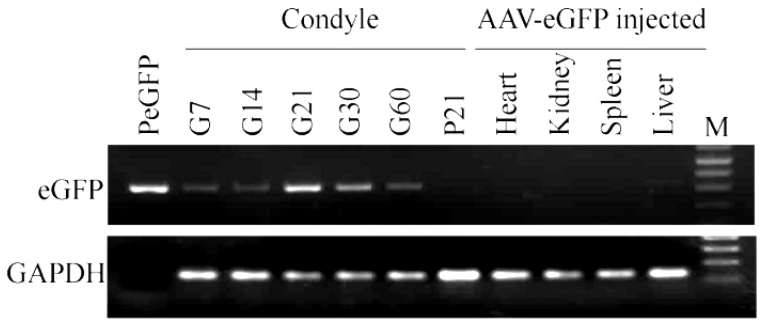

Figure 4. RT-PCR analysis for eGFP expression in the mandibular condyles and remote organs. By agarose gel electrophoresis, in the mandibular condyle of rAAV-eGFP injected rats, eGFP mRNA was clearly detected at postinjection of day7 (G7), day 14 (G14), day 21(G21), day30 (G30) and day 60 (G60). While, in PBS injected condyle at day 21 (P21), no positive band was detected. Furthermore, in the heart, kidney, spleen and liver of AAV-eGFP injected rats, transgene expression was not detected at any time points. GAPDH served as internal control and the plasmid of rAAV-eGFP construct (peGFP) as positive control.

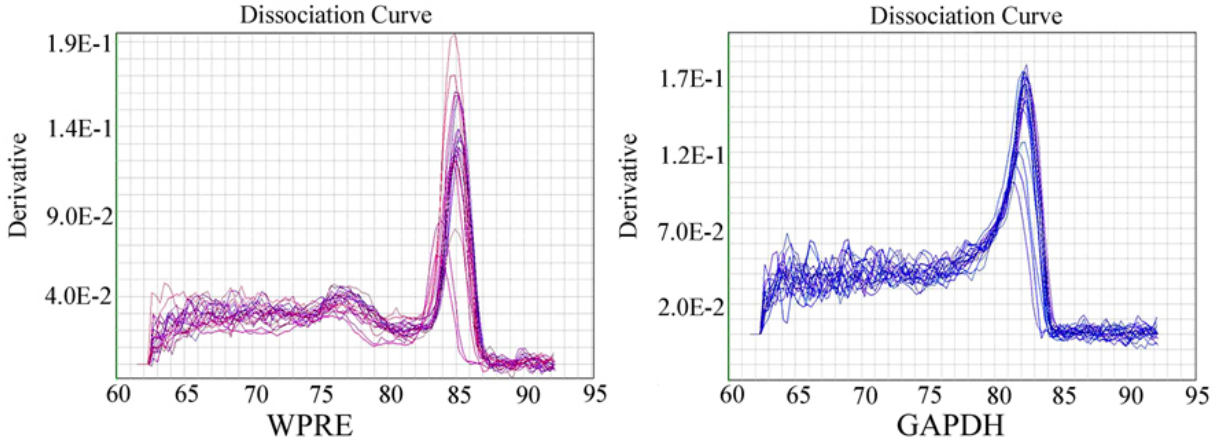

Figure 5. Specificity of the PCR amplicon was confirmed by melting curve analysis.

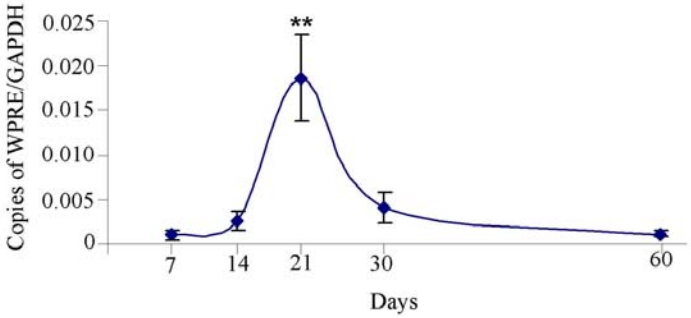

Figure 6. Real-time RT-PCR results of WPRE mRNA expression. The mean values of normalized WPRE mRNA expression and the standard deviations are shown.

(36). Furthermore, AAV must bind to a cellular receptor as well as to coreceptor for successful entry into the target cells. It was reported that AAV has one receptor of tropic heparan sulfate proteoglycan (37) and two coreceptors of fibroblast growth factor receptor 1(38) and integrin αVβ5 (39). Recently, these receptors were detected in mandibular condylar cartilage layer (40-42). Hence, our study proved that rAAV-mediated genes could be delivered into mandibular condyle *in vivo*.

One interesting finding in this study was the duration of transgene expression. By real-time PCR quantification, transgene expression *in vivo* increased after injection, reached the peak on day 21 and then decreased after day 30. On day 60, the transgene expression returned to a level similar to day 7. Since it was known that the rAAV vector exists as a single stranded DNA virus, while the viral genome is transported to the nucleus within minutes after infection (43), *in vivo* transduction take days

to weeks as a consequence of lagging second-strand synthesis (44). On the other hand, it is widely accepted that recombinant adeno-associated viral DNA does not integrate into the cellular genome, instead it remains episomal and lasts with the infected cells after initial exposure (45). During postnatal growth of the condyle, cells within condylar cartilage are spatially organized. The mesenchymal cells proliferate and differentiate into chondroblasts and chondrocytes, followed by maturation and hypertrophy in addition to synthesis of extracellular matrices (46). The cartilage template is eventually replaced by bone (47, 48). As early maturation of the chondrocytes ceases chondrogenesis and induces osteogenesis, the maintenance of the chondroblast layer, where mesenchymal cells stop proliferation and initiate differentiation, is thus a major regulatory point for continuing condylar growth. Accordingly, the recombinant AAV is eliminated along with the turnover course of the chondrocytes, suggesting a lower risk in case of unexpected event for long term expression.

Therefore, rAAV-mediated gene transduction of articular chondrocytes is a promising strategy that may allow for direct *in vivo* gene therapy through intraarticular injection and explore a useful delivery system for future regulating mandibular condylar growth.

6. ACKNOWLEDGEMENTS

We thank Dr R.A. Xu, K.X. Cai, H. Li and L.Y. Tse of institute of molecular biology for excellent virus package technology and W.T. Loo of department of surgery for cell culture. We thank Y.Y. Chui of hard tissue lab for tissue processing and histochemical staining. For statistics analysis and photo processing, we thank Shadow Yeung. This study was supported in part by a grant to The University Strategic Research Theme: Genomics, Proteomics & Bioinformatics, The University of Hong Kong, grant No.10206152.11222.21700.302.01.

7. REFERENCES

1. Dai, J., A. B. Rabie, U. Hagg & R. Xu: Alternative gene therapy strategies for the repair of craniofacial bone defects. *Curr. Gene Ther.*, 4, 469-85 (2004)
2. Zhang, F., K. Fischer & W. C. Lineaweaver: DNA strand gene transfer and bone healing. *J Long Term Eff Med Implants*, 12, 113-23 (2002)
3. Chen, Y., K. M. Cheung, H. F. Kung, J. C. Leong, W. W. Lu & K. D. Luk: *In vivo* new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. *Biochem. Biophys. Res. Commun.*, 298, 121-7 (2002)
4. Baum, B. J. & B. C. O'Connell: The impact of gene therapy on dentistry. *J. Am. Dent. Assoc.*, 126, 179-89 (1995)
5. Goater, J., R. Muller, G. Kollias, G. S. Firestein, I. Sanz, R. J. O'Keefe & E. M. Schwarz: Empirical advantages of adeno associated viral vectors *in vivo* gene therapy for arthritis. *J. Rheumatol.*, 27, 983-9 (2000)
6. Yovandich, J., B. O'Malley, Jr., M. Sikes & F. D. Ledley: Gene transfer to synovial cells by intra-articular administration of plasmid DNA. *Hum. Gene Ther.*, 6, 603-10 (1995)
7. Kuboki, T., T. Nakanishi, M. Kanyama, W. Sonoyama, T. Fujisawa, K. Kobayashi, T. Ikeda, T. Kubo, A. Yamashita & M. Takigawa: Direct adenovirus-mediated gene delivery to the temporomandibular joint in guinea-pigs. *Arch Oral Biol.*, 44, 701-9 (1999)
8. Kyrkanides, S., P. Kambylafkas, J. H. Miller & R. H. Tallents: Non-primate lentiviral vector administration in the TMJ. *J. Dent. Res.*, 83, 65-70 (2004)
9. Cone, R. D. & R. C. Mulligan: High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. U. S. A.*, 81, 6349-53 (1984)
10. Luk, K. D., Y. Chen, K. M. Cheung, H. Kung, W. W. Lu & J. C. Leong: Adeno-associated virus-mediated bone morphogenetic protein-4 gene therapy for *in vivo* bone formation. *Biochem. Biophys. Res. Commun.*, 308, 636-45 (2003)
11. Arai, Y., T. Kubo, S. Fushiki, O. Mazda, H. Nakai, Y. Iwaki, J. Imanishi & Y. Hirasawa: Gene delivery to human chondrocytes by an adeno associated virus vector. *J. Rheumatol.*, 27, 979-82 (2000)
12. Ulrich-Vinther, M., M. R. Duch, K. Soballe, R. J. O'Keefe, E. M. Schwarz & F. S. Pedersen: *In vivo* gene delivery to articular chondrocytes mediated by an adeno-associated virus vector. *J Orthop Res*, 22, 726-34 (2004)
13. Crystal, R. G., D. Sondhi, N. R. Hackett, S. M. Kaminsky, S. Worgall, P. Stieg, M. Souweidane, S. Hosain, L. Heier, D. Ballon, M. Dinner, K. Wisniewski, M. Kaplitt, B. M. Greenwald, J. D. Howell, K. Strybing, J. Dyke & H. Voss: Clinical protocol. Administration of a replication-deficient adeno-associated virus gene transfer vector expressing the human CLN2 cDNA to the brain of children with late infantile neuronal ceroid lipofuscinosis. *Hum. Gene. Ther.*, 15, 1131-54 (2004)
14. Inoue, N. & D. W. Russell: Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. *J. Virol.*, 72, 7024-31 (1998)
15. Kessler, P. D., G. M. Podsakoff, X. Chen, S. A. McQuiston, P. C. Colosi, L. A. Matelis, G. J. Kurtzman & B. J. Byrne: Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. U. S. A.*, 93, 14082-7 (1996)
16. Muzyczka, N., R. J. Samulski, P. Hermonat, A. Srivastava & K. I. Berns: The genetics of adeno-associated virus. *Adv. Exp. Med. Biol.*, 179, 151-61 (1984)
17. Flotte, T. R., M. L. Brantly, L. T. Spencer, B. J. Byrne, C. T. Spencer, D. J. Baker & M. Humphries: Phase I trial of intramuscular injection of a recombinant adeno-associated virus alpha 1-antitrypsin (rAAV2-CB-hAAT) gene vector to AAT-deficient adults. *Hum. Gene. Ther.*, 15, 93-128 (2004)
18. Rabie, A. B., L. Shum & A. Chayanupatkul: VEGF and bone formation in the glenoid fossa during forward mandibular positioning. *Am. J. Orthod. Dentofacial Orthop.*, 122, 202-9 (2002)
19. Rabie, A. B., G. H. Tang & U. Hagg: Cbfa1 couples chondrocytes maturation and endochondral ossification in rat mandibular condylar cartilage. *Arch Oral Biol.*, 49, 109-18 (2004)

20. Rabie, A. B., G. H. Tang, H. Xiong & U. Hagg: PTHrP regulates chondrocyte maturation in condylar cartilage. *J. Dent. Res.*, 82, 627-31 (2003)
21. Xu, R., C. G. Janson, M. Mastakov, P. Lawlor, D. Young, A. Mouravlev, H. Fitzsimons, K. L. Choi, H. Ma, M. Dragunow, P. Leone, Q. Chen, B. Dicker & M. J. During: Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. *Gene Ther*, 8, 1323-32 (2001)
22. Pan, R. Y., S. L. Chen, X. Xiao, D. W. Liu, H. J. Peng & Y. P. Tsao: Therapy and prevention of arthritis by recombinant adeno-associated virus vector with delivery of interleukin-1 receptor antagonist. *Arthritis. Rheum.*, 43, 289-97 (2000)
23. Douar, A. M., K. Poulard, D. Stockholm & O. Danos: Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol*, 75, 1824-33 (2001)
24. Xiao, X., J. Li & R. J. Samulski: Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.*, 72, 2224-32 (1998)
25. Arai, Y., T. Kubo, K. Kobayashi, K. Takeshita, K. Takahashi, T. Ikeda, J. Imanishi, M. Takigawa & Y. Hirasawa: Adenovirus vector-mediated gene transduction to chondrocytes: *in vitro* evaluation of therapeutic efficacy of transforming growth factor-beta 1 and heat shock protein 70 gene transduction. *J Rheumatol*, 24, 1787-95 (1997)
26. Hermens, W. T., O. ter Brake, P. A. Dijkhuizen, M. A. Sonnemans, D. Grimm, J. A. Kleinschmidt & J. Verhaagen: Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther*, 10, 1885-91 (1999)
27. Chen, Y., K. D. Luk, K. M. Cheung, R. Xu, M. C. Lin, W. W. Lu, J. C. Leong & H. F. Kung: Gene therapy for new bone formation using adeno-associated viral bone morphogenetic protein-2 vectors. *Gene Ther.*, 10, 1345-53 (2003)
28. Reiter, I., M. Tzukerman & G. Maor: Spontaneous differentiating primary chondrocytic tissue culture: a model for endochondral ossification. *Bone*, 31, 333-9 (2002)
29. Ulrich-Vinther, M., M. D. Maloney, J. J. Goater, K. Soballe, M. B. Goldring, R. J. O'Keefe & E. M. Schwarz: Light-activated gene transduction enhances adeno-associated virus vector-mediated gene expression in human articular chondrocytes. *Arthritis Rheum*, 46, 2095-104 (2002)
30. Madry, H., M. Cucchiarini, E. F. Terwilliger & S. B. Trippel: Recombinant adeno-associated virus vectors efficiently and persistently transduce chondrocytes in normal and osteoarthritic human articular cartilage. *Hum. Gene. Ther.*, 14, 393-402 (2003)
31. Rabinowitz, J. E. & R. J. Samulski: Building a better vector: the manipulation of AAV virions. *Virology*, 278, 301-8 (2000)
32. Gao, G., M. R. Alvira, S. Somanathan, Y. Lu, L. H. Vandenberghe, J. J. Rux, R. Calcedo, J. Sanmiguel, Z. Abbas & J. M. Wilson: Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci U S A*, 100, 6081-6 (2003)
33. Grimm, D., M. A. Kay & J. A. Kleinschmidt: Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. *Mol. Ther.*, 7, 839-50 (2003)
34. Mori, S., L. Wang, T. Takeuchi & T. Kanda: Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology*, 330, 375-83 (2004)
35. Watanabe, S., T. Imagawa, G. P. Boivin, G. Gao, J. M. Wilson & R. Hirsch: Adeno-associated virus mediates long-term gene transfer and delivery of chondroprotective IL-4 to murine synovium. *Mol. Ther.*, 2, 147-52 (2000)
36. Burkus, J. K., T. M. Ganey & J. A. Ogden: Development of the cartilage canals and the secondary center of ossification in the distal chondroepiphysis of the prenatal human femur. *Yale. J. Biol. Med.*, 66, 193-202 (1993)
37. Summerford, C. & R. J. Samulski: Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.*, 72, 1438-45 (1998)
38. Qing, K., C. Mah, J. Hansen, S. Zhou, V. Dwarki & A. Srivastava: Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat. Med.*, 5, 71-7 (1999)
39. Summerford, C., J. S. Bartlett & R. J. Samulski: AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat. Med.*, 5, 78-82 (1999)
40. Visnapuu, V., T. Peltomaki, O. Ronning, T. Vahlberg & H. Helenius: Distribution of fibroblast growth factors (FGFR-1 and -3) and platelet-derived growth factor receptors (PDGFR) in the rat mandibular condyle during growth. *Orthod. Craniofac. Res.*, 5, 147-53 (2002)
41. Tajima, Y., M. Kawasaki, K. Kurihara, T. Ueha & S. Yokose: Immunohistochemical profile of basic fibroblast growth factor and heparan sulphate in adult rat mandibular condylar cartilage. *Arch Oral Biol.*, 43, 873-7 (1998)
42. Molteni, A., D. Modrowski, M. Hott & P. J. Marie: Differential expression of fibroblast growth factor receptor-1, -2, and -3 and syndecan-1, -2, and -4 in neonatal rat mandibular condyle and calvaria during osteogenic differentiation *in vitro*. *Bone*, 24, 337-47 (1999)
43. Seisenberger, G., M. U. Ried, T. Endress, H. Buning, M. Hallek & C. Brauchle: Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science*, 294, 1929-32 (2001)
44. Fisher, K. J., G. P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda & J. M. Wilson: Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol*, 70, 520-32 (1996)
45. McCarty, D. M., S. M. Young, Jr. & R. J. Samulski: Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu Rev Genet*, 38, 819-45 (2004)
46. Luder, H. U., C. P. Leblond & K. von der Mark: Cellular stages in cartilage formation as revealed by morphometry, radioautography and type II collagen immunostaining of the mandibular condyle from weanling rats. *Am J Anat*, 182, 197-214 (1988)
47. Rabie, A. B. & U. Hagg: Factors regulating mandibular condylar growth. *Am J Orthod Dentofacial Orthop*, 122, 401-9 (2002)
48. Rabie, A. B., F. Y. Leung, A. Chayanupatkul & U. Hagg: The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. *Angle Orthod*, 72, 431-8 (2002)

Key Words: Gene therapy, Adeno-associated virus, Enhanced green fluorescence protein, PCR, Mandibular condyle

Send Correspondence to: Professor A. Bakr M. Rabie, Department of Orthodontics, Faculty of Dentistry, The University of Hong Kong, 2/F, Prince Philip Dental Hospital , 34 Hospital Road, Sai Ying Pun, Hong Kong, SAR, China, Tel: 852-2859 0260 Fax: 852-2559 3803, E-mail: rabie@hkusua.hku.hk

http://www.bioscience.org/current/vol12.htm