Persistence, Localization, and External Control of Transgene Expression After Single Injection of Adeno-Associated Virus into Injured Joints

Hannah H. Lee,^{1,2} Michael J. O'Malley,¹ Nicole A. Friel,¹ Karin A. Payne,^{2,3} Chunping Qiao,⁴ Xiao Xiao,⁴ and Constance R. Chu^{1,5}

Abstract

A single intra-articular injection of adeno-associated virus (AAV) results in stable and controllable transgene expression in normal rat knees. Because undamaged joints are unlikely to require treatment, the study of AAV delivery in joint injury models is crucial to potential therapeutic applications. This study tests the hypotheses that persistent and controllable AAV-transgene expression are (1) highly localized to the cartilage when AAV is injected postinjury and (2) localized to the intra-articular soft tissues when AAV is injected preinjury. Two AAV injection time points, postinjury and preinjury, were investigated in osteochondral defect and anterior cruciate ligament transection models of joint injury. Rats injected with AAV tetracycline response element (TRE)–luciferase received oral doxycycline for 7 days. Luciferase expression was evaluated longitudinally for 6 months. Transgene expression was persistent and controllable with oral doxycycline for 6 months in all groups. However, the location of transgene expression was different: postinjury AAV-injected knees had luciferase expression highly localized to the cartilage, while preinjury AAV-injected knees had more widespread signal from intra-articular soft tissues. The differential transgene localization between preinjury and postinjury injection can be used to optimize treatment strategies. Highly localized postinjury injection appears advantageous for treatments targeting repair cells. The more generalized and controllable reservoir of transgene expression following AAV injection before anterior cruciate ligament transection (ACLT) suggests an intriguing concept for prophylactic delivery of joint protective factors to individuals at high risk for early osteoarthritis (OA). Successful external control of intra-articular transgene expression provides an added margin of safety for these potential clinical applications.

Introduction

TRAUMATIC AND DEGENERATIVE INJURIES to articular cartilage are a leading cause of disability. The limited healing potential of articular cartilage contributes to development of osteoarthritis (OA). OA, whether idiopathic or posttraumatic, develops over many years and patients frequently need chronic treatment. Common medications for OA include nonselective, nonsteroidal, anti-inflammatory drugs (NSAIDs) (Recommendations for the Medical Management, 2000; Evans *et al.*, 2004). Daily long-term use of these drugs, however, is not without significant complications. NSAID therapy results in systemic side effects and was responsible for 16,500 deaths per year in the United States (Singh *et al.*, 2006). As such, there are no

disease-modifying treatments for osteoarthritis or to reestablish cartilage homeostasis (Izal *et al.*, 2008). Posttraumatic osteoarthritis affects only the injured joint. Hence, delivery of bioactive agents in a localized manner to the involved joint is an attractive therapeutic option (Singh, 2012).

A novel treatment strategy for localized treatment is gene therapy, utilizing viral vectors, such as adeno-associated virus (AAV), to deliver therapeutic proteins of interest to affected joints to enhance joint repair or to prevent cartilage degradation. Recombinant AAV is a promising delivery vehicle for articular cartilage with the advantages of sustained transgene expression, reduced potential for host immune response, and the capacity to transduce both dividing and nondividing cells *in vivo* and *in vitro* (Ulrich-Vinther *et al.*,

¹Cartilage Restoration Center, Department of Orthopaedic Surgery; ²Department of Bioengineering; University of Pittsburgh, Pittsburgh, PA 15313.

³Department of Orthopedic Surgery, University of Colorado Denver, Aurora, CO 80045.

⁴Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599.

⁵Department of Orthopedic Surgery, Stanford University, Stanford, CA 94305.

2002; Lee *et al.*, 2011). The viral vector is derived from an endemic, nonpathogenic parvovirus, and intra-articular injection of AAV has been used in clinical trials for delivery of bioactive substances (Mease *et al.*, 2010; High and Aubourg, 2011). Numerous serotypes of AAV exist, each serotype with different preferential targets (Goater *et al.*, 2000). AAV5 has recently been suggested to have better transduction efficiency in rodent arthritic joints. However, AAV2 has the best defined safety profile and it is used in clinical trials (Mueller and Flotte, 2008).

A recently published study from our laboratory has shown both stable and persistent reporter transgene expression at one year after a single intra-articular injection of AAV (Payne *et al.*, 2011). Most of the AAV transduction occurred in the intra-articular soft tissues covered with synovial cells, with limited articular chondrocyte transduction. This is consistent with other studies, as vector penetration through the dense cartilage extracellular matrix is restricted (Goater *et al.*, 2000; Evans *et al.*, 2004). However, high-level expression of transgene product was possible from the soft tissues.

To improve effectiveness of treatments in which timing of administration are important and to provide an additional margin of safety, controllable vectors have been developed to limit treatment time/dosage to the minimal needed to achieve therapeutic efficacy. One novel system is the tetracycline (Tet)-on promoter, in which control is facilitated by using tetracycline as an activator of the promoter, stimulating transgene expression only in the presence of the drug (Gossen and Bujard, 1992). Previous studies in our laboratory have successfully modulated gene expression utilizing the Tet-on system and doxycycline, a tetracycline analog (Payne *et al.*, 2011). However, as intact/undamaged articular cartilage is unlikely to require treatment, AAV administration strategies in the setting of joint injury will provide invaluable information to improve gene therapy strategies for joint restoration.

This study tests the hypotheses that (1) *in vivo* AAV transgene expression localizes to the cartilage when AAV is administered postinjury, (2) AAV injection preinjury leads to the creation of a soft tissue reservoir that persistently expresses transgene products at high levels, and (3) *in vivo* transgene signal can be externally controlled after joint injury using oral doxycycline. Two surgically created unilateral joint injuries of high clinical relevance will be studied: osteochondral defect (OCD) and anterior cruciate ligament transection (ACLT).

Materials and Methods

AAV preparation

Double-stranded AAV serotype 2 (AAV2) vectors were produced, purified, and vector genome (vg) titer determined as previously described (Rehman *et al.*, 2005). AAV2s with constitutively active cytomegalovirus (CMV) promoter included AAV2-CMV-Luciferase (Luc) and AAV2-CMV reverse-tetracycline-controlled transactivator (rtTA). AAV2 with tetracycline response element (TRE) promoter included AAV2-TRE-Luc.

Animal studies

Animal experiments were performed following a University of Pittsburgh Institutional Animal Care and Use

Committee (IACUC)-approved protocols 0901937 and 1103868. Longitudinal *in vivo* study was performed using 48 3-month-old male Sprague Dawley rats (Harlan and Hilltop Lab Animals).

In vitro explant AAV transduction

Femoral explants harvested from 3-month-old Sprague Dawley rats (Harlan) were washed in phosphate buffered saline (PBS) with 2% penicillin-streptomycin (pen/strep, both from Gibco) and incubated for 2–3 days in 37°C, 5% CO₂ in 1:1 ratio Dulbecco's modified Eagle medium (DMEM) and F-12 (DMEM/F-12, Gibco), 10% fetal bovine serum (FBS, Gibco), 2% pen/strep, and 1% amphotericin B (Invitrogen). The explants were divided into three groups in triplicate: no-AAV control, intact with AAV, and injury with AAV. The explants from the latter two groups were transduced with AAV2-CMV-Luc at a concentration of 80,000 vector genomes (vg)/cell in serum-free DMEM/F-12. After 1-hr incubation, fresh DMEM/F-12, 10% FBS, 2% pen/strep, and 1% amphotericin B were added to the culture. The culture media was changed after 2 days. The explants were imaged after 96 hours post-AAV transduction, using IVIS® Imaging System 200 (Xenogen), by adding 10 µL of 30 mg/mL D-Luciferin Potassium Salt (Caliper Life Sciences) per 1 mL of culture media. The images were taken at 1-min exposure.

Animal surgeries

Twenty-four rats underwent unilateral OCD surgeries, while the other 24 rats underwent ACLT surgeries as previously described (Williams *et al.*, 1982; Lee *et al.*, 2013). Briefly, all the procedures were carried out while the animals were anesthetized with inhalation of isoflurane (Phoenix Pharmaceuticals): 2–5% for induction and 0.25–4.0% for maintenance. Rat knee joints were exposed via medial parapatellar and medial patellar tendon approaches. The patella was dislocated laterally and the knee was flexed. For the OCD surgeries, the midpoint of the femoral trochlea was identified and a 1.5-mm carbide-tipped drill (Emil Lange/ELA) was used to create an OCD approximately 1.0-mm deep. The subchondral bone was penetrated, and bleeding bone was observed. For the ACLT surgeries, ACL and posterior cruciate ligament (PCL) were visualized with the aid of a 3× surgical loupe (Carl Zeiss). Forceps were used to protect the PCL, and the ACL was severed using a 1.5-mm Cutting Edge Beaver Needle Blade (Becton, Dickinson and Company). ACLT was confirmed with an anterior drawer test. Following injury creation for both OCD and ACLT, the joint spaces were washed with sterile saline, and both capsule and skin were sutured using monofilament 4-0 monocril (Ethicon). An intramuscular injection of 0.2–0.5 mg/kg buprenorphine (Hospira) was administered twice per day for 2 days following surgery for analgesia.

AAV injections

AAV2-CMV-Luc was used to characterize the persistence and localization of the transgene signal while AAV2-TRE-Luc was used to characterize the external controllability of the transgene signal using oral doxycycline.

Two different AAV injection time points were investigated to compare the transgene expression levels: postinjury AAV

injection and preinjury AAV injection. For the postinjury AAV injection group, the viral vectors were injected bilaterally into both injured and intact joints at 1-week postinjury for OCD rats and 3-weeks postinjury for ACLT rats. For the preinjury AAV injection group, the AAVs were injected bilaterally into the joint. After 28 days, when the transgene expression level has stabilized, the animals underwent unilateral joint surgeries, either OCD or ACLT.

Each knee was given a total of 50 μ L of AAVs, containing 2.5×10^{10} vg of either AAV2-CMV-Luc or AAV2-TRE-Luc and an equal amount of AAV2-CMV-rtTA by single intra-articular injection. During the injection procedures, animals were anesthetized with gaseous isoflurane. Six rats were used for each viral construct per injury model per AAV injection time points.

External control of transgene expression

To induce expression of luciferase in the animals injected with AAV2-TRE-Luc, the rats were administered drinking water containing doxycycline (Sigma) at a concentration of 2 mg/mL for 7 days, followed by its removal. To ensure fresh supply of doxycycline, the water was replaced every day and protected from light using amber drinking bottles. Addition of doxycycline to the drinking water was performed at 14 days, 3 months, and 6 months following AAV injection for postinjury AAV-injected animals and following surgery for preinjury AAV-injected animals. Rats injected with AAV2-CMV-Luc had regular drinking water for the duration of the study.

Longitudinal in vivo live animal imaging

At each imaging time point, rats were anesthetized with isoflurane inhalation, followed by a 50- μ L intra-articular injection of 30 mg/mL D-Luciferin Potassium Salt substrate (Caliper). After 5 min, rats were placed in and imaged with an IVIS® 200 optical imaging system. Rats injected with AAV2-CMV-Luc were imaged every week for the first month, and monthly thereafter, until 6 months post-AAV injection, when the rats were sacrificed. On the day of sacrifice following regular imaging as detailed above, rats received a 400- μ L intraperitoneal injection of 30 mg/mL D-Luciferin Potassium Salt substrate (Goldbio) to determine if any signal was present outside the joint. The joint was then opened to image the intra-articular tissues. Rats injected with AAV2-TRE-Luc were imaged three times for every oral doxycycline stimulation cycle: predoxycycline time point prior to addition of doxycycline, 7 days following doxycycline addition, and 7 days following doxycycline removal. These rats were sacrificed at 6 months post-AAV injection as well, following the third doxycycline stimulation cycle. All the rats were sacrificed via CO₂ asphyxiation and subsequent thoracotomy.

Viral genome copy determination

Following sacrifice, the knee joints of $n=3$ rats/group were further dissected to isolate chondrocytes from femoral and tibial cartilages to detect viral DNA copy as previously described (Qiao *et al.*, 2012). Briefly, cartilage pieces from each of the femoral and tibial cartilages were separately stored dry in -80°C freezer until analysis. Total DNA was extracted using DNeasy blood and tissue kit (Qiagen), and vector copy number was determined with a 7300 real-time

PCR system (Applied Biosystems). The rat glucagon gene was used as the endogenous control. The sequences for rat glucagon primers and probes were as follows: Glucagon-real-F (rat), AAG GGA CCT TTA CCA GTG ATG TG; Glucagon-real-R (rat), ACT TAC TCT CGC CTT CCT CGG; Taqman rat glucagon probe, FAM-cag caa agg aat tca-MGB. Luciferase primers and probes were designed for AAV viral genome detection: Luc-F, TTG ACC GCC TGA AGT CTC TGA; Luc-R, ACA CCT GCG TCG AAG ATG TTG; Luc-probe, FAM- ccg ctg aat tgg aat c-MGB. The genome copy number is expressed as viral genome copies per diploid nuclear genome [(vector copy number/endogenous control) $\times 2$]. The sensitivity of the assay was 0.001 vector copies per cell.

Statistical analyses

All the statistical analyses were performed using Statistical Packages for Social Studies (SPSS) 17.0 (IBM). Results were judged for significance at $p < 0.05$, unless otherwise noted. Explant data were observed in triplicate (Fig. 1, $n=3$). *In vivo* animal data were observed using independent animals, with $n=6$ per group. All the data were tested for assumptions of parametric data: Shapiro-Wilk for normality of data distribution and Levine's for homogeneity of variance. The parametric bioluminescence data for the explant study is represented as median and interquartile range boxplots and analyzed using one-way independent analysis of variance (ANOVA) with *post hoc* Tukey (Fig. 1) (OCD, Figures 2 and 3; ACLT Figures 4 and 5). The longitudinal bioluminescence data for the animal studies assessing the persistence and magnitude of the AAV transgene are represented as line graphs $\pm$ standard error of mean (SEM) and were analyzed using two-way mixed ANOVA, with main independent variables of joint injury status as paired variable and time as repeated variable (Figs. 2 and 4). Friedman's ANOVA test with a Bonferroni correction was used as a *post hoc* test to assess the effect of time on each of the injured and intact joints when the interaction effect between the joint injury status and time was significant (Fig. 4B), with $p < 0.025$ (0.05/2 comparisons) considered significant. Further *post hoc* analysis of each consecutive time point was performed using Wilcoxon test and a Bonferroni correction, with $p < 0.006$ (0.025/4 comparisons) considered significant for the intact joint (Fig. 4B). The bioluminescence data assessing controllability of transgene expression were analyzed using one-way repeated measured ANOVA (Fig. 6). Viral DNA copy number was determined from tissues from $n=2-3$ per group and shown as mean $\pm$ SEM in Table 1.

Results

In vitro explant AAV transduction

Transgene expression was observed from femoral cartilage explants transduced with AAV-CMV-Luc (Fig. 1). The bioluminescence signal was greater in no-injury/AAV ($p=0.046$) and scratch/AAV ($p=0.008$) groups compared to no-injury/no-AAV control group. While no difference was observed between the two AAV-CMV-Luc-transduced groups ($p=0.313$), the mechanical scratch injury model where the condyles were scratched with a blade showed brighter bioluminescence at both lateral and medial condyles.

FIG. 1. Explant AAV transduction of rat femoral cartilage. Rat femoral cartilage explants were either scratched or kept intact (No Injury), after which they were transduced with AAV2-CMV-Luc. Luciferase signal was detected after 96 hr. **(A)** Representative images of bioluminescence signal from the explants. **(B)** Median and interquartile range of bioluminescence for the different explant groups ($n=3$; $*p<0.05$ versus No Injury/No AAV Control group). AAV, adeno-associated virus; CMV, cytomegalovirus; Luc, luciferase.

FIG. 2. Persistence of AAV transgene in the OCD injury model. Persistence of *in vivo* transgene expression in OCD-injured joints with a single intra-articular injection of AAV-CMV-Luc post-OCD (**A** and **B**) or pre-OCD (**C** and **D**) were assessed via bioluminescence. **(A)** Representative images of transgene signal from the OCD and intact joints at 7 days, 1 month, and 6 months following AAV-CMV-Luc injection 1 week after OCD injury. **(B)** Mean and SEM of bioluminescence observed in the OCD and intact joints of all rats in the group for 6 months following AAV-CMV-Luc injection 1 week after OCD injury. **(C)** Representative images of transgene signal from the OCD and intact joints injected with AAV-CMV-Luc 4 weeks prior to OCD injury at pre-OCD, 1-month, and 6-month time points. **(D)** Mean and SEM of bioluminescence observed in the OCD and intact joints of all rats in the group for 6 months following OCD injury in joints injected with AAV-CMV-Luc 4 weeks prior ($n=6$ /group). OCD, osteochondral defect.

FIG. 3. Localization of AAV transgene in the OCD injury model. Intra-articular localization of transgene expressions in OCD-injured joints with a single intra-articular injection of AAV-CMV-Luc were assessed after exposing the joint on the day of sacrifice using the IVIS optical imaging system. **(A)** Representative intra-articular images of transgene signal from the exposed OCD and intact joints that were injected with AAV-CMV-Luc 1 week after OCD injury. **(B)** Representative intra-articular images of transgene signal from the exposed OCD and intact joints that were injected with AAV-CMV-Luc 4 weeks prior to OCD injury.

AAV transduction in OCD injury

AAV transgene expression was persistent in both the OCD and intact joints whether the AAV was injected post- or pre-OCD injury (Fig. 2). However, the magnitude of the transgene expression levels in the OCD joints was different between the two AAV injection time points. For the knees with AAV injected post-OCD, there was a significant main effect of the injury status on the bioluminescence level

($p=0.015$), where the OCD knees had lower signal than the intact knees (Fig. 2B). On the other hand, for the knees with AAV injected pre-OCD, the main effect of the injury status on the bioluminescence level was nonsignificant ($p=0.924$, Fig. 2D). In addition, the luciferase expression remained unchanged from the pre-OCD time points. For both the AAV injection time-point groups, there was a nonsignificant main effect of time on the bioluminescence level ($p=0.175$ for post-OCD AAV injection and $p=0.185$ for pre-

FIG. 4. Persistence of AAV transgene in ACLT injury model. Persistence of *in vivo* transgene expressions in ACL-transected joints with a single intra-articular injection of AAV-CMV-Luc post-ACLT (**A** and **B**) or pre-ACLT (**C** and **D**) were assessed via bioluminescence. **(A)** Representative images of transgene signal from the ACLT and intact joints at 7 days, 1 month, and 6 months following AAV-CMV-Luc injection 3 weeks after ACL transection. **(B)** Mean and SEM of bioluminescence observed in the ACLT and intact joints of all rats in the group over 6 months following AAV-CMV-Luc injection 3 weeks after ACL transection. **(C)** Representative images of transgene signal from the ACLT and intact joints injected with AAV-CMV-Luc 4 weeks prior to ACL transection at pre-ACLT, 1-month, and 6-month time points. **(D)** Mean and SEM of bioluminescence observed in the ACLT and intact joints of all rats in the group for 6 months following ACL transection in joints injected with AAV-CMV-Luc 4 weeks prior ($n=6$ /group). ACLT, anterior cruciate ligament transection.

TABLE 1. VIRAL DNA COPY NUMBER IN DIFFERENT CARTILAGE COMPONENTS OF THE KNEE JOINT

Injury	AAV injection time point	Cartilage components	Viral DNA copy number (mean $\pm$ SEM, copies/cell)
OCD	Post-OCD	Femoral cartilage	0.115 $\pm$ 0.076
		Tibial cartilage	0.151 $\pm$ 0.125
	Pre-OCD	Femoral cartilage	0.061 $\pm$ 0.033
		Tibial cartilage	0.014 $\pm$ 0.001
ACLT	Post-ACLT	Femoral cartilage	1.229 $\pm$ 1.151
		Tibial cartilage	0.602 $\pm$ 0.599
	Pre-ACLT	Femoral cartilage	0.009 $\pm$ 0.003
		Tibial cartilage	0.004 $\pm$ 0.001

Mean $\pm$ SEM of viral DNA copy number detected in chondrocytes isolated from femoral and tibial cartilage of the AAV-transduced joint. $n=2-3$ /group.

OCD, osteochondral defect; ACLT, anterior cruciate ligament transection.

OCD AAV injection): the transgene magnitude remained constant throughout the duration of the study for each of the joints. The interaction effect between the joint injury status and the time was nonsignificant in both cases as well ($p=0.182$ for post-OCD AAV injection and $p=0.921$ for pre-OCD AAV injection).

Upon exposing the intra-articular space, the location of transgene expression was different in the OCD injured knees between the two different AAV injection time points (Fig. 3). For both of the AAV injection time points, the intact joints had the diffuse and high magnitude signal in the exposed infrapatellar fat pad. However, for the OCD injured joints, the bioluminescence signal was concentrated to the injured femur in the post-OCD AAV-injected knees (Fig. 3A), whereas the signal was more diffusely found in intra-articular soft tissues, mainly the fat pad in the pre-OCD AAV-injected knees (Fig. 3B). Hence, the pre-OCD AAV-injected knees had transgene signal location similar to those of the intact joints. The analysis of the cartilage tissues confirmed the presence of viral DNA genome copies in the chondrocytes isolated from the femoral and tibial cartilages of OCD-injured joints (Table 1).

AAV transduction in ACLT injury

Similar to the OCD injury model, the transgene expression was persistent in both the ACLT and intact joints, and for both post-ACLT and pre-ACLT AAV injections (Fig. 4). The

magnitude of the bioluminescence levels in the ACLT joints was again different between the two AAV injection time points. For the joints with AAV injected post-ACLT, there was a significant main effect of the injury status ($p=0.001$). The ACLT joints had lower signal than the intact joints (Fig. 4B). For the knees with AAV injected pre-ACLT, on the other hand, the main effect of the injury status was nonsignificant, with no change in transgene expression from the pre-ACLT time points ($p=0.279$, Fig. 4D). The main effect of time on the bioluminescence level was significant for the post-ACLT AAV-injected group ($p=0.010$) and nonsignificant for the pre-ACLT AAV-injected group ($p=0.531$). The interaction effect between the joint injury status and the time was significant for post-ACLT AAV injection study group ($p=0.011$) and nonsignificant for pre-ACLT AAV injection study group ($p=0.872$). The effect of time in post-ACLT AAV injection study group did not achieve significance for the ACLT joint following *post hoc* analysis ($p=0.992$). For the intact joint, on the other hand, the effect of time remained significant ($p=0.003$), necessitating additional *post hoc* testing. Nonetheless, Wilcoxon test and a Bonferroni correction revealed no significant differences between the consecutive time points. Hence, the transgene magnitude remained constant throughout the study period for all joints in post-ACLT and pre-ACLT AAV injection groups.

FIG. 5. Localization of AAV transgene in ACLT injury model. Intra-articular localization of transgene expressions in ACLT-transected joints with a single intra-articular injection of AAV-CMV-Luc were assessed after exposing the joint on the day of sacrifice using the IVIS optical imaging system. **(A)** Representative intra-articular images of transgene signal from the exposed ACLT and intact joints that were injected with AAV-CMV-Luc 3 weeks after ACL transection. **(B)** Representative intra-articular images of transgene signal from the exposed ACLT and intact joints that were injected with AAV-CMV-Luc 4 weeks prior to ACL transection.

When the intra-articular space was exposed, the localization of bioluminescence signal was different in the ACLT joints between the two different AAV injection time points as well (Fig. 5). Consistent with the intact sides of the OCD-injury model animals, the intact joints showed diffuse signal in the exposed infrapatellar fat pad for both of the AAV injection time points. In addition, the diffuse bioluminescence signal was found in the infrapatellar fat pad for the pre-ACLT AAV-injected, ACL-transected joints, which overwhelmed signal detection from the cartilage (Fig. 5B). However, for the post-ACLT, AAV-injected, ACL-transected joints, the bioluminescence signal was lower in the intra-articular soft tissues, but some region of concentrated signal was detected in the cartilage (Fig. 5A). The analysis of the cartilage tissues confirmed the presence of viral DNA genome copies in the chondrocytes isolated from the femoral and tibial cartilages of ACLT-injured joints at both injection time points (Table 1).

External control of AAV transgene expression in injured joints

The 7-day oral doxycycline cycle to induce transgene expression was performed starting at three different time points

throughout the study in animals that received the AAV2-TRE-Luc. For all three doxycycline stimulation cycles, there was an upregulation of gene expression with the addition of Dox ($p < 0.05$) and a downregulation following its removal from the drinking water ($p < 0.05$) of both the OCD- and ACLT-injured joints, and from both the post- and preinjury AAV injection time points (Fig. 6). However, the magnitude of transgene upregulation was different between the two AAV injection time points: those from postinjury AAV-injected joints achieved lower mean fold increase in bioluminescence signal compared to those from preinjury AAV-injected joints ($p < 0.001$).

Discussion

Treatment or prevention strategies of pathologic processes within articular joints are severely limited. Hence, a successful delivery strategy for adequate levels of therapeutic molecules in a localized manner is critically important. Gene therapy strategies, therefore, have gained a lot of attention for arthritis treatment (Goater *et al.*, 2000; Evans *et al.*, 2004). Numerous vectors are available for *in vivo* gene transfer, such as naked DNA, retrovirus, adenovirus, etc. However, most are limited in terms of safety, efficacy, and

FIG. 6. Controllability of AAV transgene in joint injury models. Intra-articular transgene expression in injured joints with a single intra-articular injection of AAV-TRE-Luc postinjury (A and C) or preinjury (B and D) were assessed after three cycles of doxycycline addition and removal using the IVIS optical imaging system. (A) Representative intra-articular images of transgene signal from the OCD joints that were injected with AAV-TRE-Luc 1 week after OCD injury. (B) Representative intra-articular images of transgene signal from the OCD joints that were injected with AAV-TRE-Luc 4 weeks prior to OCD injury. (C) Representative intra-articular images of transgene signal from the ACLT joints that were injected with AAV-TRE-Luc 3 weeks after OCD injury. (D) Representative intra-articular images of transgene signal from the ACLT joints that were injected with AAV-TRE-Luc 4 weeks prior to ACL transection (* $p < 0.05$ compared to Day 0, Pre-Dox; # $p < 0.05$ compared to Day 7, Dox +; $n = 6$ /group). Tre, tetracycline response element.

duration (Ulrich-Vinther, 2007). AAV, on the other hand, is a very promising vector system that may provide the best balance between safety and *in vivo* gene delivery efficiency (Evans *et al.*, 2004). Methods to localize and control *in vivo* transgene expression are important to enhance the therapeutic efficiency and safety of intra-articular AAV gene therapy. This has been achieved in intact joints (Payne *et al.*, 2011). However, to more closely mimic the osteoarthritic environment, AAV transduction to injured cartilage and joints has been investigated.

We initially characterized the efficacy of AAV transduction in explant cultures of femoral cartilage. With 80,000 vg/cell AAV2-CMV-Luc, significant transduction was observed for rat femoral explants. There was no significant difference with transduction efficacy between the two AAV-transduced groups. However, the mechanical injury model where the condyles were scratched with a blade showed high intensity of luciferin flux at both lateral and medial condyles. This is potentially due to the scratch injury creating a more permeable path to articular chondrocytes for AAV transduction. Thus, we expected to have more AAV transduction in damaged areas of articular cartilage.

Two commonly used joint injury models of high clinical relevance were chosen: OCD and ACLT. The timing of AAV injection, pre- or post-injury, permitted us to characterize the effect of joint injury on AAV transduction and localization of transgene expression. For both injury models, AAV transduces and is mainly expressed by intra-articular soft tissues of the intact sides, as shown in the previous study (Payne *et al.*, 2011). However, if the AAV is injected after joint injury, as would likely be the case in a clinical setting, the transgene signal localizes to the vicinity of the injured cartilage. As in the case for the explant study, the injured cartilage, with more permeable extracellular matrix, would have allowed AAV to reach and transduce the chondrocytes/injury-activated repair cells. The transgene expression from the soft tissues was limited for both OCD- and ACLT- injured joints. This can also be due to other reasons, such as increased volume of synovial fluid in injured joints diluting the intra-articular viral concentrations, injury-activated macrophages clearing up the virus prior to effective transduction, or the viral gene promoter shutting off in response to the injury (Qiao *et al.*, 2011).

It is also possible that the injury may have resulted in more diffuse distribution of AAV transduction throughout the joint and hence affected our ability to measure the total signal strength if the signal strength did not reach the threshold of detection. In contrast, when the AAV is injected before injury, the transgene signal magnitude as well as localization was no different from those of the intact joints and persisted following the surgical procedure. Transgene expression was mainly found in the intra-articular soft tissues with minimally detectable viral DNA copies in chondrocytes from the injured cartilage. This preinjury AAV injection data makes the theory that the promoter shuts off in response to injury less likely.

For the postinjury AAV injection time points, we chose 1 week for the OCD model to allow time for the acute inflammatory stage from the OCD surgery to subside. In addition, we hoped to have the repair cells from the underlying bone marrow recruited to the site of defect to allow their viral transduction. On the other hand, we chose 3 weeks

postinjury time point to inject the AAV in order for the ACLT model to allow some cartilage damage from altered mechanical/biochemical forces following the joint instability.

The surgical trauma of either OCD or ACLT procedure may have influenced the accuracy of joint injection. However, we have extensive experience in accurate intra-articular (IA) injection of AAV and other substrates in rat knee joints (Chu *et al.*, 2010; Payne *et al.*, 2011). In addition, we imaged the preinjury AAV-injected joints with IA luciferin injection at a weekly interval following surgery and found no difference in signal patterns from preinjury time points. Hence, we are confident that the joint injections with either AAV or luciferin substrate have been accurate following joint injury.

In order to image and quantify the luciferase transgene signal, we utilized the IA route of luciferin substrate administration. While the IA route introduces an unnecessary variable compared to subcutaneous or intraperitoneal (IP) injections, we chose the IA route due to cost and time restrictions. Compared to the IA route, IP injection required eight-fold more volume of luciferin substrate, as well as five-fold longer luciferin distribution time for optimal imaging. Nonetheless, we have imaged rats in groups injected with AAV2-CMV-Luc with IP administration of luciferin at 35 days postinjury. The bioluminescence signal pattern was no different from those imaged and quantified using the IA luciferin administration route the week prior (28-day post-injury or the 1-month timepoint). As previously mentioned, IA injections into rat stifle joints have been extensively and reliably utilized in our previous studies (Chu *et al.*, 2010; Payne *et al.*, 2011).

The controllability of gene expression in intact and injured joints is also important since gene expression may not be needed for a prolonged period of time to have a beneficial effect, and it provides an added means of safety. We have shown this controllability in all the injury models, whether the AAV was injected post- or pre-injury using oral administration of doxycycline in drinking water. The magnitude of doxycycline-induced transgene upregulation, however, was different between the two AAV injection time points. The transgene signal was lower for the postinjury AAV-injected animals compared to preinjury AAV-injected animals, which is consistent with the transgene magnitude found in AAV-CMV-Luc-injected animals. Doxycycline is an analog of tetracycline, an antimicrobial agent that is safely used in humans. It has a long-term record of relative safety and few recognized adverse effects, namely sun sensitivity, monilial vaginitis, and nonspecific gastrointestinal symptoms. In a human trial that used doxycycline for 30 months, only these three side effects were observed (Brandt *et al.*, 2005). No serious adverse events, such as death, hospitalization, and development of a life-threatening or debilitating condition, were attributable to doxycycline. In addition, the use of doxycycline as the transgene inducer molecule may have added beneficial effect for cartilage protection. Doxycycline inhibits collagen-degrading enzymes such as matrix metalloproteinases (MMPs), and has shown beneficial effects in both OCD and ACLT injury models by preventing MMP upregulation (Yu *et al.*, 1992; Lee *et al.*, 2013).

The differential transgene location between the two AAV injection time points can be used to optimize AAV-mediated cartilage repair strategies. The highly localized gene expression following AAV injection postinjury is advantageous for

gene therapy strategies that deliver anabolic growth factors that can aid in cartilage repair, especially genes that need to specifically target chondrocytes to be effective. Such strategies may include RNA interference against various cartilage catabolites, such as aggrecanases/ADAMTSs (Song *et al.*, 2007), or chondrogenic growth factors that have adverse effects on other intra-articular soft tissues, such as transforming growth factor (TGF)- β_1 . TGF- β_1 has been shown to increase chondrocyte matrix synthesis when its gene vector was injected in the joint; however, its overexpression from the synovium resulted in adverse fibrosis (Mi *et al.*, 2003). Our data showing external controllability of intra-articular transgene expression through oral doxycycline supports use of a tet-on construct to additionally minimize undesired effects by regulating temporal exposure to anabolic agents.

Diffuse signal, seen when AAV is injected before creation of injury, is also of potential value, as it provides a model to study the delivery of protective factors to individuals that may be at high risk for joint injury or cartilage degeneration. Although the virus does not specifically transduce chondrocytes, a high level of transgene expression from surrounding soft tissues still allows for preventative therapeutic approaches by using the soft tissue as a drug depot to release protective molecules. The high intra-articular concentrations of secreted products, such as cytokine antagonists, can compete with inflammatory mediators that are upregulated in disease states, to protect the cartilage. Synoviocytes release numerous proinflammatory molecules in arthritic conditions (Pasztoi *et al.*, 2009). Hence, a strong tropism of AAV for the synovium can be utilized to suppress expression of any of these injurious compounds.

A cytokine antagonist interleukin-1-receptor antagonist protein (IRAP) is an extensively studied molecule for such reasons (Arend *et al.*, 1998). This compound is especially attractive due to its lack of any known agonist activities. Adenoviral delivery of IRAP has led to chondroprotective effect in equine osteoarthritic model of osteochondral fragmentation (Frisbie *et al.*, 2002; Morisset *et al.*, 2007). Additional *in vivo* studies using prophylactic AAV-IRAP are planned using small animal joint-injury models. Successful external control of intra-articular transgene expression with oral doxycycline provides an added margin of safety for potential prophylactic clinical applications.

The studies investigating AAV transduction in injured joints are limited with prior work focused more on rheumatoid arthritis (Pan *et al.*, 1999; Goater *et al.*, 2000; Zhou *et al.*, 2011). Two groups used a transgenic mouse model that spontaneously develops arthritis due to tumor necrosis factor (TNF)- α overexpression (Goater *et al.*, 2000; Zhou *et al.*, 2011). Goater *et al.* (2000) reported increased AAV transduction efficiency with increased degree of the joint disease following an intra-articular injection of AAV, with synoviocytes as the primary target. In addition, *in vitro* transduction efficiencies of synoviocytes extracted from the transgenic mice increased with the treatment of these cells with damaging agents, such as irradiation and TNF- α . However, their study was done using single-stranded AAV, where the second-strand synthesis is the limiting step in AAV transgene expression. Hence, the injurious agents promote DNA synthesis and thus transgene expression as well. The use of double-stranded or self-complementary AAV has been shown to improve AAV transgene expression (Kay *et al.*,

2009). Zhou *et al.* (2011) showed persistent transgene expression for 120 days following AAV-Luc injection in the transgenic mouse joints; however, they did not compare their bioluminescence level to intact joints, preventing a comparison to our study. Another study investigated intra-articular AAV transduction in Sprague Dawley rats damaged via a joint injection of lipopolysaccharide (LPS) (Pan *et al.*, 1999). This study showed positive correlations between disease severity and AAV transduction and between disease severity and transgene expression. Again, however, these injury models mimic inflammatory/rheumatoid arthritis rather than osteoarthritis.

In summary, this study evaluated localization, persistence, and controllability of AAV-induced intra-articular transgene expression in two clinically relevant joint injury models. While AAV transgene expression was localized to the soft tissues of the uninjured joint and injured joints when AAV was injected preinjury, AAV was able to transduce articular chondrocytes within the cartilage matrix when injected following injuries. The explant data showing AAV transduction of articular chondrocytes in damaged cartilage further suggests that cartilage matrix injury may have facilitated entry of more AAV into the cartilage. Differential transgene expression can be used to optimize AAV-mediated cartilage repair strategies: postinjury AAV injection approaches would directly target articular chondrocytes in diseased and injured cartilage, and preinjury AAV injection would allow intra-articular delivery of secreted anti-inflammatory factors for sustained release from soft tissues to protect the joint. Overall, these data support further study of the *in vivo* therapeutic potential of AAV for safe and localized delivery of bioactive substances to injured joints. This can aid in cartilage repair as well as restoration and maintenance of joint homeostasis as strategies to delay or prevent the onset of debilitating osteoarthritis.

Acknowledgments

This work was funded by the National Institutes of Health (NIH) RO1 AR051963 (CRC) and T32-EB001026 (HHL/SSM), the Albert Ferguson Endowed Chair (CRC), and the Albert B. Ferguson, M.D., Orthopaedic Fund of the Pittsburgh Foundation (MJO/CRC).

Author Disclosure Statement

The authors declare no conflicts of interest.

References

- Arend, W.P., Malyak, M., Guthridge, C.J., and Gabay, C. (1998). Interleukin-1 receptor antagonist: role in biology. *Annu. Rev. Immunol.* 16, 27–55.
- Brandt, K.D., Mazzuca, S.A., Katz, B.P., *et al.* (2005). Effects of doxycycline on progression of osteoarthritis - Results of a randomized, placebo-controlled, double-blind trial. *Arthritis and Rheumatism* 52, 2015–2025.
- Chu, C.R., Coyle, C.H., Chu, C.T., *et al.* (2010). In vivo effects of single intra-articular injection of 0.5% bupivacaine on articular cartilage. *J. Bone Joint Surg. Am.* 92, 599–608.
- Evans, C.H., Gouze, J.N., Gouze, E., *et al.* (2004). Osteoarthritis gene therapy. *Gene Ther* 11, 379–389.
- Frisbie, D.D., Ghivizzani, S.C., Robbins, P.D., *et al.* (2002). Treatment of experimental equine osteoarthritis by *in vivo*

- delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther.* 9, 12–20.
- Goater, J., Muller, R., Kollias, G., *et al.* (2000). Empirical advantages of adeno associated viral vectors in vivo gene therapy for arthritis. *Journal of Rheumatology* 27, 983–989.
- Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5547–5551.
- High, K.A., and Aubourg, P. (2011). rAAV human trial experience. *Methods Mol Biol.* 807, 429–457.
- Izal, I., Acosta, C.A., Ripalda, P., *et al.* (2008). IGF-1 gene therapy to protect articular cartilage in a rat model of joint damage. *Arch. Orthop. Trauma Surg.* 128, 239–247.
- Kay, J.D., Gouze, E., Oligino, T.J., *et al.* (2009). Intra-articular gene delivery and expression of interleukin-1Ra mediated by self-complementary adeno-associated virus. *J. Gene Med.* 11, 605–614.
- Lee, H.H., Haleem, A.M., Yao, V., *et al.* (2011). Release of bioactive adeno-associated virus from fibrin scaffolds: effects of fibrin glue concentrations. *Tissue Eng. Part A* 17, 1969–1978.
- Lee, H.H., O'Malley, M.J., Friel, N.A., and Chu, C.R. (2013). Effects of doxycycline on mesenchymal stem cell chondrogenesis and cartilage repair. *Osteoarthritis and Cartilage* 21, 385–393.
- Mease, P.J., Wei, N., Fudman, E.J., *et al.* (2010). Safety, tolerability, and clinical outcomes after intraarticular injection of a recombinant adeno-associated vector containing a tumor necrosis factor antagonist gene: results of a phase 1/2 study. *Journal of Rheumatology* 37, 692–703.
- Mi, Z., Ghivizzani, S.C., Lechman, E., *et al.* (2003). Adverse effects of adenovirus-mediated gene transfer of human transforming growth factor beta 1 into rabbit knees. *Arthritis Res. Ther.* 5, R132–R139.
- Morisset, S., Frisbie, D.D., Robbins, P.D., *et al.* (2007). IL-1ra/IGF-1 gene therapy modulates repair of microfractured chondral defects. *Clin. Orthop. Relat. Res.* 462, 221–228.
- Mueller, C., and Flotte, T.R. (2008). Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther.* 15, 858–863.
- Pan, R.Y., Xiao, X., Chen, S.L., *et al.* (1999). Disease-inducible transgene expression from a recombinant adeno-associated virus vector in a rat arthritis model. *J. Virol.* 73, 3410–3417.
- Pasztoi, M., Nagy, G., Geher, P., *et al.* (2009). Gene expression and activity of cartilage degrading glycosidases in human rheumatoid arthritis and osteoarthritis synovial fibroblasts. *Arthritis Res. Ther.* 11, R68.
- Payne, K.A., Lee, H.H., Haleem, A.M., *et al.* (2011). Single intra-articular injection of adeno-associated virus results in stable and controllable in vivo transgene expression in normal rat knees. *Osteoarthritis and cartilage/OARS, Osteoarthritis Research Society* 19, 1058–1065.
- Qiao, C., Yuan, Z., Li, J., *et al.* (2011). Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. *Gene Ther.* 18, 403–410.
- Qiao, C., Yuan, Z., Li, J., *et al.* (2012). Single tyrosine mutation in AAV8 and AAV9 capsids is insufficient to enhance gene delivery to skeletal muscle and heart. *Hum. Gene Ther. Methods* 23, 29–37.
- Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines. (2000). *Arthritis Rheum.* 43, 1905–1915.
- Rehman, K.K., Wang, Z., Bottino, R., *et al.* (2005). Efficient gene delivery to human and rodent islets with double-stranded (ds) AAV-based vectors. *Gene Ther.* 12, 1313–1323.
- Singh, J.A. (2012). Stem cells and other innovative intraarticular therapies for osteoarthritis: what does the future hold? *BMC Med.* 10, 44.
- Singh, G., Fort, J.G., Goldstein, J.L., *et al.* (2006). Celecoxib versus naproxen and diclofenac in osteoarthritis patients: SUCCESS-I Study. *Am. J. Med.* 119, 255–266.
- Song, R.H., Tortorella, M.D., Malfait, A.M., *et al.* (2007). Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. *Arthritis Rheum.* 56, 575–585.
- Ulrich-Vinther, M. (2007). Gene therapy methods in bone and joint disorders. *Acta Orthopaedica* 78, 2–64.
- Ulrich-Vinther, M., Maloney, M.D., Goater, J.J., *et al.* (2002). Light-activated gene transduction enhances adeno-associated virus vector-mediated gene expression in human articular chondrocytes. *Arthritis Rheum.* 46, 2095–2104.
- Williams, J.M., Felten, D.L., Peterson, R.G., and O'Connor, B.L. (1982). Effects of surgically induced instability on rat knee articular cartilage. *J. Anat.* 134, 103–109.
- Yu, L.P., Smith, G.N., Brandt, K.D., *et al.* (1992). Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. *Arthritis & Rheumatism* 35, 1150–1159.
- Zhou, Q., Guo, R., Wood, R., *et al.* (2011). Vascular endothelial growth factor C attenuates joint damage in chronic inflammatory arthritis by accelerating local lymphatic drainage in mice. *Arthritis Rheum.* 63, 2318–2328.

Address correspondence to:

Dr. Constance R. Chu

Professor and Vice Chair Research

Orthopedic Surgery

Stanford University

450 Broadway Street, Pavilion C, 4th Floor

Redwood City, CA 94063-6342

E-mail: chucr@stanford.edu

Received for publication June 8, 2012;

accepted after revision March 7, 2013.

Published online: March 15, 2013.