

Multiple Recombinant Adeno-Associated Viral Vector Serotypes Display Persistent *In Vivo* Gene Expression in Vector-Transduced Rat Stifle Joints

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Abstract

Our aim was to investigate serotype-specific cell and tissue-transduction tropisms, transgene expression levels and longevity, and immunogenicity of candidate rAAV serotypes in rat osteochondral cells, tissues, and stifle joints. *In vitro*, we used six rAAV serotypes and two promoters to transduce synoviocytes and chondrocytes. Serotypes rAAV2/5 and 2/2 yielded the highest transduction efficiency 4 days after transduction. No differences were detected between cytomegalovirus and chicken β -actin promoters. *In vivo*, intra-articular injection was used to introduce four rAAV serotypes into 4-month-old rats in the left stifle joint. Eleven months later, serotype 2/5 vector, diluted with saline or surfactant, was injected into the right stifle joint of the same rats. Rats were analyzed up to 12 months after initial injection. Bioluminescence was detected at 7 days and all serotypes tested displayed bioluminescence above controls after 1 year in the left stifle. Gene expression was detected in the right stifle joints of all rats with the exception of rats previously injected with serotype 2/5. We observed no difference irrespective of whether the luciferin was injected subcutaneously or intraperitoneally. However, surfactant-diluted vectors led to increased gene expression compared with saline-diluted vectors. Cell- and tissue-specific transduction was observed in rat stifles injected with an *nLacZ*-containing rAAV. Transduction was greatest in stromal tissues and mesenchymal cell types. Exposure to a specific serotype did not inhibit subsequent transduction with a different serotype at a second vector injection. Including surfactant as a vector diluent increased gene expression within the stifle joint and should be considered for *in vivo* gene therapy applications.

Introduction

MOST OSTEOARTHRITIS THERAPIES treat symptoms of the disease and are not curative. Current therapies for osteoarthritis include the use of analgesics, nonsteroidal anti-inflammatory drugs, or intra-articular injections of hyaluronan or corticosteroids for temporary relief of pain and inflammation. Such treatments, however, can be associated with numerous side effects, including gastrointestinal erosion or hemorrhage, impairment of renal function, osteoporosis, and hypertension (Fraenkel *et al.*, 2004). In patients who fail to respond to these conventional measures, surgical intervention is required to provide relief from pain and disability (Walker-Bone *et al.*, 2000; Hunter and Felson, 2006).

Gene therapy is currently being investigated as an alternative approach to the treatment of arthritis. Osteoarthritis is

an appropriate target for a gene therapy approach because it is common, poorly treatable, and a leading cause of disability. Gene therapy can be used *in vivo* to modify the intra-articular environment of joints without disrupting the native joint architecture. Despite the promise of gene therapy, there are three major disadvantages that must be overcome before these therapies could be clinically viable: limited transduction efficiency of dense articular tissues (cartilage, ligament), transient transgene expression, and elicitation of immunologic responses (Evans *et al.*, 2006; Saraf and Mikos, 2006).

The use of an adeno-associated virus (AAV) to deliver the therapeutic gene may help us overcome these disadvantages. Limited transduction efficiency is often blamed on induction of a host immune response against the vector and/or viral proteins or on nonhomologous transgenes. AAVs are less immunogenic than commonly used adenoviral vectors, and

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most serotypes appear to have minimal immunogenicity in humans (Tenenbaum *et al.*, 2003; Cottard *et al.*, 2004; Wang *et al.*, 2011).

Articular joint gene delivery strategies have traditionally targeted the synovium (Hung *et al.*, 1994). A consensus of published studies has determined that transgene expression often persists for only 2–3 weeks after inoculation (Khoury *et al.*, 2006). In these studies, the vast majority of transduced cells are CD90⁺ synovial fibroblasts. Although CD90⁺ synovial fibroblasts are highly vulnerable to genetic modification, this cell population is also transient, with an apparent half-life in normal joints of less than 1 month (Gouze *et al.*, 2007). This half-life decreases further in diseased joints (Remmers *et al.*, 1990; Kaklamanis, 1992; Pan *et al.*, 1999). If the majority of vector-transduced cells in a target joint are transient, the long-term therapeutic effects in the joint will be based on the small remaining nontransient cell population. Small populations of nontransient cells do reside within synovial joints and provide alternative targets for transduction. Targeting cell types other than CD90⁺ synovial fibroblasts may provide a better opportunity for a long-term therapeutic effect. The different cell types present in articular joints may also respond differently to the introduction of a transgene. This observation points to the importance of establishing cell or tissue tropisms for the candidate vectors.

In the current experiment, we used six different rAAV serotypes (2/1, 2/2, 2/5, 2/6.2, 2/8, and 2/9) to transduce monolayer cells *in vitro*. In rat stifle joints *in vivo*, we used four different rAAV vector serotypes (2/2, 2/5, 2/8, and 2/9). Our aim was to investigate the serotype-specific cell and tissue-transduction tropisms, transgene expression levels and longevity, and immunogenicity of each serotype. Among the different serotypes, rAAV5 and rAAV2 have been reported to have superior transduction efficiency in rodent arthritic joints and rAAV2 has been used in rat *in vivo* studies (Payne *et al.*, 2011). Serotypes rAAV2/8 and 2/9 have enhanced *in vivo* transduction properties in our laboratories despite their lackluster *in vitro* performance (Allocca *et al.*, 2007; Keswani *et al.*, 2012; Mason *et al.*, 2012). In addition to serotype comparisons over a 1-year period, we investigated the use of an increased concentration of surfactant as a diluent for vector delivery. Finally, we examined the parameters required for accurate and consistent analysis of *in vivo* rAAV-transduced rat stifles.

Materials and Methods

Vector production

Recombinant AAV2-cytomegalovirus (CMV)–firefly luciferase (*ffl*), AAV2-CMV-*nLacZ*, AAV2-chicken β -actin (CB)–*ffl*, and the rAAV2/1, 2/2, 2/5, 2/6.2, 2/8, and 2/9 packaging constructs were generated, and vectors were produced by triple transfection and purified as previously described (Xiao *et al.*, 1998; Auricchio *et al.*, 2001; Hildinger *et al.*, 2001; Gao *et al.*, 2002). Briefly, a CMV- or CB-driven transgene cassette encoding *ffl* or *nLacZ* flanked by AAV2-inverted terminal repeats was co-transfected with a packaging plasmid encoding the *rep* and *cap* genes. Adenoviral helper function was delivered *in trans* from a third helper plasmid, p Δ F6. Recombinant AAV vectors were purified as previously described (Gao *et al.*, 2002). All vector preparations were banded on three, sequential CsCl density gradients. Vector titers were evaluated

by TaqMan real-time polymerase chain reaction with primers and a probe specific for the polyadenylation signal in the vector transgene cassette (bGH forward, GCCAGCCATCT GTTGT; bGH reverse, GGAGTGGCACCTTCCA; bGH probe, 6FAM-TCC CCC GTG CCT TCC TTG ACC-TAMRA).

Experiment one

All experiments were conducted in accordance with protocols and procedures approved by the Institutional Animal Care and Use Committee of The University of Pennsylvania. Cartilage and synovial tissues were collected aseptically from the stifle joints of four 3-month-old male Sprague-Dawley rats. Cartilage was collected from the tibial plateau and femoral condyle and consisted of cartilage tissue from the tangential layer to the tidemark. Synovial tissue was collected from the areas surrounding the patella and articular surfaces and consisted of synovial, adipose, and fibrous capsular tissue. Samples were collected within 30 min after euthanasia. The tissue was placed in 50 ml centrifuge tubes with PBS+ (PBS with CA2⁺ and Mg2⁺) that contained a 2 \times antimicrobial solution and was maintained at room temperature (21°C) until all tissues were collected. Cells were isolated from explant tissue and cultured as described elsewhere (Mason *et al.*, 2012). Cells were plated at 3.3 \times 10³ cells/cm² in 150 mm tissue culture-treated dishes and cultured with DMEM/F12 culture medium with 10% fetal bovine serum. An additional 50 μ g of ascorbate-2-phosphate/ml was added to chondrocyte cultures. All cells were used at passage three for transduction assays. Non-transduced control cells were cultured and assayed in parallel with experimental groups. For *in vitro* transduction experiments, 1.0 \times 10³ mitomycin-C-inactivated cells were plated directly in 96-well, opaque-walled assay plates (day 0) with and without surfactant (0.01% final surfactant concentration [Pluronic F68]) and transduction was initiated the following day (day 1).

Monolayer-cultured chondrocytes and synoviocytes were transduced with six rAAV serotypes (2/1, 2/2, 2/5, 2/6.2, 2/8, and 2/9) and two promoters (CMV and CB) with *ffl* as the marker gene. Cells were transduced at 1.0 \times 10² genome copies (GCs)/cell in 100 μ l of a basal medium consisting of DMEM/F12 with no protein added. One hour after initiation of transduction, an additional 100 μ l of a serum-containing culture medium was added to each well. Two hours after initiation of transduction, cells were rinsed with PBS⁺ solution and the wells were filled with 200 μ l of the serum-containing culture medium and replaced in the incubator (37°C at 5% CO₂).

Four days after initiation of transduction, the cells were lysed and bioluminescence was measured 2 sec after addition of 100 μ l of luciferase assay substrate (Promega) in a bioluminescence-imaging plate reader and recorded as relative light units per well. Transduction efficiency for monolayer-cultured cells was defined as bioluminescence per cell (BPC). Corrections were not necessary for cell growth over the 4-day culture period between vector inoculation and the assessment of gene expression since the cells were mitotically inactivated before plating and transduction.

Experiment two

An intra-articular injection was used to introduce rAAVs to the left stifle joint of 16 four-month-old male

Sprague-Dawley rats. Rats were anesthetized by intraperitoneal (IP) injection of ketamine (30 mg/kg) and xylazine (10 mg/kg). The injection site was clipped and aseptically prepared, and 1.0×10^{10} GCs of vector that contained an *ffl* marker gene diluted to a 50 μ l volume with saline was injected intra-articularly into the left stifle joint using a Hamilton syringe with a 30-gauge needle. A 50 μ l volume of saline was injected intra-articularly into the right stifle joint as an injection control. As in the *in vitro* experiments, we used *ffl* as our marker gene. Live animal bioluminescence imaging was used to determine the extent of gene expression of reporter genes in the injected joints. To collect quality data from our *in vivo* bioluminescence imaging experiment, the parameters for collection of data were closely defined for treatment or experimental group. We used a Xenogen VivoVision IVIS Lumina optical imaging system. Animals were anesthetized and injected with luciferin (150 mg/kg) through either a subcutaneous (SC) or IP route. Image acquisition began 1 min after luciferin injection and continued for 61 min. Data were collected as bioluminescence (p/sec/cm²/sr) in a designated region of interest (ROI) with a unique ROI for each stifle joint and for control regions used for normalization of bioluminescence values. Bioluminescence data were also plotted against time. Peak and time-to-peak bioluminescence values were extrapolated from the plots (Fig. 1). Imaging began at 7 days after vector injection and continued at increasing intervals throughout 1 year.

Experiment three

Eleven months after the initial vector injection in the left stifle, all the rats used for *in vivo* bioluminescence imaging in experiment two were reinjected in the right stifle with 1.0×10^{10} GCs of an *ffl*-containing serotype 2/5 vector. For this experiment, the injected vector was diluted with either saline or surfactant (0.01%). At the end of the experiment, rats were anesthetized injected with luciferin as previously described, and both stifles were imaged. After both stifles were imaged in the live rats, the rats were euthanized and the stifles were further imaged during dissection.

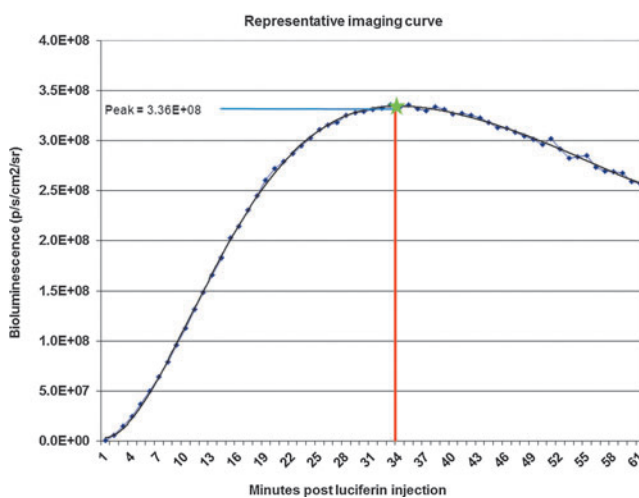


FIG. 1. Representative imaging curve. Bioluminescence readings at 1 min intervals were plotted for each animal beginning at the time of luciferin injection.

Experiment four

Naïve rats were injected in both left and right stifles with 1.0×10^{10} GCs of an *nLacZ*-containing serotype 2/9 vector. The vector was again diluted with either saline or surfactant as in experiment three. Three weeks after vector injection, confirmatory tissue sections from sacrificed animals were analyzed for depth of vector penetration. Stifle joints were removed and were stained for 48 hr for beta-galactosidase (β -gal) expression. After staining for β -gal expression, the rat stifle joints were fixed in 10% neutral buffered formalin (NBF) for 48 hr. The joints were subsequently decalcified. Briefly, the joints were washed free of NBF with double-deionized (DDI) water and placed in 5% formic acid that was changed daily. After 5 days in formic acid, the joints were washed with DDI and soaked in DDI overnight, after which they were immersed in 70% ETOH and submitted for histology. The joints were bisected coronally at the mid-point of the joint. The cranial section containing the patella was subsequently bisected in a sagittal orientation. The sections were routinely processed and embedded for analysis.

It has been reported that synoviocytes may produce significant endogenous levels of lysosomal galactosidase, an enzyme that may react with X-Gal during the β -gal staining procedure and produce false-positive signals (Roessler *et al.*, 1993). We used a nuclear-targeted *nLacZ* gene to more easily identify β -gal staining that was caused by vector injection in contrast to endogenous β -gal-positive signals from lysosomal galactosidase in synoviocytes. Nontransduced tissues were included during the staining procedures as negative controls.

Statistical analysis

Experimental data were analyzed preliminarily by one-way ANOVA and linear regression analysis. Significance tests for each predictor in the multiple linear regression model were implemented with the Student's *t*-test, two-tailed, unequal distribution of variance assumed and Chi-square (χ^2) analysis where appropriate. Test results were considered significant for P-values $P < 0.05$.

Results

Transduction of monolayer cell cultures

Cells were isolated from the tissues, plated in monolayer cultures, and transduced with six different rAAV vector serotypes. Four days after initiation of transduction, the highest ranking serotypes were clearly separated from the lowest ranking serotypes on the basis of a considerably higher BPC. Cells transfected with serotypes rAAV2/5 and 2/2 displayed the greatest BPC in chondrocyte and synoviocyte cultures (Fig. 2A). In all serotypes tested, synoviocytes were more readily transduced than chondrocytes. No differences were detected between cells transduced with vectors containing a CB promoter, compared with a CMV promoter (Fig. 2B), or in cell cultures with or without surfactant (data not shown).

In vivo subcutaneous versus intraperitoneal luciferin delivery

Because of the possibility that repeated intra-articular injection of luciferin over the period of 1 year might alter normal stifle joint physiology and pathology, we opted for an indirect luciferin delivery method. We compared both SC

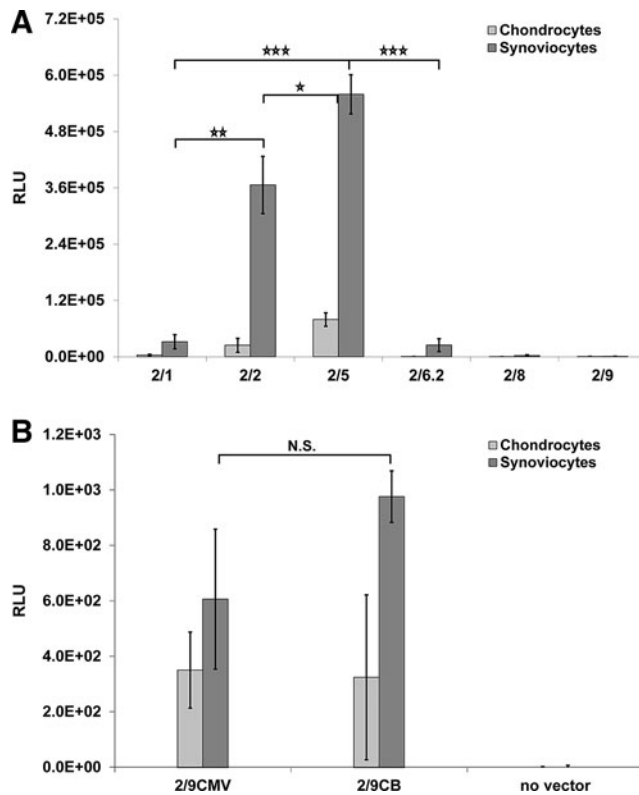


FIG. 2. Mean RLU results for serotype transduction in control and vector-inoculated monolayer-cultured cells. **(A)** Four days after vector inoculation at 1.0×10^2 GCs/cell in monolayer-cultured synoviocytes and chondrocytes, serotype rAAV2/5 displayed the highest BPC, followed by serotypes rAAV2/2 and rAAV2/1. **(B)** No difference was detected between cells transduced with CMV or CB promoter-containing vectors. Error bars represent SE ($*p < 0.1$, $**p < 0.05$, $***p < 0.01$). BPC, bioluminescence per cell; CB, chicken β -actin; CMV, cytomegalovirus; GCs, genome copies; N.S., not significant; RLU, relative light units; SE, standard error.

and IP luciferin delivery and found that luciferin effectively reached the joint capsule with both delivery methods.

Very little difference in bioluminescence signal was observed irrespective of whether the luciferin was injected SC or IP. However, the dynamics of luciferin-induced bioluminescence was significantly different between vector serotypes (Fig. 3). Serotype 2/5 reached its peak level of expression sooner than the other serotypes, and serotype 2/2 took almost twice as long as the other serotypes to reach its peak expression level. This trend was maintained whether the luciferin was injected SC or IP.

In vivo bioluminescence is often measured shortly after luciferin injection. We measured the slope of increasing bioluminescence from 1 to 10 min after luciferin injection to capture the linear range of the bioluminescence curve in all animals imaged. The ranking of the slopes of increasing bioluminescence did not correlate well with the ranking of the peak bioluminescence values (no correlation between slope and peak in 17 of the 28 curves, 61%).

Temporal gene expression for a 1-year period

In vivo bioluminescence persisted for 1 year after initial vector inoculation. Bioluminescence was detected at the first

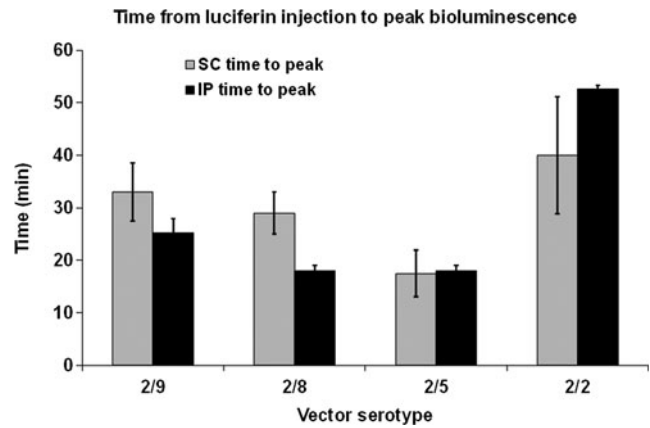


FIG. 3. Time from luciferin injection to peak bioluminescence. Animals were anesthetized and injected with luciferin through either an SC or IP route, and images were collected beginning at 1 min after luciferin injection and imaging continued for 61 min. Bioluminescence is plotted against time. Peak and time-to-peak values were extrapolated from the plots. Error bars represent SE. IP, intraperitoneal; SC, subcutaneous.

imaging time point of 7 days and all four vector serotypes tested displayed bioluminescence levels above controls at 1 year after inoculation. Vector serotype 2/9 was initially the highest expressing serotype and remained the highest expressing serotype throughout the experiment (Fig. 4). Serotype 2/9 was also the only vector serotype to display a significant decrease in gene expression after the 1-month imaging time point.

Localization of gene expression

During the year-long experiment, marker gene expression was never detected outside of the injected stifle joint capsule

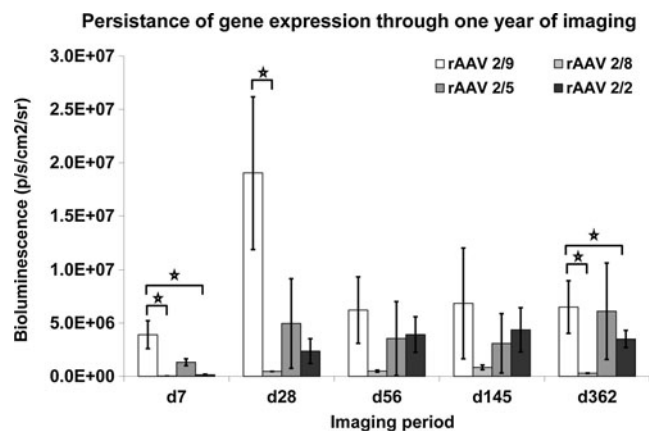


FIG. 4. Persistence of gene expression through 1 year. Imaging began at 7 days after vector injection and continued at increasing intervals through 1 year. We used a Xenogen VivoVision IVIS Lumina optical imaging system to detect bioluminescence from an ROI encompassing the vector-injected stifle joint. Data are presented as mean bioluminescence (p/sec/cm²/sr) from designated ROIs (normalized with ROIs from nontransduced tissues). Error bars represent SE ($*p < 0.1$). ROI, region of interest.

using *in vivo* bioluminescence imaging. At euthanasia, the stifle joints were imaged during dissection to localize bioluminescence within the dissected joint tissues. Bioluminescence was detected in most tissues surrounding the joint, but was never detected on intact cartilage surfaces (Fig. 5).

To increase the precision of the localization of vector transduction within the injected stifle joints over what was capable with *in vivo* bioluminescence imaging, localization was additionally observed in histological sections from rats injected with an *nLacZ*-containing rAAV.

Gene expression 3 weeks after vector inoculation revealed that transduction was greatest in stromal tissues and mesenchymal cell types (Fig. 6). In these representative sections, transduced cell types included, but were not limited to, osteoblasts, osteoclasts, adipocytes, chondrocytes, muscle cells, synovial fibroblasts, and macrophages. Transduction appeared to favor more primitive cell types.

Reinjection of AAV vector

Eleven months after the initial marker gene vector injection in the left stifle, the same rats used for the initial *in vivo* bioluminescence imaging were reinjected with vector serotype 2/5 in the right stifle with and without surfactant. All rats displayed detectable gene expression above background in the right stifle by 14 days after injection (Fig. 7). The one exception to this was the rats previously injected in the left stifle using serotype 2/5. None of these previously 2/5-serotype, left-stifle-injected rats displayed any detectable gene expression in the 2/5-injected right stifle. However, all of these rats did maintain gene expression in the left stifle previously injected with the 2/5 vector. The efficiency of rAAV2/5 vector for right-stifle-directed gene transfer of the luciferase marker gene was not impacted by preimmunization with the other rAAV serotypes tested in the left stifle. At

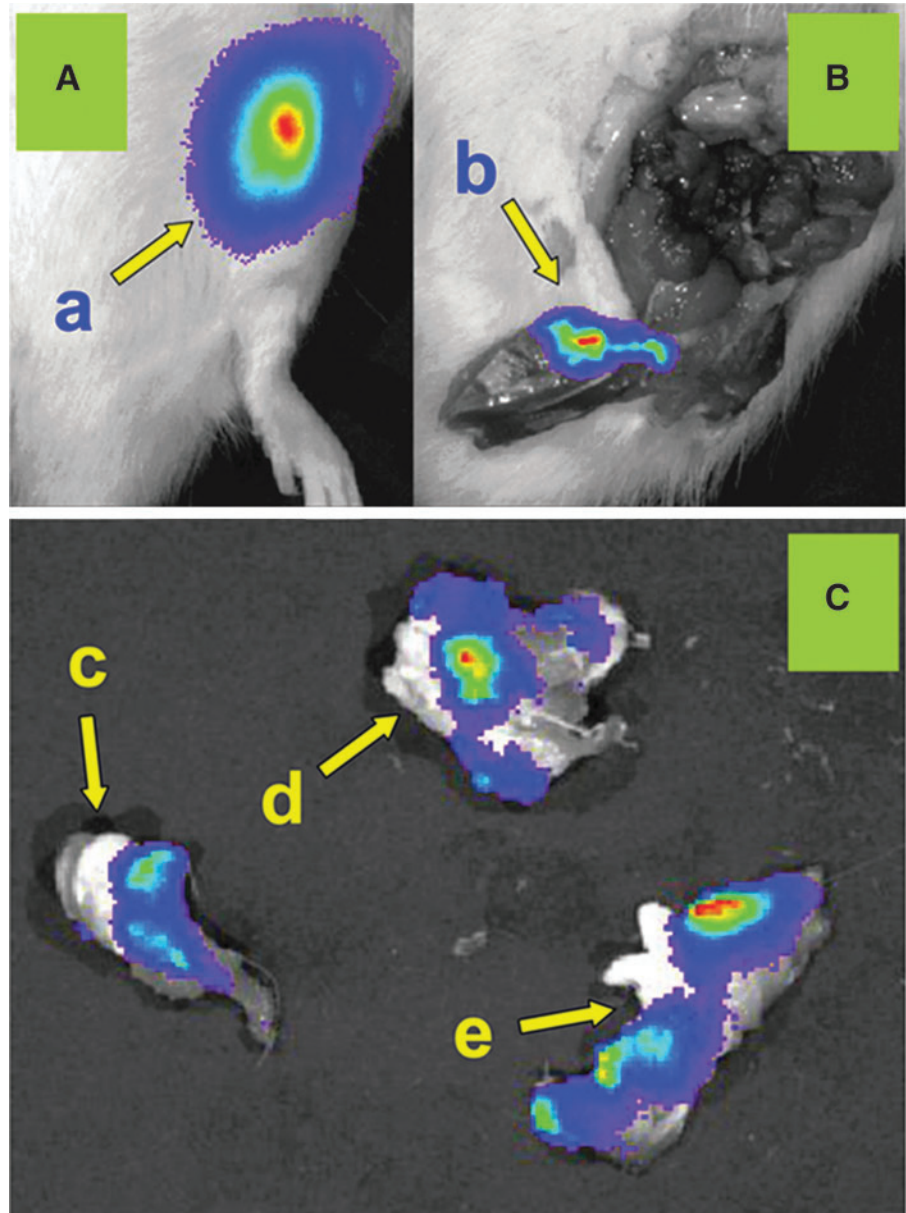


FIG. 5. *In vivo* bioluminescence produced from luciferase gene expression within the vector-injected stifle joint. **(A)** *In vivo* bioluminescence emanating from the *ffl* vector-injected left stifle joint. (a) Representative *in vivo* luciferin-injected stifle before dissection. **(B)** Postdissection bioluminescence after removal of the stifle joint. (b) Bioluminescence from skin and connective tissue that previously covered the patellar region. **(C)** Bioluminescence was detected abundantly within joint tissues with the exception of the cartilage surfaces. Cartilaginous surfaces with no detectable bioluminescence signal included (c) femoral condyles, (d) tibial plateau, and (e) patellar cartilage surface.

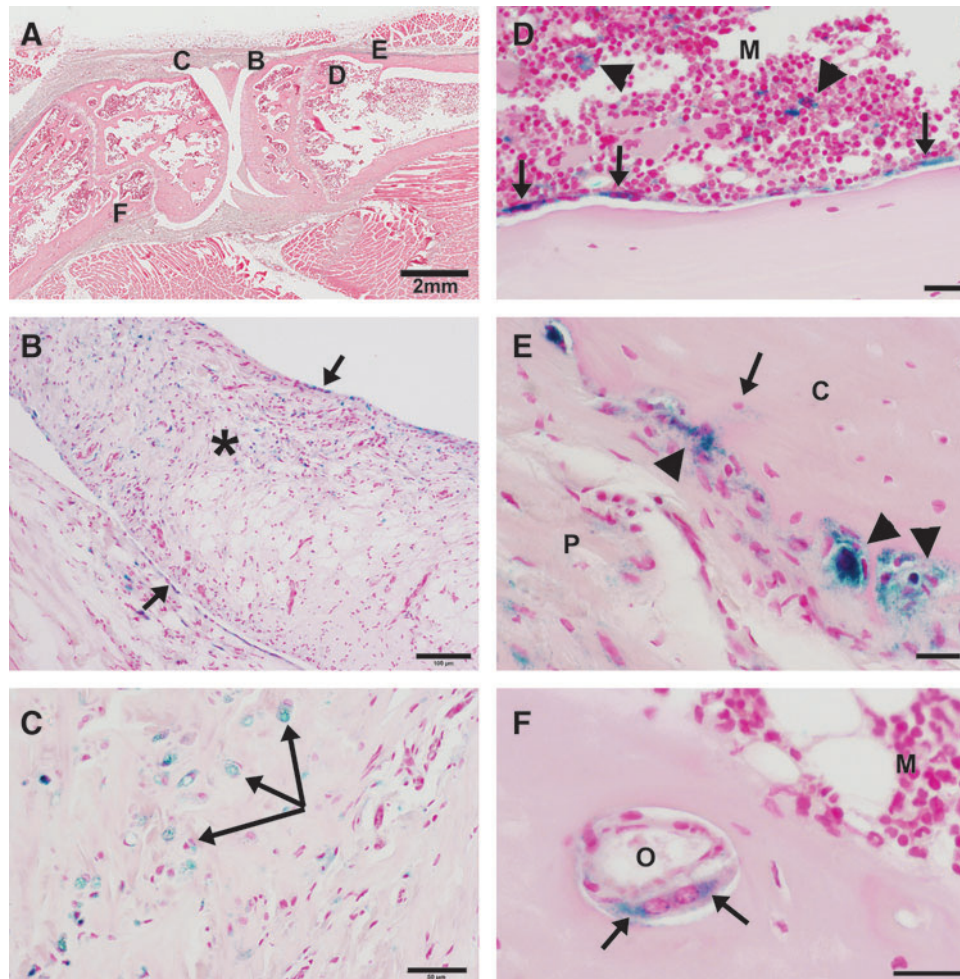


FIG. 6. Representative beta-galactosidase staining of stifle joint tissues injected *in vivo* with an *nLacZ* marker-containing vector. **(A)** Low-magnification (scale bar = 2 mm), hematoxylin & eosin–stained photomicrograph of a sagittal section of the femorotibial joint (left, distal femur; right, proximal tibia). **(B–F)** Regions of the section highlighted in the remaining panels in this composite figure. **(B)** Higher magnification (scale bar = 100 μ m) of region B in panel A shows β -gal stain within cells lining the synovial membrane (arrows) and within cells of the subsynovial fibroadipose tissue (asterisk). **(C)** Higher magnification (scale bar = 50 μ m) of region C in panel A shows β -gal stain within chondrocytes (arrows) and spindle cells of the fibrocartilage within the patellar tendon. **(D)** Higher magnification (scale bar = 25 μ m) of region D in panel A shows β -gal stain within bone-lining cells along the endosteal surface (arrows) and within cells of the bone marrow (M). **(E)** Higher magnification (scale bar = 20 μ m) of region E in panel A of the periosteum from the proximal cranial tibial cortex. C indicates β -gal stain within cells lining the bone (morphology consistent with osteoblasts and multinucleate osteoclasts; arrow heads), an osteocyte (arrow), and within spindle cells of the periosteum (P). **(F)** Higher magnification (scale bar = 20 μ m) of region F in panel A of bone from the distal caudal femoral cortex shows β -gal stain within spindle cells (arrows) lining the endoseum of an osteonal canal (O) in proximity to the medullary cavity (M).

the end of the study, all rats tested displayed neutralizing antibodies (Nab) to the vector serotypes they had been injected with, but not to any of the other serotypes we used. Rats injected twice with the 2/5 serotype, once in each stifle, had no greater 2/5 Nab load than rats injected only once with this serotype.

Surfactant as a vector diluent

The use of a surfactant as the vector diluent instead of saline increased gene expression in the stifle joint as measured with *in vivo* bioluminescence imaging. All rat stifle joints that received vector diluted with surfactant displayed greater bioluminescence than joints that received vector diluted in saline (Fig. 7).

Discussion

In the current *in vivo* experiments, we used rAAV vector serotypes 2/2, 2/5, 2/8, and 2/9. Serotypes 2/2 and 2/5 have been used previously to transduce articular joints *in vivo* and are consistently top-performing *in vitro* serotypes when using articular joint cells and tissues. Serotypes 2/8 and 2/9 are often poor *in vitro* transducers, but were chosen for this experiment because of their past performance with *in vivo* experiments in our laboratory. At 7 days after vector injection, the level of gene expression detected for serotype 2/9-injected rats was fourfold above compared with that for serotype 2/5-injected rats, the only two detectable expressing serotypes above control levels of bioluminescence at 7 days after vector inoculation. This is in contrast to previous

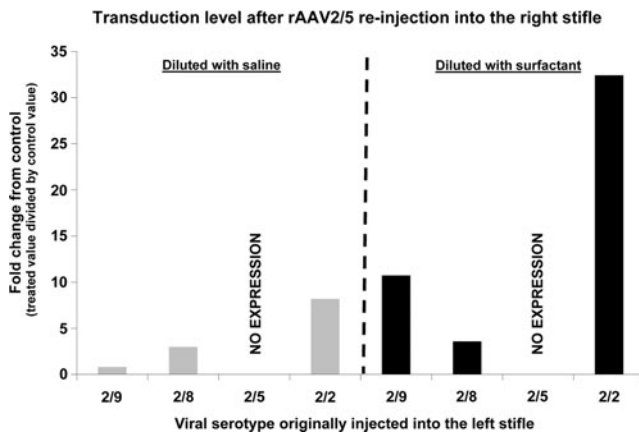


FIG. 7. Neutralizing antibodies and surfactant dilution. Eleven months after the initial marker gene injection with various serotypes in the left stifle (previously injected serotype along the x-axis), the rats used for *in vivo* bioluminescence imaging were re-injected with 1.0×10^{10} genome copies of vector serotype 2/5 in the right stifle. For this experiment, the injected vector was diluted with either saline or surfactant (0.01% final surfactant [F68] concentration). Unfortunately, not all animals initially injected with the various serotypes were available for the second injection 11 months later. For serotype 2/9 animals, three were re-injected with serotype 2/5 vector diluted with saline and two with vector diluted with surfactant. For serotype 2/8 animals, one was re-injected with vector diluted with saline and two with vector diluted with surfactant. For serotype 2/5 animals, two were re-injected with vector diluted with saline and one with vector diluted with surfactant, and for serotype 2/2 animals, one was re-injected with vector diluted with saline and two with vector diluted with surfactant. These results reflect what has been observed when using surfactant supplementation in other animal models, and the use of surfactant supplementation has become standard practice in our laboratory. No transduction in the right stifle was observed in rats previously injected in the left stifle with serotype 2/5.

reports where bioluminescence in the rat stifle joint was first reported 14 days after vector injection (Payne *et al.*, 2011). However, in the Payne *et al.*'s study, serotype 2/2 was utilized and their results were similar to ours for this serotype. Serotype 2/2-injected animals in our study also did not display detectable gene expression until 14 days after injection. After 4 weeks, serotypes 2/5 and 2/2 both displayed stable gene expression well above control levels and just below the levels displayed by 2/9-injected rats. These levels of expression persisted throughout the remainder of the experiment, suggesting that similar cell types were transduced by these vector serotypes and that they were not transient cell populations, but more stable, resident cell populations.

Vector serotype 2/9 displayed a significant decrease in gene expression between 4 and 8 weeks after vector injection. This observed decrease may have been caused by initial high transduction of a transient cell population, such as $CD90^+$ synovial fibroblasts, which have a high turnover rate and may have contributed to the high initial rate of gene expression seen with serotype 2/9 (Gouze *et al.*, 2007). In previous reports (Goater *et al.*, 2000), transgene expression within the joint was lost within 3 weeks, suggesting that the vectors used in that study elicited a strong immune re-

sponse or that the vectors transduced only a transient cell population.

Serotype 2/9 has previously displayed long-term gene expression in many tissue types, including cardiac tissue (Fechner *et al.*, 2008; Jiang *et al.*, 2009), nervous system (Rahim *et al.*, 2011; Mattar *et al.*, 2012), vascular tissue (Kotchey *et al.*, 2011), lung (Bell *et al.*, 2011; Pfeifer *et al.*, 2011), liver (Chen *et al.*, 2009; Luo *et al.*, 2011), and skeletal muscle (Koo *et al.*, 2011; Pulicherla *et al.*, 2011; Wang *et al.*, 2011). When delivered systemically (tail vein injection), the viral genome clearance rate from the blood was slower for serotype 2/9 than any other serotype tested (Zincarelli *et al.*, 2008; Kotchey *et al.*, 2011).

The superiority of serotype 2/9 *in vivo* is in contrast with our current *in vitro* observations and in contrast with previous observations using articular joint cells and tissues (Mason *et al.*, 2012). In the current study, serotype 2/9 displayed the lowest *in vitro* gene expression among all serotypes tested. As seen in previous work, synoviocytes displayed superior *in vitro* gene expression levels compared with chondrocytes.

In vivo bioluminescence is frequently measured soon after luciferin injection, often within 5–10 min. In the current experiment, values from this early, linear portion of the bioluminescence curve correlated with the ultimate peak bioluminescence value in only 11 of 28 curves plotted (39%). This observation suggests that peak bioluminescence may be a better measure of differences in gene expression between treatments than an earlier reading taken during the linear portion of the curve.

SC and IP luciferin delivery allowed us to capture the peak bioluminescence from the joint because of the time delay during diffusion of luciferin from the site of injection into the joint. *In vivo* SC and IP luciferin delivery was technically less demanding than intra-articular delivery and eliminated any chance of the injection needle causing joint damage after repeated intra-articular injections over the course of the experiment. Additionally, because luciferin was distributed throughout the body, we would have detected any extra-articular bioluminescence, which would be difficult to detect using intra-articular luciferin delivery. Previous reports have addressed the issue of SC versus IP luciferin injection and the issue of the timing of *in vivo* bioluminescence imaging, but the issue of luciferin delivery within the stifle joint of mature male rats had not been addressed (Inoue *et al.*, 2009, 2010). In our rat stifle joints, very little difference in bioluminescence signal was detected whether luciferin was injected through an SC or an IP route. By injecting luciferin IP or SC, rather than intra-articularly, we were able to confidently scan the entire animal for bioluminescence every time we imaged.

Marker gene bioluminescence was never detected outside of the injected stifle joint capsule using *in vivo* bioluminescence imaging. Our rats were imaged in three distinct orientations to detect bioluminescence from any region of the animal, including, but not limited to, the contralateral stifle, IP luciferin injection site, SC luciferin injection site, abdomen (liver region), head, and testis. Bioluminescence was occasionally detected from the metallic ear tags used to identify each animal. These observations suggest limited systemic exposure and limited gene transfer outside the joint capsule, which may potentially help to reduce an animal's total viral load. The volume of luciferin we used was calculated from

literature values for similar studies and from previous *in vivo* imaging in our laboratory. The concentration and/or volume of luciferin used can affect imaging parameters, but should not have had an effect on the vector serotype hierarchy we observed in time-to-peak bioluminescence.

While we noticed very little difference in bioluminescence caused by the luciferin injection route, we did discover a significant difference in the timing of the response to luciferin depending on the vector serotype used. Vector serotype 2/5 reached peak bioluminescence earlier than any of the other serotypes, and serotype 2/2 took roughly twice as long as the other serotypes to reach peak bioluminescence. These differences in the timing of peak bioluminescence likely reflected the specific cell types transduced and/or differences in systemic circulation patterns dictating access of luciferin to the specific cells and tissues transduced by each serotype.

Various AAV vector serotypes are known to possess specific cell and tissue tropisms or the ability to transduce different populations of cells or tissue types with a range of efficiencies. *In vivo* bioluminescence is very good at providing a qualitative assessment of gene expression for an ROI in an animal, but to determine specific cell and tissue types transduced, we used basic histology of tissue sections from rats injected with an *nLacZ*-containing rAAV to acquire a more precise depiction of transduction effectiveness within the stifle joint. Gene expression 3 weeks after vector inoculation revealed that transduction was greatest in stromal tissues and mesenchymal cell types and appeared to favor more primitive cell types. This may be one explanation for the longevity of gene expression observed in our vector-injected stifle joints. However, this 3-week time point was before the post-4-week drop in gene expression detected with serotype 2/9 in the year-long *in vivo* bioluminescence experiments and was therefore likely to include transient as well as stably transduced cell populations.

The host immune response to AAV capsid can be species specific and is mediated principally by circulating antibodies, which may prevent initial or repeated AAV administrations (Manno *et al.*, 2006). Among individuals that are seropositive for AAV9, most present with low titers (Boutin *et al.*, 2010). To test if our vectors injected into the left stifle joint and expressing only in the left stifle joint had produced a systemic immune response great enough to prevent repeat AAV administration with other serotypes, 11 months after the initial marker gene vector injection, the same rats previously injected with various serotypes in the left stifle were re-injected with vector serotype 2/5 in the right stifle. All rats displayed detectable gene expression above background by 14 days after injection with the exception of rats previously injected in the left stifle with serotype 2/5. None of these previously 2/5-injected rats displayed any detectable gene expression in the right stifle, but did maintain gene expression in the previously injected left stifle. Preimmunization with serotypes other than 2/5 did not impact the efficiency of stifle-directed gene transfer of the luciferase marker gene by a serotype 2/5 vector.

Delivery of AAV vector *in vitro* or *in vivo* is often compromised by fractional loss of the vector to the devices used to handle and/or deliver the vector. In a previous study (Bennicelli *et al.*, 2008), vector recovery was assessed *in vitro* in the presence or absence of surfactant after vector dilution and passage through injection devices. In this study, recov-

ery of the vector approximated 100% in the presence of surfactant and ranged from 24% to 51% without the use of surfactant. When used *in vivo*, surfactant treatment significantly increased β -gal expression in mouse adipose tissue (Zhang *et al.*, 2011). *In vitro*, we found no difference in gene expression when cells were transduced in the presence of surfactant compared with saline. However, *in vivo* with our 11-month reinjection experiment, all rats that received vector diluted with surfactant displayed greater bioluminescence than rats that received vector diluted in saline. In addition, rat joints injected with an *nLacZ*-marker gene that was diluted with surfactant subjectively appeared to display a superior range of tissue gene expression within the joint compared with saline-diluted vector. This surfactant effect is further supported by results from the use of surfactant dilution in other animal models of *in vivo* transduction in our laboratory. The use of a surfactant as the vector diluent instead of saline significantly increased the gene expression in the stifle joint as measured with *in vivo* bioluminescence imaging. The appearance of the β -gal-stained tissues appeared to suggest that surfactant may have increased the invasiveness of the viral particles, but further experiments will be required to determine if the increase in observed gene expression with surfactant was caused by a superior range of tissues transduced or simply a decrease in vector loss at delivery, which resulted in a greater dose of vector being delivered to the tissue.

In summary, differences in the dynamics of *in vivo* bioluminescence were detected between the various vector serotypes tested. This suggests that the serotype used should be considered during collection of *in vivo* bioluminescence data. No differences were detected irrespective of whether luciferin was injected using an IP or SC luciferin injection route. This observation presents the option to choose from multiple methods of luciferin delivery based on specific experimental requirements. All serotypes tested displayed year-long persistence of gene expression, which was restricted to the injected stifle joint, and serotype 2/9 displayed the highest gene expression of all serotypes tested. However, gene expression was not detected at the cartilage surfaces, but tended to favor more primitive cell types within the joint. Exposure to a specific serotype did not inhibit subsequent transduction with an alternate serotype at a second vector injection. Successive rAAV treatments can be a complication for some *in vivo* rAAV vector delivery schemes. Multiple treatments may be successful if alternate vector serotypes are utilized. Including surfactant as a vector diluent can increase measurable gene expression within the stifle joint and should be considered for *in vivo* gene therapy applications.

Acknowledgments

We gratefully acknowledge Drs. Peter Bell, affiliated with Gene Therapy Program, and Steven Berkowitz, associated with Unionville Equine Associates, Oxford, PA, 19363; Ms. Deirdre McMenamin and Ms. Christine Draper, affiliated with Gene Therapy Program, and Mr. Ryan Delaney, affiliated with Department of Clinical Studies, for their intellectual support and technical assistance. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number

1F32AR056936-01A2. Research was also supported by The University of Pennsylvania Vector Core Facility and a grant to J.M.W. (NIH Grant 2-P30-DK047757-16). Recombinant AAV preparations were provided by The University of Pennsylvania Vector Core Facility (Philadelphia, PA). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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Received for publication October 11, 2012;
accepted after revision April 24, 2013.

Published online: May 9, 2013.