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AAV-MEDIATED, *IN VIVO* GENE DELIVERY TO THE ROTATOR CUFF

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Abstract

Tendon injuries present a considerable clinical challenge due to the limited regenerative capacity of tendons. The use of gene transfer to deliver growth factors to sites of tendon damage has been suggested as a promising strategy for improving tendon regeneration. A major issue for this approach is to identify clinically acceptable vectors that can deliver genes to the cells of the tendon, preferably by *in vivo* delivery. Adeno-associated virus (AAV) has many advantages in this regard, including a favourable safety profile and the ability to sustain long-term transgene expression. Here we explored the use of AAV to deliver marker genes to the supra- and infra-spinatus tendons of the rotator cuff in the rat by injection into the subacromial space. First, we screened various AAV serotypes for their transducing ability towards rat and human tenocytes *in vitro*. Of the 10 serotypes tested, AAV2.5 and AAV2 exhibited the highest *in vitro* transduction efficiency in both rat and human tenocytes. *Ex vivo* transduction of cells within explants of isolated, intact tendon was also demonstrated. Injection of AAV2.5 encoding luciferase into the subacromial space confirmed gene delivery to the infra-, but not supra-, spinatus tendon *in vivo* with transgene expression persisting for 7 days post-transduction. These data demonstrate the ability of AAV2.5 to deliver genes to the infraspinatus tendon, leading to sustained local expression following *in vivo* delivery. Our findings suggest that AAV2.5 has several advantages as vector for stimulating tendon regeneration by local, *in vivo*, gene transfer.

Keywords: Tendon healing, supraspinatus tendon, infraspinatus tendon, rotator cuff, tenocytes, adeno-associated virus, adeno-associated virus 2.5, gene therapy for tendon healing.

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List of Abbreviations

AAV	adeno-associated virus
MMPs	matrix metalloproteinases
TIMPs	tissue inhibitors of metalloproteinases
FDA	U.S. Food and Drug Administration
PBS	phosphate-buffered saline
P/S	penicillin/streptomycin
DMEM	Dulbecco's modified Eagle's medium
FBS	foetal bovine serum
PEI	polyethylenimine
GFP	green fluorescent protein
CMV	cytomegalovirus
qPCR	quantitative polymerase chain reaction
ITR	Inverted terminal repeat
ROI	region of interest
dsDNA	double-stranded DNA
IgG	immunoglobulin G
IVIS	*in vivo* imaging system
EMA	European Medicines Agency
b-FGF	basic fibroblast growth factor
TGF-β1	transforming growth factor beta 1
VEGF	vascular endothelial growth factor
GDF-5	growth/differentiation factor 5
PDGF-B	platelet-derived growth factor subunit B
BMP	bone morphogenetic protein
IL	Interleukin
TNF-α	Tumor necrosis factor alpha

Introduction

Tendons are complex structures that play a vital role in facilitating joint movement and transmitting forces from muscle to bone. Tendon injuries have become more prevalent due to various factors, including overuse or repetitive stress, age-related degeneration and inadequate recovery periods after strenuous exercise (Aicale *et al.*, 2018; Andarawis-Puri *et al.*, 2015; Wu *et al.*, 2017). These factors can lead to structural changes in the tendon, resulting in degeneration, inflammation and tears that cause pain, functional limitations and impaired quality of life (Ackerman *et al.*, 2021; Docheva *et al.*, 2015). Tenocytes are specialized fibroblast-like cells that play a crucial role in the structure and function of tendons (Kannus, 2000; Li *et al.*, 2021). Scleraxis and tenomodulin are specific molecular markers of tenocytes that are likely to play major roles in tendon regeneration (Aslan *et al.*, 2008). Tenocytes produce extracellular matrix proteins including collagen types I and III, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs), which are involved in the healing and remodeling processes in the tendon (Birch *et al.*, 2013; Subramanian and Schilling, 2015).

Rotator cuff tendons, forearm extensors, Achilles tendon, tibialis posterior and patellar tendons are particularly prone to injury. Shoulder disorders account for over 4.5 million patient visits annually in the United States (Gomoll *et al.*, 2004; Linaker and Walker-Bone, 2015). Indeed, injuries to the rotator cuff are among the most common musculotendinous conditions treated by orthopaedic surgeons. The rotator cuff comprises multiple tendons; healing of damaged tendons is typically slow and often leads to suboptimal outcomes in terms of structural integrity and mechanical strength (Dang and Davies, 2018). This poses a significant clinical challenge with a considerable impact on the well-being of patients, highlighting the need for effective treatment strategies to address tendon injuries and alleviate the associated burden (Jain *et al.*, 2013; Teunis *et al.*, 2014).

Various growth factors show promise as mediators of tendon regeneration (Docheva *et al.*, 2015) but they are difficult to deliver in a sustained fashion to sites of tendon damage. Gene delivery has been suggested as a means to overcome the delivery problem but little progress has been made in applying this concept to tendons; there have been few large animal studies and no clinical trials (Evans and Huard, 2015). Selection of the vector to use is a critical pre-clinical decision when developing a translational gene therapy programme. Efficacy, safety and cost are all key factors; for a common, non-lethal condition such as tendon injury; safety and cost are particularly important. Vector deployment strategies also need careful consideration, with *in vivo* gene delivery being much more practical and less expensive than *ex vivo* protocols that require the expansion of autologous cells (Evans *et al.*, 2021; Evans *et al.*, 2007).

In this context, adeno-associated virus (AAV) is an attractive vector. It is widely utilized in human gene therapy applications due to its safety, broad tropism and low immunogenicity (Evans and Huard, 2015; Greelish *et al.*, 1999). This vector possesses the ability to transduce both dividing and nondividing cells while demonstrating limited cytotoxicity; it rarely integrates into the host chromosomes yet can produce long-term expression of the transgene (Hastie and Samulski, 2015; Li and Samulski, 2020). Several AAV-based gene therapies have received marketing approval from the U.S. Food and Drug Administration (FDA).

Various native and artefactual AAV serotypes have been identified (Issa *et al.*, 2023; Zincarelli *et al.*, 2008). The *in vivo* transduction tropisms of these vectors relies on the properties of their capsids and the route of administration (Li and Samulski, 2020). Certain serotypes of AAV have demonstrated efficient transduction of cells within musculoskeletal tissues, including bone (Atasoy-Zeybek and Kose, 2018; Lakhan *et al.*, 2015; Lee *et al.*, 2019; Yazici *et al.*, 2011), cartilage (Chen *et al.*, 2020; Evans *et al.*, 2023; Watson Levings *et al.*, 2018; Yoon *et al.*, 2021), and skeletal muscle (Greelish *et al.*, 1999; Harper *et al.*, 2002; Muraine *et al.*, 2020).

In the present study, we investigated the *in vitro* transduction efficiency of AAV vectors of different serotypes using tenocytes derived from rat supraspinatus and human Achilles tendons and used the most potent of these to deliver a marker gene to the rat rotator cuff *in vivo.* Because transduction efficiency *in vitro* is not always predictive of transduction efficiency *in vivo*, we undertook additional studies in the rat aimed at determining the ability of AAV2.5 to transduce cells within the rotator cuff *in vivo*. This also allowed us to measure the duration of transgene expression *in vivo* and to investigate the localization of transgene expression.

Materials and Methods

In vitro studies

Cell culture

This study investigated transduction of the supra- and infra-spinatus tendons of the rotator cuff through *in vitro, ex vivo*, and *in vivo* approaches. For *in vitro* studies, tenocytes were isolated from two male F344 rats, aged between 5 and 7 months. Animal studies were conducted following the guidelines of Mayo Clinic's Institutional Animal Care and Use Committee (Protocol Number: A00004279). Euthanasia of the animals was carried out in accordance with the institutional humane euthanasia policy, using carbon dioxide inhalation. Immediately after confirming expiry, tenocytes were isolated from harvested supraspinatus tendon. Briefly, the tissue samples were washed multiple times with phosphate-buffered saline (PBS) (Gibco, Gaithersburg, MD, USA) containing 1 % penicillin/streptomycin (P/S) (Gibco). The supraspinatus tendons were then dissected into small pieces (1-2 mm) and incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5 % foetal bovine serum (FBS) (Gibco), 1 % P/S and 0.25 % w/v collagenase type II (Gibco) for 2-3 hours at 37 °C. Following digestion, the tendon fragments were filtered through a 100 μm cell strainer and centrifuged at 400 g for 5 minutes. The cells were transferred to a tissue culture flask at a density of 2×10^4 cells/cm^2 and cultured in growth medium (high-glucose DMEM supplemented with 2 % L-glutamine, 10 % FBS, and 1 % P/S). Cells were maintained at 37 °C, 95 % humidity, and 5 % carbon dioxide, with a medium change every 2-3 days. Once the cultured cells reached 80-90 % confluence, they were detached with TrypLE™ (Thermo Fischer Scientific, Waltham, MA, USA) and subcultured at a density of 1×10^4 cells/cm^2 until passage 4-5.

Human tenocytes isolated from Achilles tendon were purchased from ZenBio (ZenBio Inc., Durham, NC, USA; Cat No # TEN-F; Lot No TEN012121A). According to the suppliers, the cells expressed types I and III collagen, thrombospondin 4 and scleraxis, as well as CD44 and CD90. They were negative for CD45 and CD31. Cells were expanded in Tenocyte Growth Medium (ZenBio Inc.; Cat No # TEN-F1) supplemented with 10 % MSC-grade FBS (Takara Bio, San Jose, CA, USA), and 1 % P/S (Gibco) following the manufacturer's instructions (ZenBio Inc.; instruction manual zbm0075.04). For all experiments, human tenocytes at passage 2–4 were utilised.

Generation of AAV serotypes

Polyethylenimine (PEI) was used to transfect 293T cells with the following 3 plasmids: helper

adenovirus plasmid, RepCap pXR (R representing the serotype of choice – serotypes 1, 2, 2.5, 3, 4, 5, 6, 8, 9, rh10), and the self-complementary transgene plasmid encoding a green fluorescent protein (GFP)-luciferase fusion protein reporter with gene expression driven by a cytomegalovirus (CMV) promoter. Twenty-four hours post-transfection the medium (low glucose DMEM, 10 % FBS, 1 % P/S) was changed, and the cells harvested 72 hours post-transfection. Following three rounds of freeze-thaw with dry ice and an ethanol bath, the resulting lysate was added to an iodixanol density column and ultra-centrifuged at 62,500 g for 2 hours. The fraction of the iodixanol gradient containing the 40 % density layer was pulled and was diluted with PBS prior to concentration via 100 kDa Amicon filters (Millipore Sigma, St. Louis, MO, USA). Once concentrated, the AAV was titrated using quantitative polymerase chain reaction (qPCR) with Sybr Green and primers targeting the inverted terminal repeat (ITR), (forward: ITR primer, 5′-GGAACCCCTAGTGATGGAGTT, reverse: ITR primer, 5′-CGGCCTCAGTGAGCGA).

For *ex vivo* tissue explant culture experiments and for *in vivo* experiments, AAV2.5 vectors expressing GFP or luciferase were obtained from a commercial source (Welgen Inc., Worcester, MA, USA).

Transduction of tenocytes with AAV

A total of 10,000 rat or human cells were seeded into each well of a 96-well plate, with 100 µL of growth medium. The next day, the cells were transduced with AAV serotypes to be tested (1, 2, 2.5, 3, 4, 5, 6, 8, 9, rh10) at a concentration of 1×10^4 vector genome copies (vg) in 25 µL of serum-free DMEM. After a 2-hour incubation period, an additional 75 µL of growth medium were added to each well. Following overnight transduction, the medium containing the virus was replaced with fresh growth medium. Cells were harvested 72 hours post-transduction.

Transduction efficiency

To assess the transduction efficiency of individual AAV serotypes, the number of GFP+ cells were counted in three different microscopic fields within a defined region of interest (ROI), Counting was performed in triplicate samples for each serotype and the average number of GFP+ cells per field was determined. Fluorescence microscopy (Olympus, Center Valley, PA, USA) at 10× magnification was used to visualize and count.

Firefly luciferase activity

Luciferase activity in the cell lysates was determined following the instructions provided in the user's manual (Promega, Madison, WI, USA). Briefly, 100 µL of the Glo-lysis buffer were used to lyse the cells. Subsequently, 30 µL of the cell lysate were transferred to 96-well plates in triplicate. Then, 30 µL Luciferase Assay Reagent (Bright-Glo™ Luciferase assay system, Promega) were added to each well, and the plates were incubated for 10 minutes at room temperature. Firefly luminescence was measured using a microplate luminometer (Varioskan Lux, Thermo Fisher Scientific).

Picogreen DNA assay

The total DNA content of the cell lysate was quantified using the QuantiT PicoGreen double-stranded DNA (dsDNA) Assay Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. To generate a standard curve for quantification, a series of DNA standard solutions was prepared. In a black 96-well plate, 100 µL of each sample at a dilution of 1:10, DNA standards and a blank control were transferred to wells. Then, 100 µL of the picogreen assay reagent were added to each well. The plate was incubated for 5 minutes to allow the picogreen dye to bind to the DNA. The fluorescence intensity was measured using a fluorescence plate reader (Varioskan Lux, Thermo Fisher Scientific) at the excitation wavelength of 485 nm and emission wavelength of 535 nm. The resulting fluorescence measurements were used to determine the amount of DNA in each sample, and the luciferase activity data were normalised to nanogrammes of DNA.

Transduction of explanted tissue

The supraspinatus tendon of one rat, including paratenon, and muscle, was carefully isolated from the surrounding tissue. The isolated tissue was then thoroughly washed with sterile PBS to eliminate debris and remove any residual blood.

Next, the tendon was transferred to ultra-low attachment 96-well plates. For AAV2.5-GFP (Welgen Inc.) transduction, 5×10^9 vg were added to each well in 50 µL of serum-free DMEM. To enhance transduction efficiency, spinoculation was performed at 2000 g for 20 minutes at 37 °C. Following a 2-hour incubation period, an additional 100 µL of growth medium were added to each well. After overnight transduction, the medium was replaced with fresh growth medium. At 48 hours post-infection, the tissue explants were fixed for subsequent immunohistochemical analysis.

Immunohistochemical staining

Tendons were fixed in 10 % neutral buffered formalin for 48 hours (n = 1). Fixed specimens were dehydrated in a series of graded ethanols and embedded in paraffin. 5-µm sections were cut using an automatic microtome (HM 355S, Thermo Fischer Scientific) and mounted onto positively charged slides (Superfrost™ Plus Microscope Slides, Thermo Fisher Scientific). Expression of GFP was detected by enzymatic immunohistochemistry following a previously published protocol (De La Vega *et al.*, 2018). Briefly, the formalin-fixed tissue sections were deparaffinised, rehydrated, and washed twice in PBS for 5 minutes. Endogenous peroxidase activity was blocked with peroxidase blocking reagent (Vectastain® Elite®, Vector Laboratories, Newark, CA, USA; Cat no # PK-6105). Non-specific binding was blocked with 5 % rabbit serum. Subsequently, the slides were incubated overnight at 4 °C with a rabbit polyclonal anti-GFP antibody (Abcam, Boston, MA, USA; Cat no # Ab27478) at a dilution of 1:1000. On the following day, slides were washed with PBS, and incubated with biotinylated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Vector Laboratories; Cat no # BA-1000), followed by detection with an avidin-biotin-based peroxidase kit (Vectastain Elite ABC HRP Kit™; Vector laboratories). Slides were incubated in 3,3′ diaminobenzidine (ImmPACT DAB, Vector Laboratories), a peroxidase substrate that yields a brown product, followed by counterstaining with haematoxylin. Positive controls were formalin-fixed, paraffin-embedded femora from transgenic rats expressing GFP constitutively (De La Vega *et al.*, 2018). Non-transduced tendon was used as negative control and an isotype control for anti-GFP antibody (Abcam; Cat no # Ab240) was used to distinguish non-specific background signal from GFP signal. The stained sections were visualised using an Olympus BX43 microscope equipped with an Olympus SC50 camera (Olympus, Center Valley, PA, USA).

The same protocol was used for immunohistochemical staining of luciferase (n = 2), but in this case an anti-luciferase antibody (Novus Biologics, Minneapolis, MN, USA; Cat no # NB100-1677) at a dilution of 1:1000 was used as the primary antibody. Rabbit anti-goat IgG antibody (Novus Biologics; Cat no # NBP1-74526) was employed as the secondary antibody.

In vivo studies

Surgery

Animal care protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic (A00007034-23).

Surgical procedures were conducted on a total of five skeletally mature male Fischer 344 rats, 8 months old, weighing between 300 and 450 g (Charles River Laboratories, Wilmington, MA, USA). Rats were housed in Mayo Clinic animal care facility with 12-hour light cycles and were given chow and water *ad libitum*. Prior to surgery, all animals received a pre-operative subcutaneous injection of 1 mg/kg buprenorphine-ER 0.5 mg/mL analgesic for pain management. Anaesthesia was induced using inhaled isoflurane at a concentration of 1-3 %. Both shoulders were shaved and sterilised using 70 % ethanol and iodopovidone. Under sterile conditions and with continuous anaesthesia using inhaled isoflurane, a 1-2 cm incision was made on the lateral right shoulder, proximal to the clavicle to expose the shoulder region. Injections were performed using a 25 µL Hamilton® syringe (Hamilton, Reno, Nevada, USA; Cat no # 7636-01; 25 µL, Model 702 RN SYR) with a 28-gauge needle, under direct visualisation into the subacromial space above the supraspinatus tendon. Correct subacromial positioning was confirmed visually before injecting the vector. No muscles were detached. Since the injection was performed into subacromial space, it is likely that the bursa was also exposed to the virus, but we did not verify this.

The right shoulder was injected in the subacromial space with AAV2.5 encoding luciferase (Welgen Inc.), consisting of 1 × 10^8 vg/μL suspended in 15 μL of sterile PBS (Thermo Fisher Scientific). The left shoulder was prepped and injected in an identical fashion to the right shoulder but received an injection of sterile PBS alone. The cutaneous skin incision was closed using continuous sutures technique with a 4-0 Vicryl suture (Ethicon™, Inc., Somerville, NJ, USA). Two additional rats underwent this survival procedure, receiving the same concentration of virus in the right shoulder, and sterile PBS for control in the left shoulder. A fourth rat underwent this surgery receiving virus in both shoulders, and a fifth rat underwent this surgery receiving sterile PBS in both shoulders. Rats were injected this way to be as efficient as possible in acquiring adequate samples for both imaging and histology.

Bioluminescence imaging

Two rats injected with AAV2.5 encoding luciferase were subjected to bioluminescence imaging using an *in vivo* imaging system (IVIS) spectrum (PerkinElmer Health Sciences Inc., Waltham, MA, USA) at designated time points following the surgical procedure. Rats were anaesthetised with 1-3 % isoflurane and administered an intraperitoneal injection of D-Luciferin (GoldBio, St. Louis, MO, USA) diluted in 15 mg/mL of PBS at a final dose of 150 mg/kg. Rats were positioned within the IVIS under continued isoflurane anaesthesia (2 %-2.5 % in 1 L/min O_2) and imaged 20 minutes after D-luciferin delivery. The resulting bioluminescence signal was superimposed onto a photograph of the rat, and signal intensity was quantified using Living Image software 4.7.2 (PerkinElmer Health Sciences Inc). Immediately after imaging, the rat selected for additional, *ex vivo* experimentation was euthanised using 3.5-4 L/min of CO_2 for 5 minutes. Both shoulders were harvested while isolating the supraspinatus and infraspinatus tendons, as well as adjacent cartilage, muscle and bone, which were placed into individual wells in a 12-well plate containing D-luciferin (300 μg/mL). IVIS images were taken and the resulting bioluminescence signal (1 min, 8 min) superimposed onto a photograph of the tissue within the well-plate.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 9.2.0 (GraphPad, Boston, MA, USA). There were 3 replicates (n = 3) for each experiment. All data are represented as average ± SD, as indicated in the figures. The Kolmogorov–Smirnov with Dallal-Wilkinson-Liliefor *p*-value and Shapiro–Wilk tests were performed to determine the data distribution. For normally distributed data, an unpaired *t*-test with Welch's correction was used to compare two independent groups. In multiple comparisons, one-way-ANOVA followed by Tukey's post hoc test was used. For non-normally distributed data, Mann–Whitney U-test was used to compare two independent samples. A p-value < 0.05 was considered statistically significant.

Results

AAV2.5 demonstrated highest transduction efficiency in both rat and human tenocytes

A GFP-luciferase fusion protein under the transcriptional control of the CMV promoter was used to examine the tropism of the ten different scAAV serotypes listed in the methods section. Table 1 shows the titres that were obtained for each of these serotypes. When observed by fluorescence microscopy, cultures of rat tenocytes transduced by AAV2 and AAV2.5 contained the greatest number of GFP+ cells (Fig. 1**A,B**). Other serotypes, in contrast, had little or no transducing ability. Measurement of GFP fluorescence suggested that AAV2.5 produced greater GFP expression than AAV2, with AAV6, AAV9 and AAVrh10 producing low levels of GFP expression that were not seen by eye (Fig. 1**C**) (**p < 0.01; ***p < 0.001). Measurement of luciferase activity also suggested superior transgene expression using AAV2.5 (Fig. 1**D**) (*p < 0.05). Similar results were obtained for the human tenocytes (Fig. 2). Based on GFP expression, AAV2 and AAV2.5 had approximately equal transducing potency and were clearly superior to other serotypes (Fig. 2**A,B,C**). As with the rat tenocytes, when measuring luciferase activity AAV2.5 was superior to AAV2 and other serotypes (Fig. 2**D**) (*p < 0.05; **p < 0.01).

On the basis of these results, we selected AAV2.5 to take forward into subsequent *ex vivo* and *in vivo* experiments.

Table 1. Titers of AAV serotypes.

Serotype	AAV Titer (vg/μL)
AAV1	5.44×10^7
AAV2	1.47×10^7
AAV2.5	3.03×10^6
AAV3	3.04×10^7
AAV4	2.73×10^6
AAV5	3.43×10^7
AAV6	4.43×10^6
AAV8	3.71×10^8
AAV9	2.28×10^8
AAVrh10	3.92×10^8

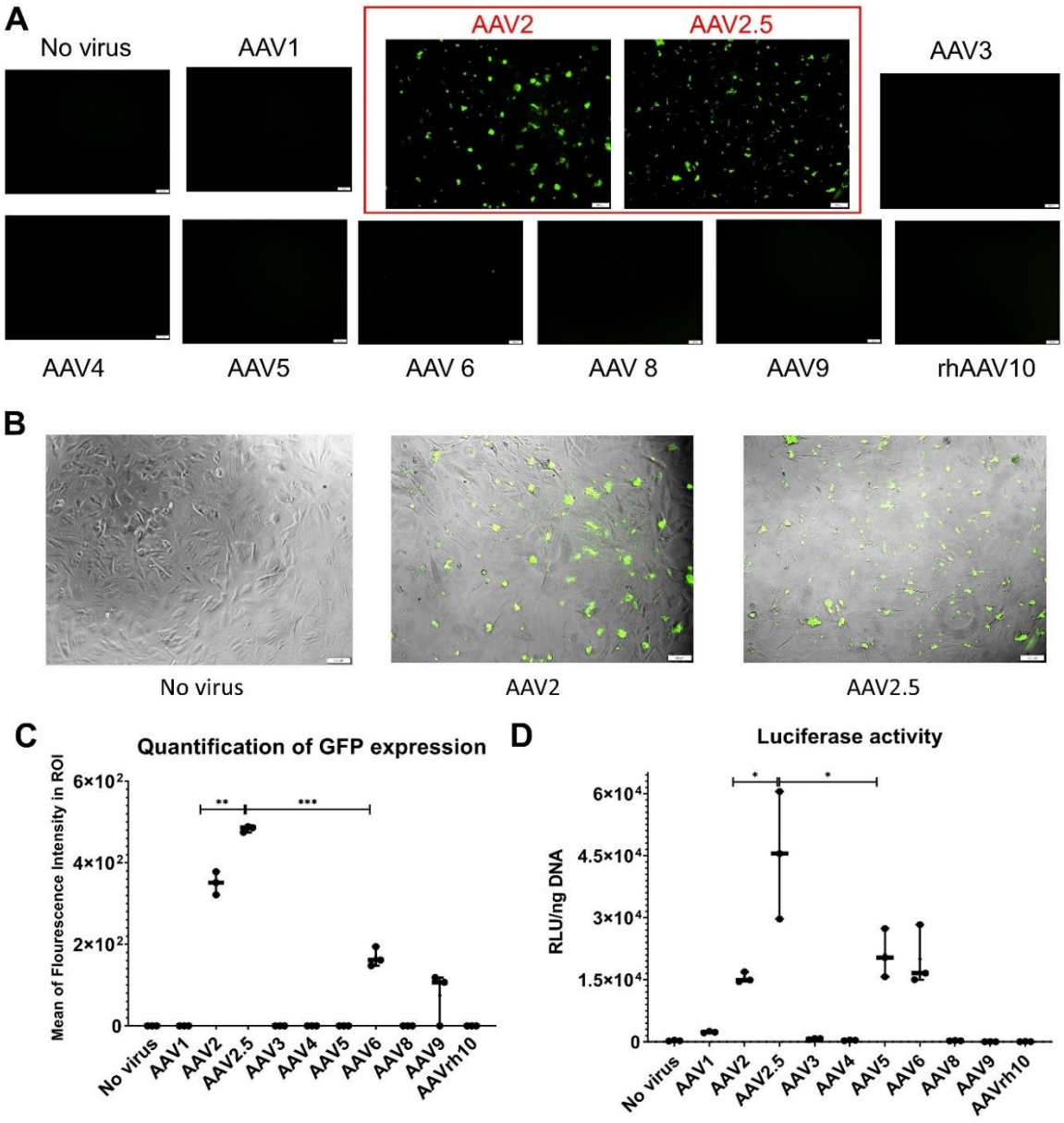

Fig. 1. Transduction of rat supraspinatus tenocytes by different AAV serotypes. (**A**) GFP imaging by fluorescence microscopy 72 hours post-transduction with different AAV serotypes. The two most

effective serotypes are highlighted. (**B**) Higher magnification, 72-hour images of tenocytes transduced with AAV2 and AAV2.5. Combined fluorescence and brightfield imaging. (**C**) Quantification of GFP+ positive cells with microscope software. (**D**) Luciferase activity assay of tenocytes transduced by each AAV serotype. Each experimental group comprises three replicates. Scale bar in panels A and B: 100 μm. Quantitative data are presented as mean ± SD, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

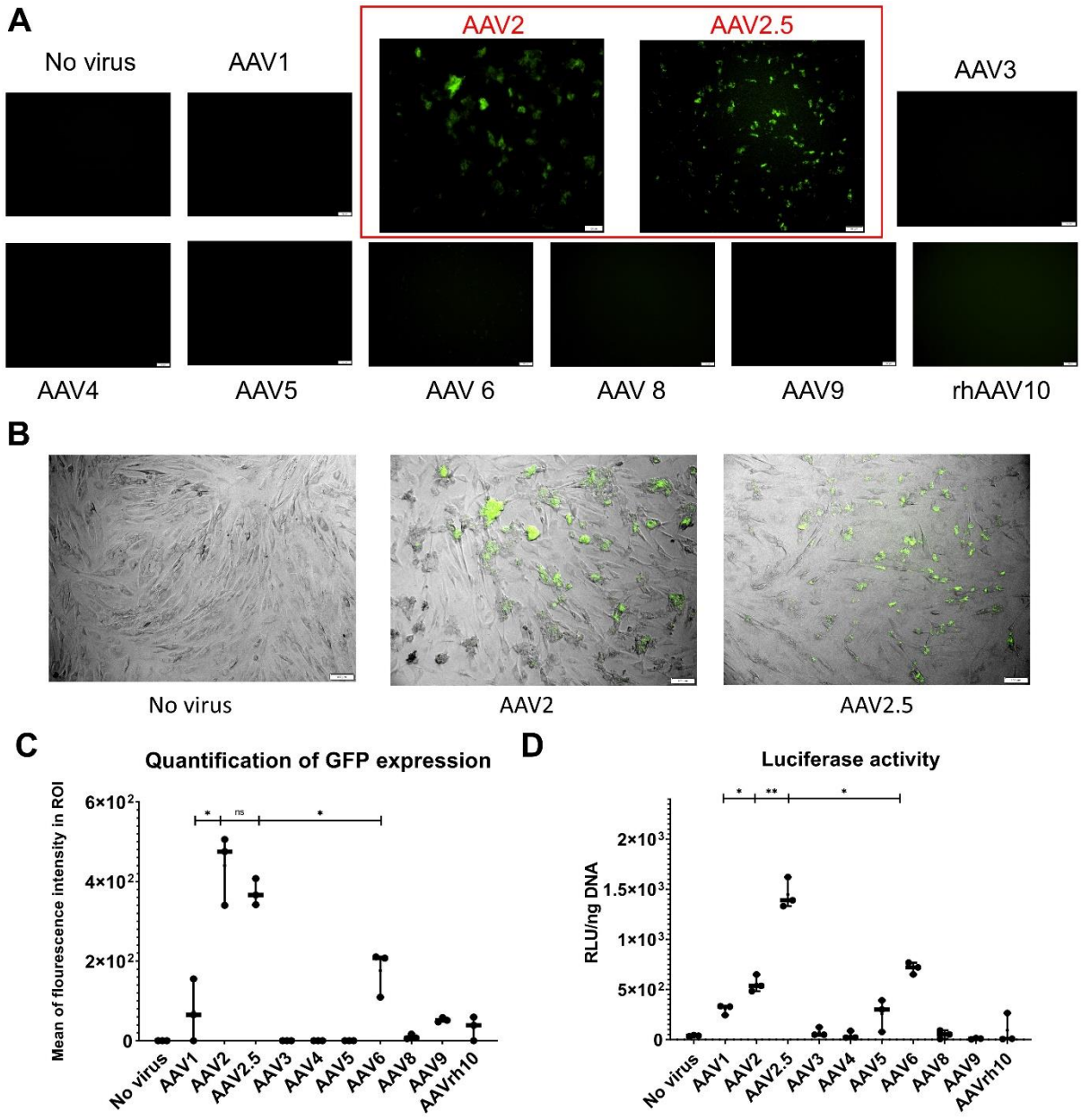

Fig. 2. Transduction of human tenocytes with AAV serotypes. (**A**) GFP imaging by fluorescence microscopy 72 hours post-transduction with different AAV serotypes. The two most effective serotypes are highlighted. (**B**) Higher magnification, 72-hour images of tenocytes transduced with AAV2 and AAV2.5. Combined fluorescence and brightfield imaging (n = 3). (**C**) Quantification analysis of GFP+ positive cells with microscope software. (**D**) Luciferase activity assay of tenocytes transduced by each AAV serotype. Each experimental group comprises three replicates. Scale bar in panels A and B, 100 μm. Quantitative data are presented as mean ± SD, ns: not-significant; *$p < 0.05$; **$p < 0.01$.

***Ex vivo* AAV2.5 transduction of the rat supraspinatus revealed the presence of GFP-positive cells not only in tenocytes but also in the paratenon and surrounding muscle**

Intact supraspinatus tendon was dissected from rat shoulders and transduced in organ culture with AAV2.5.GFP to confirm transduction of cells *in situ* and to locate transduced cells. GFP fluorescence imaging (Fig. 3**A**) demonstrated successful transduction of tenocytes, with a notable presence of GFP-positive cells in the paratenon (Fig. 3**B**). Additionally, immunohistochemical analysis confirmed the presence of GFP-positive cells within the tendon, paratenon, and as well as the adjacent muscle (Fig. 3**C**).

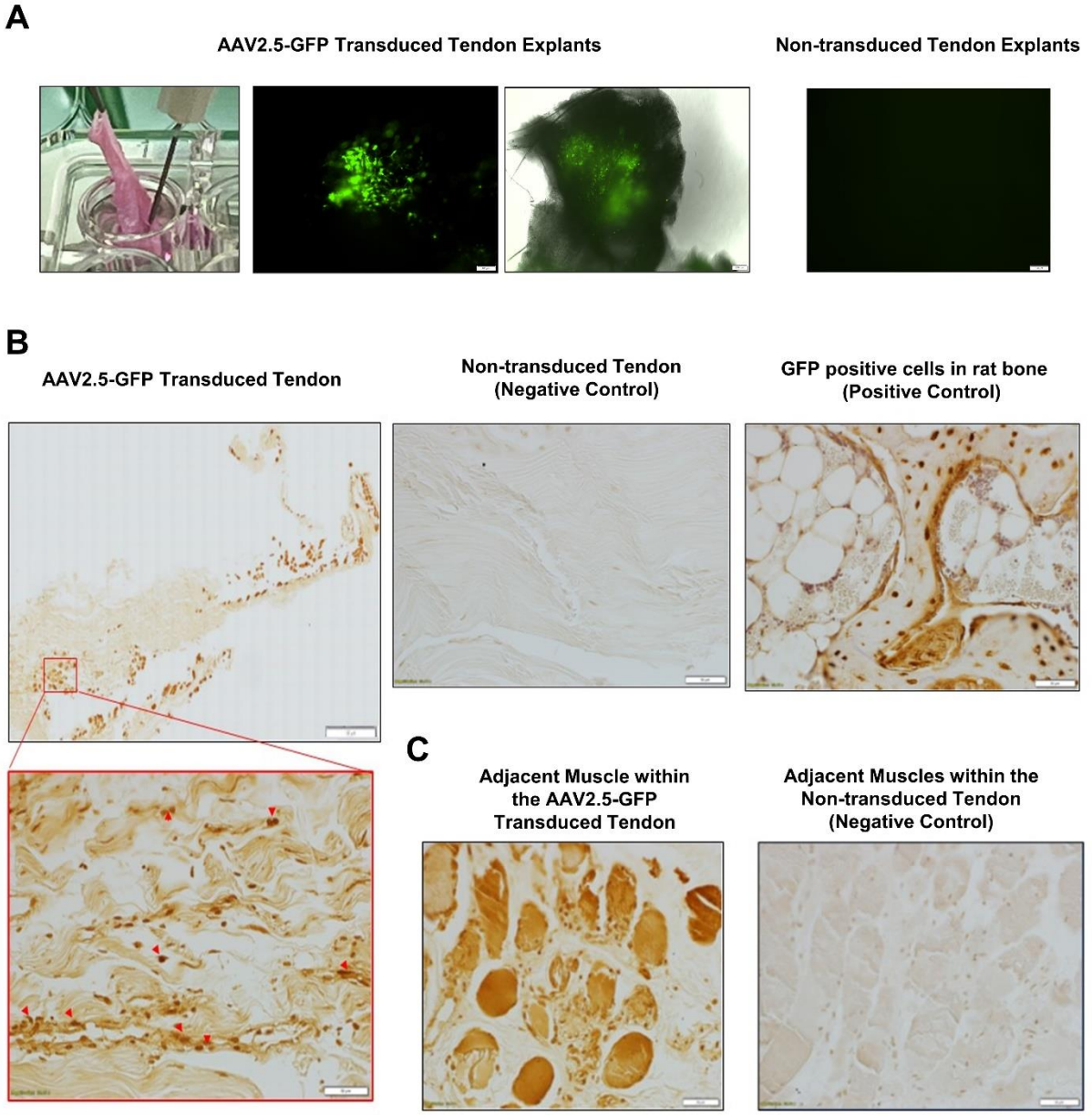

Fig. 3. *Ex vivo* transduction of rat supraspinatus tendon with AAV2.5-GFP. (**A**) GFP imaging of rat supraspinatus tendon 72 hours after transduction (n = 1). (**B**) Immunohistochemical analysis of AAV2.5-GFP transduced tendon (scale bar: 200 μm), non-transduced tendon (negative control, Scale bar: 50 μm) and bone from a GFP-expressing rat (positive control, Scale bar: 50 μm). GFP+ positive cells stain dark brown. The square insert shows a higher magnification image of the indicated area. Red arrowheads indicate GFP-expressing cells. Scale bar: 50 μm. (**C**) GFP+ positive cells within the adjacent muscle. Scale bar: 50 μm.

Rat infraspinatus efficiently transduced by AAV2.5 *in vivo*

To confirm transduction *in vivo*, AAV2.5 luciferase was injected into the subacromial space of the shoulders of rats. Transgene expression was then analysed using an *in vivo* live imaging system. After a 48-hour delay, robust luciferase expression was visualized until day 7 (Fig. 4**A**). Highest luminescence intensity occurred on day 6 (Fig. 4**B**). Luciferase expression was limited to the subacromial space of the shoulder. Immunohistochemical analysis from this *in vivo* study demonstrated luciferase expression in rotator cuff (Fig. 4**C**). In a subsequent experiment, each component of the rotator cuff from the *in vivo* study was individually dissected and subjected to IVIS analysis. *Ex vivo* bioluminescence imaging localised transgene expression to the infraspinatus tendon with no signal observed from the supraspinatus tendon (Fig. 4**D,E,F**), (**$p < 0.01$), cartilage, muscle or bone (data not shown). Subsequent, immunohistochemistry analysis confirmed transgene expression in rat infraspinatus (Fig. 4**G**).

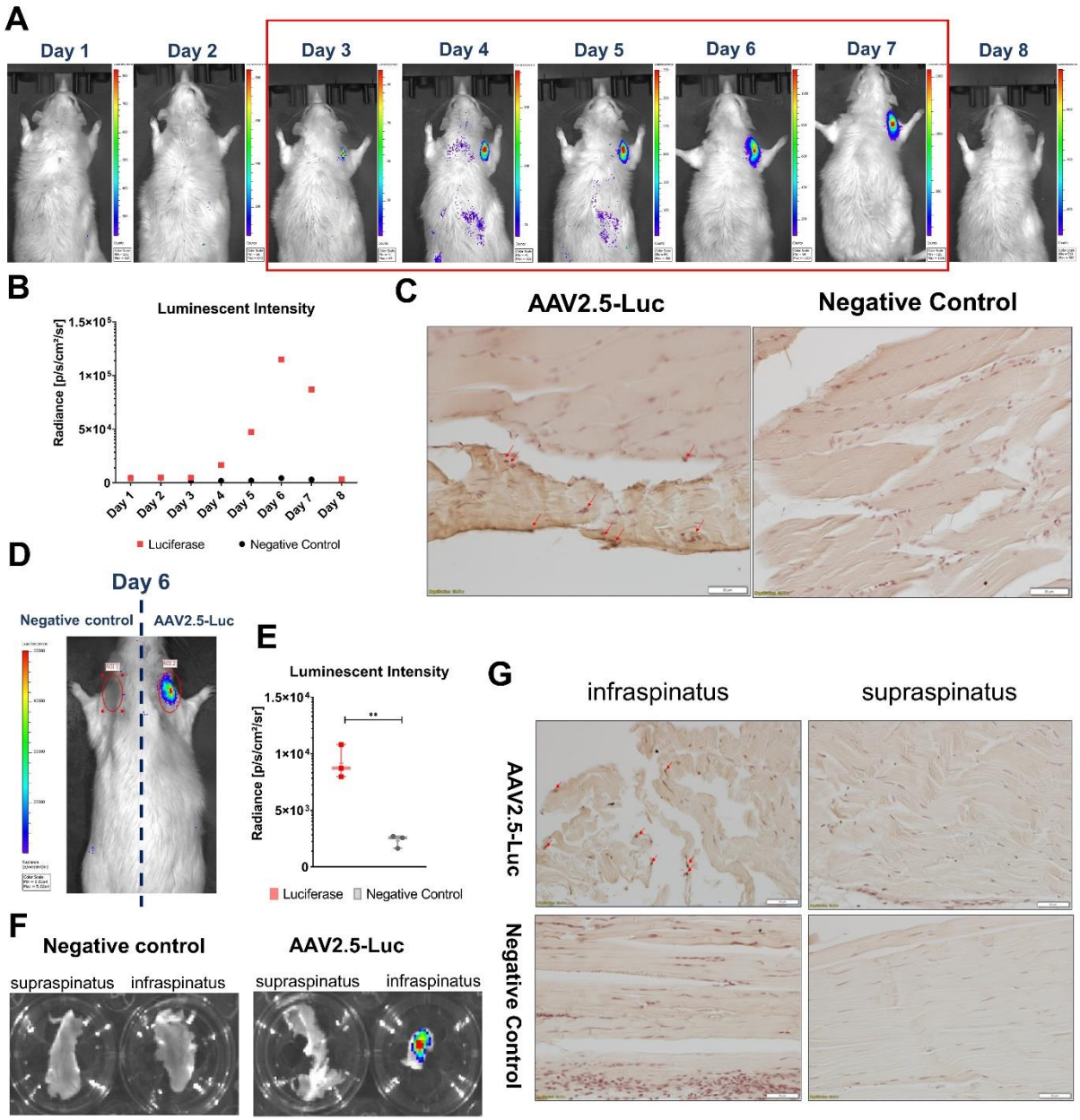

Fig. 4. *In vivo* expression of luciferase following injection of AAV2.5-Luciferase into subacromial space of the rat shoulder. (**A**) IVIS images of rats at various times following injection of AAV2.5-

Luciferase (n = 1). Boxed images show *in vivo* expression of luciferase. (**B**) Quantification analysis of luminescent intensity in AAV2.5-Luciferase injected rats for up to 8 days. (**C**) Immunohistochemistry staining of luciferase activity in infraspinatus tendon at day 6 (n = 1). (**D**) IVIS image of peak luciferase expression at day 6, compared to control (n = 3). (**E**) Quantification of IVIS imaging at day 6. (**F**) *Ex vivo* live imaging and (**G**) immunohistochemistry analysis of luciferase activity in the supra- and infra-spinatus tendons harvested from AAV2.5-Luciferase injected shoulders at day 6. Scale bar, 50 μm. Quantitative data are presented as mean ± SD. $^{**}p < 0.01$.

Discussion

The concept of using gene delivery to promote tendon healing and regeneration is well established and both non-viral (Jin *et al.*, 2022) and viral vectors have been studied for this purpose (Docheva *et al.*, 2015). Although some progress has been reported with non-viral vectors (Jin *et al.*, 2022) transfection efficiency is poor and transgene expression is low and transient. Viral vectors (Watson-Levings *et al.*, 2022) are much more effective in gene transfer but are more complicated and expensive to manufacture and raise safety concerns. Because tendon injuries are not life-threatening, safety is a major issue that rules out the use of integrating vectors such as retrovirus and lentivirus. Adenovirus has been the most studied as a vector for gene therapy to tendon cells using a variety of different transgenes (Haddad-Weber *et al.*, 2010; Lou *et al.*, 2001). While the data are encouraging, no progress has been made in clinical translation. Among the disadvantages of adenoviral vectors is their antigenicity, especially with regard to activation of the innate immune system and consequent inflammation which is inimical to healing (Watson-Levings *et al.*, 2022). In this context AAV offers several advantages as a vector. It is considered safer than other viral vectors because the wild-type virus causes no known disease. Although AAV generates a humoral, serotype-specific immune response, it does not typically activate cell-mediated responses. Several AAV-based gene therapies have been approved by the regulatory authorities and AAV has been approved for use by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA); AAV is in clinical trials of gene therapies for osteoarthritis, a non-lethal disease (Evans *et al.*, 2023).

AAV exists in a number of different natural and artefactual serotypes with different tropisms (Issa *et al.*, 2023). In the present study we compared the abilities of several of these serotypes to transduce tendon cells *in vitro, ex vivo* and *in vivo.* The data confirm the ability of AAV2 and, especially, AAV2.5 to transduce tenocytes within the rat infraspinatus tendon *in vitro, ex vivo* and *in vivo*, and to transduce human tenocytes *in vitro.* Other serotypes were less effective or ineffective. The relative potencies of AAV2 and AAV2.5 were not always consistent between the two reporter sequences. The marker gene encodes a GFP-luciferase fusion protein, so the discrepancies cannot reflect transduction efficiency or transgene expression. GFP has a longer half-life than luciferase which may account for the differences in expression recorded in Fig. 1**C,D** and Fig. 2**C,D**. Alternatively, differences in the measurement methods—fluorescence microscopy for GFP and enzyme assay for luciferase—may well account for this.

The effectiveness of AAV as a vector for *in vivo* delivery is a major practical advantage for future clinical development, and the expression period of 7 days may be appropriate for triggering a lasting regenerative response. AAV2.5 is a recently developed serotype created by incorporating 5 amino acids from AAV1 into the capsid protein of AAV2. This modification was primarily intended to decrease susceptibility to neutralizing antibodies that specifically target AAV2 which are present in approximately 30-60 % of the general population due to previous AAV2 infections (Abdul *et al.*, 2023; Calcedo and Wilson, 2013). AAV2.5 has been used in clinical trials for muscular dystrophy (Bowles *et al.*, 2012) and osteoarthritis (ClinicalTrials.gov Identifier: NCT02790723). Our study further demonstrated the potential of AAV2.5 in enhancing the transduction ability for the rotator cuff.

The previous literature on the use of AAV for tendon healing is sparse. Only a few studies have investigated the feasibility of gene transfer in tendons. Various serotypes of AAV exhibit differences in their tropism for target cells (Issa *et*

al., 2023). AAV2, in particular, has been extensively studied and demonstrates a broad infectivity profile. For example, Wang *et al*. (2007) also found that AAV2 delivered exogenous genes to cultures of rat intrasynovial tenocytes, whereas AAV1, AAV3, AAV4, AAV5, AAV7, and AAV8 were ineffective. AAV serotype 2.5 was not tested. When AAV2 was used to transfer basic fibroblast growth factor (b-FGF) to tenocytes in cell culture, the investigators observed a significant increase in the expression of type I and III collagen, consistent with a regenerative response (Wang *et al.*, 2005). The same group went on to construct AAV2 encoding a microRNA that silenced transforming growth factor beta 1 (TGF-β1). This vector suppressed adhesion formation in a chicken model of flexor tendon healing, when injected into the injured tendons intra-operatively (Wu *et al.*, 2016). In later work they used AAV2 to deliver vascular endothelial growth factor (VEGF) in the same chicken flexor tendon healing model and noted increased strength of the healing tendon (Mao *et al.*, 2017). In a different approach to AAV-mediated tendon healing, Basile *et al*. (2008) coated AAV2 encoding growth/differentiation factor 5 (GDF-5) onto freeze-dried murine digital flexor tendon and implanted the construct in a model of flexor tendon healing. Here the intent was not to transduce tenocytes, because the allograft lacked living cells. Instead, after implantation the vector transduced cells surrounding the defect which secreted GDF-5 locally where it enhanced the healing process. In a subsequent study this group used AAV2.5 expressing GDF-5 in the freeze-dried allograft method, producing results that were broadly similar to those obtained with AAV2 (Hasslund *et al.*, 2014).

We found that AAV2.5 preferentially transduced cells within the paratenon region surrounding the supraspinatus, compared to other areas of the tendon. Some transduction of cells was also observed in the adjacent supraspinatus muscle. The paratenon region of the tendon is more cellular than the main body of the tendon (López De Padilla *et al.*, 2021) and plays a role in providing blood vessels and progenitor cells to the tendon (Müller *et al.*, 2018). While the exact function of the paratenon in tendon healing is not fully understood, lineage tracing studies in mice have demonstrated the presence of a population of progenitor cells within the paratenon expressing smooth muscle actin, which proliferate during injury and contribute to the healing process of injured tendons (Dyment *et al.*, 2014). Previous research by Müller *et al*. (2018) demonstrated an important role for the paratenon in healing an Achilles tendon defect model in rats. The ability of AAV2.5 to transduce cells within the paratenon may therefore be of high significance. Additional cell types are likely to be present in the setting of a tendon injury, but it is not possible to predict whether these will be transduced by AAV2.5. In addition to tenocytes, AAV2.5 is known to transduce chondrocytes, synovial fibroblasts (Watson Levings *et al.*, 2018) and, as confirmed here, muscle cells.

Although AAV2.5 was found to transduce cells within both the infra- and supra-spinatus tendons of explanted rat tissue, only the infraspinatus tendon was transduced following *in vivo* injection into the subacromial space. The explanation for this presumably resides with the anatomy of the rat shoulder (Edelstein *et al.*, 2011). The supraspinatus tendon sits more anteriorly in the subacromial space, so it may be partially shielded or less accessible to vectors injected into this area (Andarawis-Puri *et al.*, 2009). Thus, the infraspinatus tendon may receive more direct exposure compared to the supraspinatus with this technique. However, the close proximity of the infra- and supra-supinatus tendons within the rotator cuff should ensure that healing of the supraspinatus tendon is enhanced by growth factors secreted from the adjacent infraspinatus tendon after *in vivo* transduction by AAV.

Although prior rotator cuff research has predominantly focused on the supraspinatus, some studies suggest the infraspinatus may play a more significant role in rotator cuff rupture (Jernheden and Szaro, 2022; Kato *et al.*, 2012; Williams *et al.*, 2023). For example, Mochizuki *et al*. (2008) showed tears involving the anterior greater tuberosity have substantial infraspinatus involvement, rather than just supraspinatus damage. Subsequently, Kato *et al*. (2012) also found a close relationship between the transverse infraspinatus and supraspinatus anatomically and functionally.

While tendon injuries are not life-threatening, they can still have a major impact on quality of life. Rotator cuff injury that fails to heal properly often leads to chronic pain, reduced mobility, and decreased strength and function (Canosa-Carro *et al.*, 2022). Previous studies have demonstrated the potential of various therapeutics biologics, such as TGF-β1, platelet-derived growth factor subunit B (PDGF-B), GDF-5, e (BMP)-12, and BMP-14 utilizing gene delivery methods to enhance tendon repair (Bolt *et al.*, 2007; Docheva *et al.*, 2015; Hildebrand *et al.*, 2004; Jin *et al.*, 2022; Lou *et al.*, 2001). Antagonists of inflammatory cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α), might also be beneficial to modulate the inflammatory response and prevent adhesion formation. This is particularly relevant as these cytokines play a role in the pathophysiological mechanism of subacromial inflammation (Sachinis *et al.*, 2022). AAV2.5 is thus of considerable interest as a vector for delivering genes to regenerate injured tendons; which genes to transfer is a matter for future research.

Conclusions

Our comprehensive analysis of AAV serotypes in rat and human supraspinatus tenocytes identifies AAV2.5 as a highly efficient vector for gene transfer to tenocytes. This vector is able to transduce infraspinatus cells *in situ* following *in vivo* delivery into the subacromial space of the rat shoulder, delivering genes to both the main body of this tendon as well as the paratenon. Given the important role of the paratenon in tendon healing, the latter capability may be of high significance. *In vivo* transgene expression in the rat shoulder persists for 7 days, a period of time that may be well suited to initiating a robust regenerative response. Future research is needed to determine which transgenes provide the greatest regenerative response.

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Author contributions

R.E.S designed and performed experiments and wrote the manuscript. A.A.Z. established the primary cells, contributed technical support to the experiments, data analysis, and edited the manuscript. E.B.M generated the adeno-associated used in this work and edited the manuscript. M.A.B. supervised the generation of adeno-associated virus and edited the manuscript. C.M.L.D.P performed immunohistochemistry and manuscript editing. D.R.M. provided project development, data management, manuscript editing, and study supervision. C.H.E. investigated the research, designed experiments, analyzed data, and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal studies were conducted following the guidelines of Mayo Clinic's Institutional Animal Care and Use Committee (Protocol Number: A00004279). Animal care protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic (A00007034-23).

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Conflict of interest

The authors declare no conflict of interest. C.H.E. is serving as one of the editorial board members of this journal. We declare that C.H.E. had no involvement in the peer review of this article and have no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Juerg Gasser.

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