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ORIGINAL ARTICLE

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A comparison of in vivo viral targeting systems identifies adeno-associated virus serotype 9 (AAV9) as an effective vector for genetic manipulation of Leydig cells in adult mice

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Abstract

Background: Despite the increasing popularity of deliverable transgenics, a robust and fully validated method for targeting Leydig cells, capable of delivering long-term transgene expression, is yet to be defined.

Objectives: We compared three viral vector systems in terms of their cell targeting specificity, longevity of gene expression and impact on targeted cell types when delivered to the interstitial compartment of the mouse testis.

Materials & Methods: We delivered lentiviral, adenoviral and adeno-associated (AAV) viral particles to the interstitial compartment of adult mouse testis. Immunolocalization and stereology were performed to characterize ability of vectors to target and deliver transgenes to Leydig cells.

Results: Viral vectors utilized in this study were found to specifically target Leydig cells when delivered interstitially. Transgene expression in lentiviral-targeted Leydig cells was detected for 7 days post-injection before Leydig cells underwent apoptosis. Adenoviral-delivered transgene expression was detected for 10 days post-injection with no evidence of targeted cell apoptosis. We found serotype differences in AAV injected testis with AAV serotype 9 targeting a significant proportion of Leydig cells. Targeting efficiency increased to an average of 59.63% (and a maximum of 80%) of Leydig cells with the addition of neuraminidase during injection. In AAV injected testis sections, transgene expression was detectable for up to 50 days post-injection.

Discussion & Conclusion: Lentivirus, Adenovirus and Adeno-Associated virus delivery to the testis resulted in key variances in targeting efficiency of Leydig cells and in longevity of transgene expression, but identified AAV9 + Neuraminidase as an efficient vector system for transgene delivery and long-term expression. Simple viral delivery procedures and the commercial availability of viral vectors suggests AAV9 + Neuraminidase will be of significant utility to researchers investigating the

genetics underpinning Leydig cell function and holds promise to inform the development of novel therapeutics for the treatment of male reproductive disorders.

KEYWORDS
lentivirus, adenovirus, adeno-associated virus, Leydig cell, testis, viral vector

1 | INTRODUCTION

Developments in technologies for the investigation and editing of the mammalian genetic code are triggering a surge in the exploration of genetic disorders and, in parallel, into the field of gene delivery vectors.

Over the past 30 years, the production of transgenic and gene knockout mice has permitted the functional investigation of thousands of genes. However, these methods often require multiple backcrosses of genetic mutants, a process which is costly in time, money and in the number of animals required to obtain a consistent pedigree. In addition to this, dissecting gene function in a mature adult system from its function in a developing tissue can be challenging, because developmental impacts often preclude appropriate comparisons at later ages.

Development of tools capable of gene manipulation at any given time of life obviate developmental issues and permit rapid production of genetic models from wild-type stock, with gene manipulation occurring only at specific time points of interest. With the increasing demand for deliverable gene manipulation technologies, mass produced and bespoke delivery vectors are now commercially available—permitting their use in a wide range of biological fields including in the context of novel therapeutic development. These vectors vary widely in their targeting preferences and delivery characteristics and, therefore, require careful consideration depending on the purpose of their use. A prospective user must consider the required longevity of delivered transgene expression, the immunogenicity of their chosen vector, how the vector will be administered, whether the vector should be constructed to target in a cell-specific manner and the possibilities and consequences of off-target effects.

Gene delivery vectors, in particular viral vectors, have been widely utilized for the delivery of gene manipulation technologies in a number of biological systems including the neurological,[1,2] digestive (liver, small intestine),[3-5] ophthalmic,[6] respiratory[7-9] and endocrine (pancreas, male and female reproductive organs)[10-17] systems. The use of viral vectors in these systems range from delivery of transgenes capable of labelling specific cells, transgenes able to knockdown specific genes and transgenes capable of rescuing gene and tissue function.

Viral vectors have been utilized for the targeting and delivery of transgenes to numerous testicular cell types. The majority of studies utilizing viral vectors to target testicular cells have been focussed on targeting spermatogonial stem cells and their lineage with an overarching aim of rapid production of transgenic lines.[18-24] This has resulted in varied levels of success.[18-24] Conversely, methodologies to target Sertoli cells for robust, long-term delivered transgene expression are now well established with delivery of viral vectors to the Sertoli cells within the seminiferous tubules being almost routine.[13,15,25,26]

The Leydig cells have been targeted with lentiviral vectors,[27,28] adenoviral vectors[16] and adeno-associated viral vectors.[17,19] However, no single study has demonstrated robust and long-term transgene expression in Leydig cells in adulthood. Loss of delivered transgene expression is observed after 12 days following adenoviral injection,[16] and long-term expression data are limited for lentiviral vectors[27,28] or Adeno-Associated viral vectors,[17,19] as reported experiments were ended after just a few days. The Leydig cells reside in the interstitial compartment of the testes where peritubular myoid cells, macrophages, blood and lymphatic vessels can also be found. The dynamic nature of this environment provides both a compelling cause for and an increase in complexity when introducing viral vectors. In particular, this is due to the intricate network between each of the resident cell populations contributing to the regulation and composition of testicular interstitial fluid, known to be influenced by androgens and by multiple testicular and lymphatic components.[29,30] Therefore, a robust and fully validated methodology for targeting the Leydig cell population with the goal of delivering long-term-transgene expression would be of significance.

In this study, we characterize and compare three commonly used and readily available viral vectors, testing their efficacy for delivery of transgenes to the Leydig cells in the interstitial compartment of the adult testis, with the aim of defining cell types targeted, efficiency of targeting and longevity of delivered gene expression. With the wide availability of off the shelf and custom viral vectors now available and ease of delivery to the interstitial compartment of the testis, deployment of the consensus protocol described in this study will permit rapid and robust generation of genetically modified Leydig cells in the testes of adult mice in vivo. This will facilitate rapid generation of somatic cell transgenics, circumventing developmental phenotypes whilst simultaneously reducing time taken, and numbers of animals required, to produce experimental cohorts. Furthermore, the opportunities provided by routine targeting of Leydig cells will support Leydig cell-specific gene rescue studies and could subsequently contribute to the development of bespoke therapeutics for the treatment of male reproductive and endocrine disorders.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animals were maintained under standard conditions of care. All experimental procedures were performed in strict compliance with the UK Animal Scientific Procedures Act (1986), under UK Home Office project licences 60/4200 and 70/8804 held by LBS or in accordance with NSW Animal Research Act 1998, NSW Animal Research Regulation 2010 and the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Ed, as approved by the University of Newcastle Animal Care and Ethics Committee (Approval Number A-2018-827).

2.2 | Viral vectors

Lentiviral particles containing transgenese expressing green fluorescent protein (GFP) downstream of a Cytomegalovirus (CMV) promoter. Shuttle vectors were packaged with a third-generation lentiviral vector plasmid pseudotyped for VSV-G, concentrated to a viral titre of $\geq 5 \times 10^9$ TU/ml in serum-free media. Lentiviral particles were obtained from SuRF Biomolecular Core Facility, University of Edinburgh, UK.

Adenoviral vectors containing either GFP alone (Cat. No: 1060) or GFP with Cre-recombinase (separated with an IRES subunit) downstream of a CMV promoter (Cat. No: 1710) were obtained from Vector Biolabs (Philadelphia, PA, USA). Viral particles were supplied at a titre of 1×10^{10} PFU/ml.

Adeno-Associated viral particles (serotypes 8 (Cat. No: AB801) and 9 (Cat. No: AC001) containing GFP expressing transgenes downstream of a CMV promoter were obtained from GeneCopoeia (via United Bioresearch, AU). Viral particles were supplied at a titre of $\geq 5 \times 10^{12}$ GC/ml.

Details of viral vectors used in this study can be found in Table S2. The present study utilized viral vectors expressing fluorescent reporters downstream of the powerful CMV promoter with the knowledge that all transduced cells would express the delivered transgenes. This provided us with the opportunity to identify any 'off-target' homing of the vectors alongside determining cell specificity of viral vectors in the testes with the consistent use of the same promoter for all groups.

2.3 | Testicular injections

Viral particles were introduced into the interstitial compartment of adult (>70 days post-natal) testis using an Ultra-Fine 23 gauge 0.3-ml insulin needle, injecting close to the rete testis. A maximum volume of 20µl was delivered (volume determined using previously described methods[16,27,28]). Delivery of the particles was monitored with the addition of Trypan Blue dye to the viral particles (0.04%). Contralateral testis was injected with vehicle (serum-free media) with Trypan Blue Dye (0.04%). Injections were performed via an incision in the abdomen through which the testes were exposed, which, following injection was closed using sterile sutures. Mice were injected subcutaneously with 0.05mg/kg buprenorphine (Vetergesic; Ceva Animal Health Ltd, UK) whilst still anaesthetized and allowed to recover on a heat pad whilst being monitored. Adeno-associated virus injections were performed with and without Neuraminidase (NA). AAV9 particles were diluted with 100 milli-units (mU) of Neuraminidase (from *Vibrio cholera*, Type III) (Sigma-Aldrich, US) within each 20-µl testicular injection and both injected simultaneously into the interstitial compartment. A protocol describing the experimental process from generation of hypothesis to collection of tissues is described in Figure S1.

2.4 | Tissue collection and imaging

Freshly dissected organs were imaged in cold phosphate-buffered saline with a Leica MZFLIII microscope with an epifluorescent green filter. Testes were then fixed separately in Bouin's fixative (Clin-Tech Ltd, UK) for 6 hours before being transferred to 70% ethanol prior to processing into paraffin wax and sectioning to 5 µm for histological analysis.

2.5 | Immunostaining and stereology

Localization of proteins in tissue sections was performed using fluorescent immunostaining procedures as described previously.[31] Details of antibodies used can be found in Table S1. Images were captured using an Olympus Provis AX70 microscope (Olympus, Japan) with Zen imaging software (Carl Zeiss Ltd, UK). A minimum of three different animals from each group were utilized to ensure reproducibility of results. Negative controls lacking primary antibody were used throughout.

Estimates of viral targeting of Leydig cells were determined using whole slide scans (taken using a Zeiss Axio Scan.Z1 (Lentiviral and adenoviral-injected tissue) or with a Leica Aperio AT2 (adeno-associated virus injected tissue) of testis sections immunolabelled with GFP and a Leydig cell-specific marker (HSD3β1 or CYP17a1). Grids were then placed over the top of the scanned image, and both GFP- Leydig cells and GFP + Leydig cells were counted in alternate squares within the grid. A minimum of two testis sections per testis and three animals per experimental group were counted.

2.6 | Statistical analysis

Statistical analysis was performed with Prism 8.3.0 software (GraphPad Software Inc, USA). Data sets were tested for normality (assuming the data approximates a Gaussian distribution) using the

D'Agostino and Pearson Normality Test. Data were compared with either an unpaired two-tailed Student's t test or a Mann-Whitney *U* test dependent upon normality of data.

For these studies, a *P* value of ≤ .05 was considered statistically significant for both parametric and non-parametric analyses.

3 | RESULTS

3.1 | Lentivirus

3.1.1 | Lentiviral-delivered transgene expression is observed in 8% of Leydig cells at 7 days post-injection but absent by 10 days

Lentiviral vectors have previously been utilized to target Leydig cells in the interstitial compartment of the testes.[27,28] However, despite the ability of lentiviral vectors to induce long-term delivered transgene expression and the low turnover rate of the adult Leydig cell population,[32] studies have reported a decrease in delivered transgene expression nine days post-injection with a lentiviral vector.[27] To understand this, we delivered a VSV-G pseudotyped lentiviral vector carrying a green fluorescent protein (eGFP) transgene downstream of a CMV promoter (LV-eGFP) directly to the interstitial compartment of an adult testis (Figure 1A + B). The contralateral testis was utilized as an internal control, injected with vehicle (Figure 1A).

To determine whether Leydig cells were being targeted by the delivered lentiviral vectors, fluorescent immunolocalization for GFP and for a well established Leydig cell marker (HSD3ß) was performed on both vehicle and LV-eGFP-injected testes 3-, 7- and 10-day post-injection (dpi). These collection points were chosen based on previous studies detecting lentiviral-delivered GFP at 3 dpi and reporting a decrease in expression at 9 dpi.[27] GFP expression was solely limited to HSD3ß expressing cells demonstrating that LV-eGFP did not target other testicular cell types following delivery to the interstitial compartment. Stereological quantification of lentiviral-targeted Leydig cells (by counting GFP-positive Leydig cells vs GFP-negative Leydig cells) revealed an average of 8.3% of the Leydig cell population expressing GFP at 7 dpi (at highest concentration delivered) (Figure S2B). As a result of this low targeting efficiency, presence of GFP was not detectable in whole freshly dissected testis when illuminated at the appropriate wavelength immediately after dissection (Figure S2A).

Co-localization of GFP and HSD3ß was visible in LV-eGFP-injected testis sections at 3 dpi and 7 dpi (Figure 1C). However, despite lentiviral vectors having the capacity to permanently integrate into the transduced host cell genome, no GFP expression was evident at 10 dpi (Figure 1C).

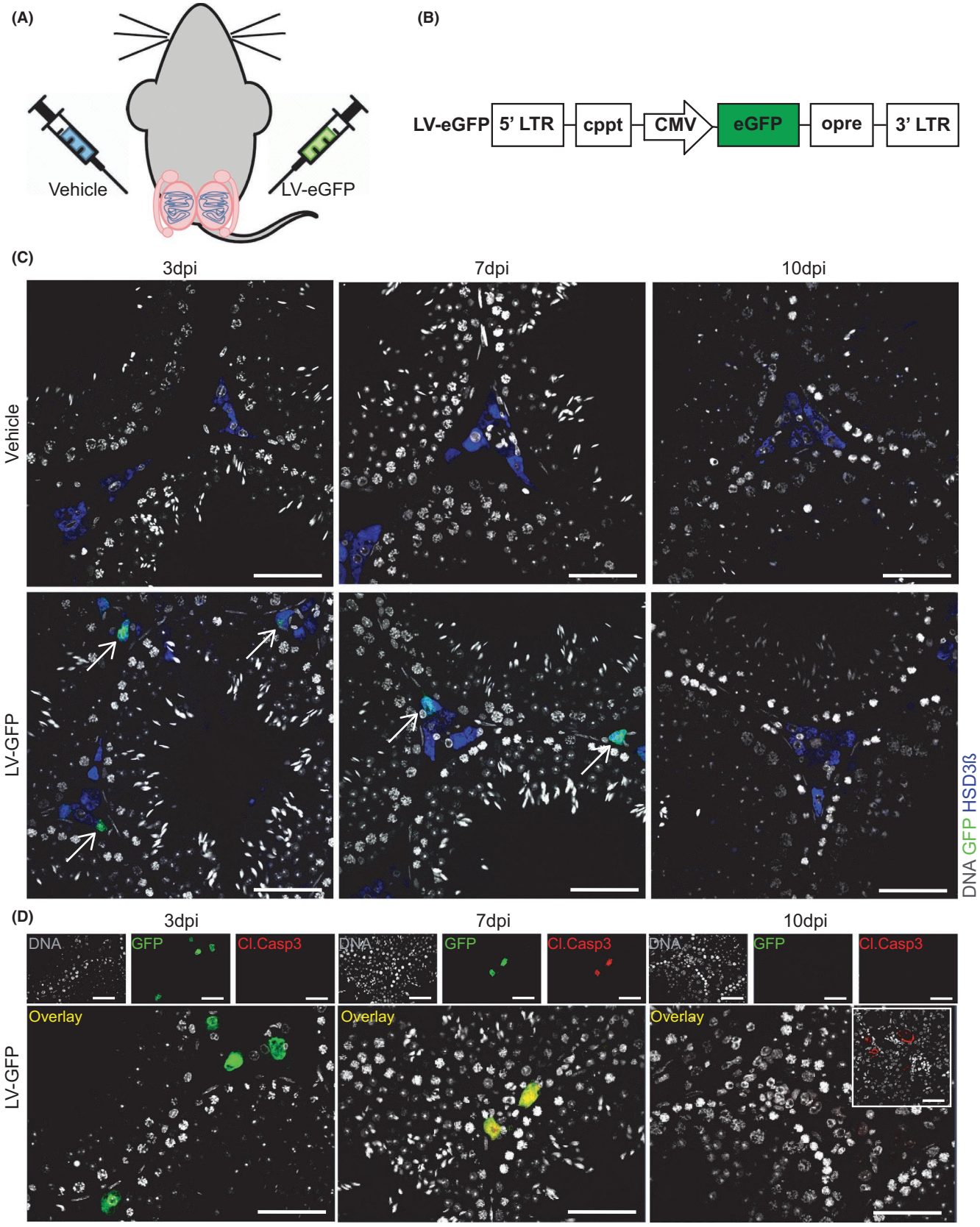
3.1.2 | Leydig cells undergo apoptosis following targeting with lentiviral vectors

As a key characteristic of lentiviral vectors is their ability to integrate into the host cell genome (even in non-dividing cells), absence of transgene expression at 10 dpi was unexpected. The two most likely explanations for the loss of transgene expression are silencing of the lentiviral transgene or death of targeted cells. Both the published literature,[33-35] and our own experience suggested that transgene silencing was unlikely (as lentivirus integrates at different genomic locations), so to determine whether the loss of GFP expression at 10 dpi in cells targeted with the LV-eGFP was due to the cells undergoing apoptosis, fluorescent immunostaining of GFP alongside a marker for apoptosis, activated cleaved caspase 3 (Cl.Casp3) was performed on LV-eGFP-injected testis. Co-localization of GFP and Cl.Casp3 revealed that LV-eGFP-targeted Leydig cells had initiated apoptosis by 7 dpi and that this process was complete by 10 dpi, providing an explanation as to the loss of GFP-positive Leydig cells at 10 dpi (Figure 1D).

To determine whether the cause of this apoptosis could be due to toxicity as a result of the high titre of viral vectors being delivered to the interstitial compartment, we repeated the study, injecting lower titres of LV-eGFP (3.75×10^7 TU/ml and 5×10^7 TU/ml) into the interstitial compartment (maintaining injection volumes at 20 μl). However, the only difference observed was that fewer GFP-positive cells were detectable at 7 dpi with reduced concentrations of LV-eGFP (Figure S2B). Again, GFP-positive cells were undergoing apoptosis at 7 dpi and no GFP-positive cells were detected at 10 dpi (data not shown) indicating that targeted cells had undergone cell death.

Together, these data suggest that VSV-G pseudotyped lentiviral vectors are not suitable for the delivery of transgenes requiring long-term expression to the Leydig cells of the adult testis. However, if short-term transgene expression in a small percentage of the Leydig cell population is desirable, lentiviral vectors may be a suitable vector choice, though consideration must be given to whether the eventual targeted cell apoptosis observed is welcome or problematic.

FIGURE 1 Leydig cells targeted by lentiviral vectors undergo apoptosis. A, Schematic diagram depicting the interstitial injections performed with vehicle in one testis and lentivirus carrying a transgene expressing green fluorescent protein (GFP) (LV-GFP) in the contralateral testis. B, Schematic illustrating the transgene expressing GFP downstream of a Cytomegalovirus (CMV) promoter. C, HSD3β and GFP expression in WT testis 3, 7 and 10 days post-injection (dpi) with either vehicle or with LV-GFP. Representative images demonstrate targeting of lentivirus to the Leydig cells at 3 and 7 dpi (white arrows) with no evidence of GFP expression at 10 dpi. D, GFP and cleaved caspase 3 (Cl.Casp3) expression in vehicle or LV-GFP-injected testis 3, 7 and 10 dpi. Overlay of DNA, GFP and Cl.Casp3 channels/images to illustrate co-localization of proteins. Insert = Cl.Casp 3 tissue-positive control (liver). Representative images reveal apoptosis of Leydig cells targeted with lentivirus. Scale Bars = 20 μm


(A)
Vehicle
LV-eGFP
(B)
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5' LTR
cppt
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7dpi
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Vehicle
LV-GFP
DNA GFP HSD3ß
(D)
3dpi
7dpi
10dpi
DNA
GFP
Cl.Casp3
Overlay
LV-GFP

3.2 | Adenovirus

3.2.1 | Delivery of adenoviral vectors to the interstitial compartment

Adenoviral vectors have previously been utilized for the delivery of transgenes to the adult Leydig cell population.[16] To confirm and further characterize targeting of Leydig cells with adenoviral vectors, C57BL6J testes were injected with adenoviral particles carrying GFP downstream of a CMV promoter (AdV-GFP) into the interstitial compartment (Figure 2A). Imaging of freshly dissected testes (Figure 2B) and immunofluorescent staining of GFP and CYP17A1 (a well-characterized steroidogenic enzyme) (Figure 2C) revealed Leydig cell-specific transgene expression in the interstitial compartment of the testis, that was detected at 3 dpi and persisted for up to 10 dpi. In all samples, GFP expression was limited exclusively to cells expressing CYP17A1. At 35 dpi, Leydig cell-specific transgene expression was evident in four of the five testes injected with AdV-GFP. GFP-positive Leydig cells at 35 dpi were found to be in close proximity to the seminiferous tubules within the interstitial compartment (Figure 2C). As these cells also expressed steroidogenic enzyme CYP17A1 (characteristic of Leydig cells), we postulated that these could be stem Leydig cells.

This confirmed that adenoviral vectors target the Leydig cells when delivered to the interstitial compartment of the adult testes.

3.2.2 | Adenoviral-delivered transgene expression is observed in 19% of Leydig cells, which do not undergo apoptosis

Stereological quantification of GFP-positive Leydig cells indicated that an average of 19.31% of Leydig cells were targeted by the adenoviral vectors and that there were no differences in the number of GFP-positive Leydig cells in testis at 3 dpi and at 10 dpi (Figure 2D). This suggested that, unlike the Leydig cells targeted with Lentiviral vectors, Leydig cells targeted with adenoviral vectors had not initiated apoptosis at 7 dpi. To confirm this, co-immunolocalization with GFP and Cl.Casp3 was performed on AdV-GFP-injected testes at 3 dpi, 10 dpi and 35 dpi. No Cl.Casp3 was detected in any AdV-GFP-injected testis sections (Figure 2E), confirming that adenoviral targeted Leydig cells did not undergo apoptosis by 35 dpi.

3.2.3 | Utility of adenoviral vectors for transient transgene expression

Adenoviral vectors are known to have an increased immunogenicity compared to alternative vectors and do not integrate delivered transgenes into the host cell DNA.[36] As a result, adenoviral vector-delivered transgene expression is often short-lived, particularly in highly proliferative cell populations as the delivered transgene may not be replicated during cell division.[37] A loss of transgene expression in the testes at around 16 dpi following a delayed acute immune response has also previously been reported.[16] We occasionally observed immune cell infiltration around the periphery of adenovirus-injected testes at 10 dpi, consistent with previous publications identifying these cells as T lymphocytes.[16] Therefore, adenoviral vectors may not be a desirable delivery vector if sustained transgene expression is required. However, as targeted cells do not undergo apoptosis, they may be suitable in circumstances where sustained transgene expression would be undesirable, for example the delivery of gene editing machineries such as Cre-Recombinase and CRISPR/Cas9 technology.

To demonstrate this capability, we injected the testes of mice carrying the red fluorescent protein (RFP) reporter allele *Gt(ROSA)*$^{26Sortm14(CAG-tdTomato)Hze}$ with adenoviral vectors carrying either GFP alone (AdV-GFP) or with Cre-recombinase (AdV-CRE-GFP), both downstream of a CMV promoter (Figure 3A). Use of this reporter line permits induction of RFP expression exclusively in cells expressing Cre-recombinase. In RFP reporter testis injected with AdV-CRE-GFP, RFP expression was detectable in freshly dissected testis (Figure 3B) at 3 dpi and 10 dpi, suggesting successful delivery and subsequent activity of Cre recombinase expressing transgene. Closer inspection of viral injected testes sections immunostained for GFP/tRFP alongside Leydig cell marker CYP17A1 confirmed Leydig cell targeting and Cre-recombinase activity following injection with AdV-CRE-GFP at 3 dpi and 10 dpi (Figure 3C). In addition to the Leydig cell-specific recombination observed in the testis following injection of AdV-CRE-GFP into RFP reporter testis, RFP expression was also detectable in freshly dissected 'non-target' tissues, in particular in the liver and in the adjacent epididymis to the injected testis (Figure S3).

These data suggest that adenoviral vectors may not be suitable for the delivery of transgenes requiring sustained expression. However, in circumstances where short-term transgene expression is necessary, such as with the delivery of some gene modification technologies, adenoviral vectors may be a suitable option though consideration should be given to the 'off-target' recombination possibility in other 'non-target' organs.

3.3 | Adeno-associated virus

3.3.1 | Adeno-Associated viral vector-delivered transgenes are expressed for over 30 days post-injection in Leydig cells

Adeno-associated viral vectors have been shown to be capable of long-term transgene expression and to be of low immunogenicity.[38] They have also recently been exploited for the targeting of testicular cell types including in immature Leydig cells and in spermatogonial stem cells.[18,19]

To determine the targeting capability of adeno-associated viruses in the adult testis in comparison to the capabilities of lentiviral and adenoviral vectors, C57BL6J testes were injected with adeno-associated viruses of either serotype 8 (AAV8) or 9

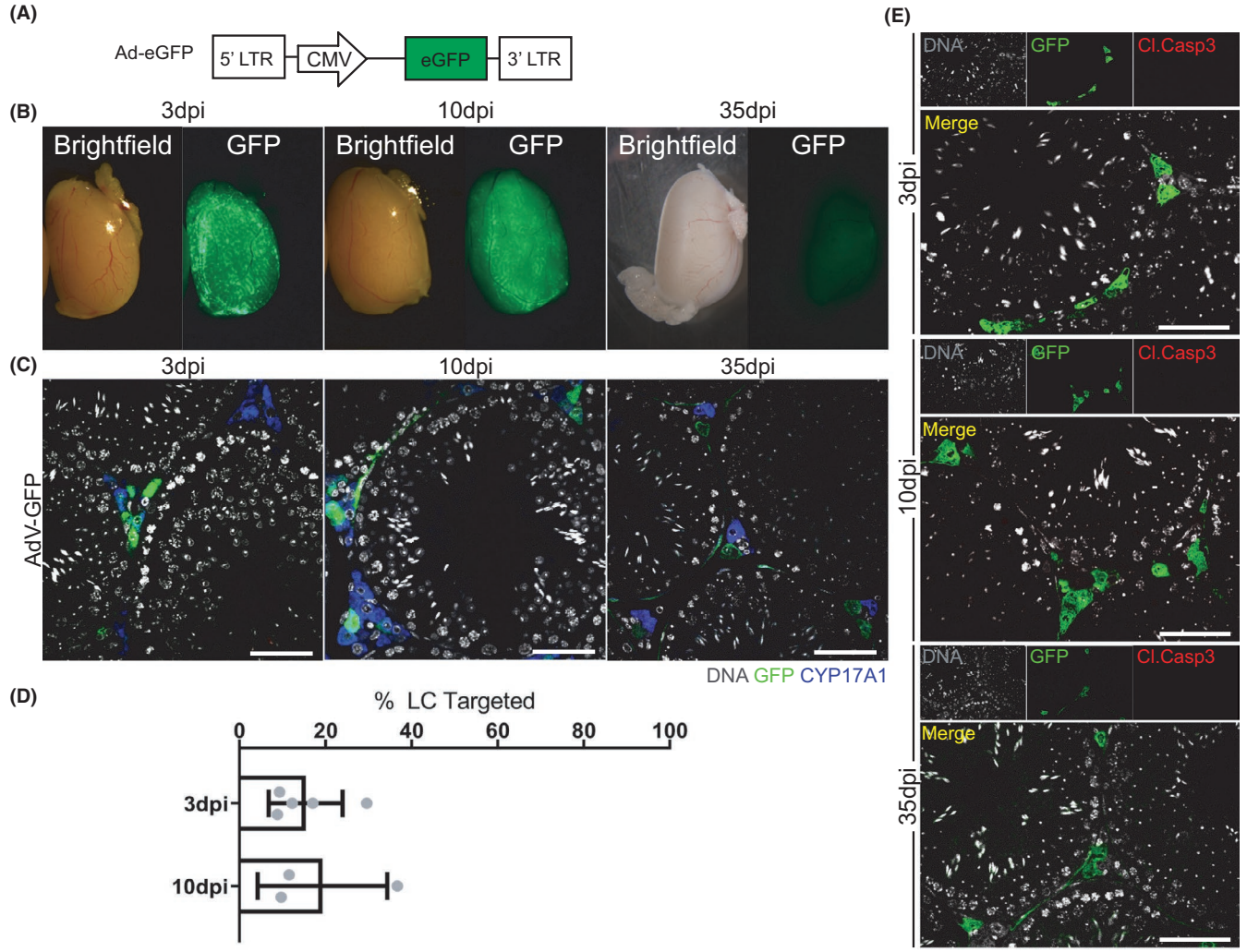


FIGURE 2 Adenoviral vectors target Leydig cells in the testis and do not result in apoptosis of targeted cells. A, Schematic diagrams of adenoviral construct used illustrating the transgene expressing GFP downstream of a CMV promoter. B, Representative images of freshly dissected C57BL6J WT testis under brightfield and GFP fluorescent filters at 3, 10 and 35 days following injection with AdV-GFP (Brightfield images taken at 0.5 seconds of exposure, fluorescent images taken after 5.5 seconds of exposure). C, GFP and CYP17A1 expression in WT testis sections 3, 10 and 35 dpi following injection with AdV-GFP. D, Percentage of cells co-expressing delivered fluorescent protein and Leydig cell marker CYP17A1; 3 dpi (n = 5) and 10 dpi (n = 3). (Unpaired t test; $P = .6479$). Bars represent average ± SEM. E, GFP and cleaved caspase 3 (Cl.Casp3) expression in AdV-GFP-injected testis 3, 10 and 35 dpi. Single channel images (DNA, GFP, Cl.Casp3) below larger overlayed channel image. Overlay of DNA, GFP and Cl.Casp3 channels/images to illustrate co-localization of proteins. Representative images reveal survival of Leydig cells targeted with adenovirus Scale bars = 20 μm

(AAV9), both carrying transgenes expressing GFP downstream of a CMV promoter (Figure 4A). To determine whether adeno-associated viral vectors are capable of delivering sustained transgene expression, we chose to include later time points of 50 dpi (AAV8) and 30 dpi (AAV9) considering these serotypes have previously been reported to target spermatogonial stem cells.[19] As studies have also demonstrated the ability to increase targeting efficiencies of adeno-associated viral vectors using the enzyme neuraminidase,[19,39] we chose to include groups in which testes were co-injected with neuraminidase (NA) alongside AAV8-GFP and AAV9-GFP (AAV8-GFP + NA and AAV9-GFP + NA, respectively) to determine whether numbers of targeted cells could be increased.

GFP expression was observed in freshly dissected AAV8-GFP and AAV9-GFP-injected testes at 7 dpi, 30 dpi (AAV8-GFP) and 50 dpi (AAV9-GFP) (Figure 4B and C). The addition of neuraminidase to injections of AAV8-GFP and of AAV9-GFP appeared to increase observed fluorescence in freshly dissected testes at 30 dpi and 50 dpi, respectively, compared to those injected with adeno-associated viral particles alone (Figure 4B and C).

To determine whether the adeno-associated viral particles were specifically targeting Leydig cells when delivered to the interstitial compartment, co-localization of GFP and HSD3ß was examined. In testes injected with either AAV8-GFP and AAV9-GFP, GFP expression was specific to HSD3ß-expressing cells, indicating that adeno-associated viral vectors specifically target Leydig cells when

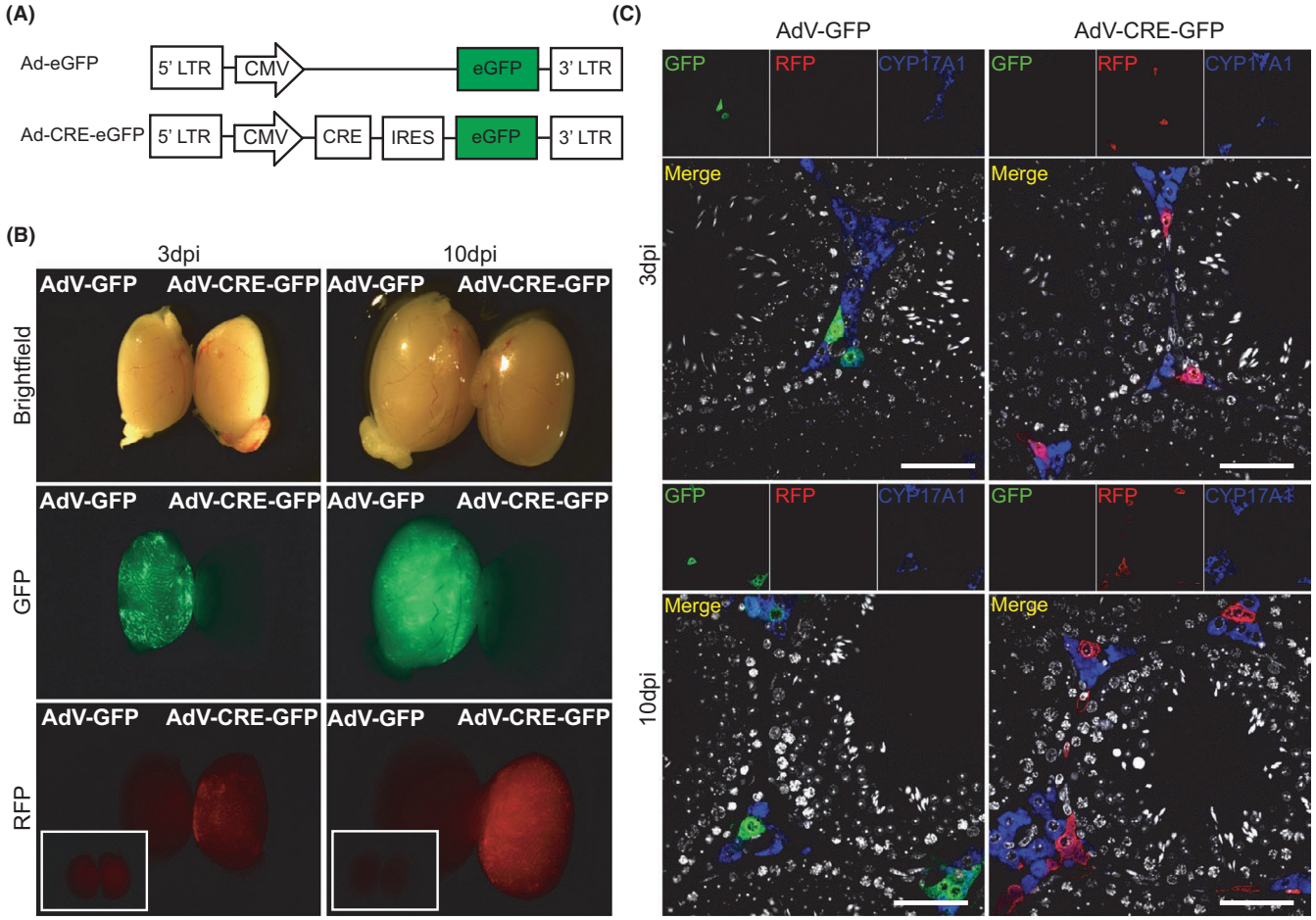
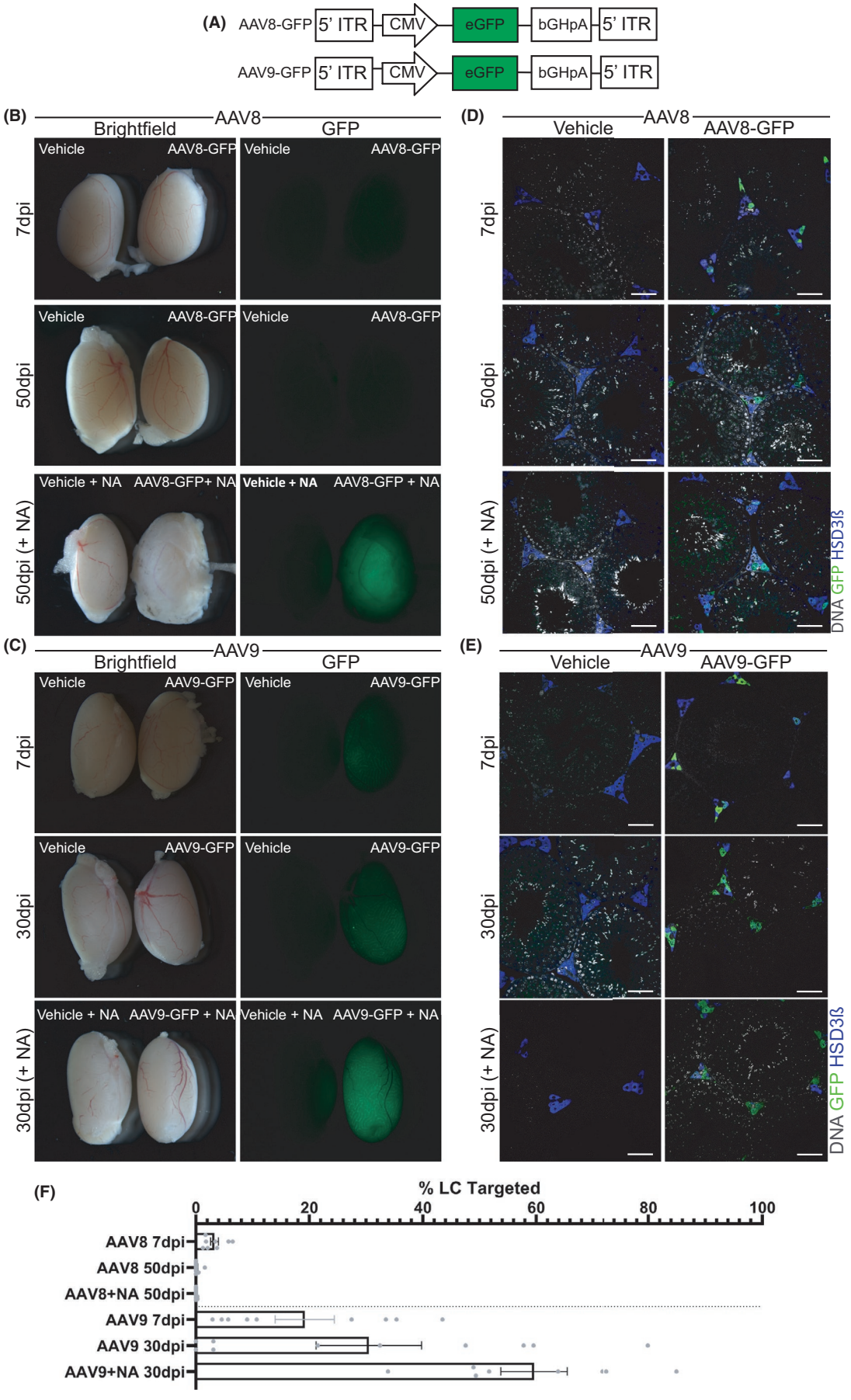


FIGURE 3 Adenoviral vectors can be utilized for the delivery of site-specific recombinases. A, Schematic diagrams of adenoviral constructs used illustrating the transgene expressing GFP downstream of a CMV promoter (Ad-eGFP) and the transgene expressing CRE and GFP (separated with an IRES component) downstream of a CMV promoter. B, Representative images of freshly dissected Gt(ROSA)$^{26Sortm14(CAG-tdTomato)Hze}$ (RFP reporter) testis 3 and 10 dpi with AdV-GFP or AdV-CRE-GFP. Inserts = AdV-CRE-GFP or AdV-GFP-injected C57BL6J testis imaged under the RFP filter demonstrating lack of RFP/autofluorescence. (Brightfield images taken at 0.5 seconds of exposure, fluorescent images taken after 5.5 seconds of exposure). C, GFP, RFP and CYP17A1 co-localization in RFP reporter testis sections 3 and 10 dpi with AdV-GFP or AdV-CRE-GFP. Overlay of GFP, RFP and CYP17A1 channels/images with DNA counterstain to illustrate co-localization of proteins. Representative images reveal activity of adenovirus delivered cre recombinase specifically in Leydig cells. Scale bars = 20 μm

delivered to the interstitial compartment and no other testicular cell population (Figure 4D and E). This cell specificity was not altered with the addition of neuraminidase into the injections, with GFP expression also exclusively located in HSD3ß expressing cells in testes injected with either AAV8-GFP + NA or AAV9-GFP + NA (Figure 4D and E). Minimal GFP expression was also observed in the liver (Figure S4). GFP expression was also observed in AAV8-GFP and AAV9-GFP-injected testis collected at 50 dpi and 30 dpi (AAV8/9-GFP and AAV8/9-GFP + NA) (Figure 4B–E). This demonstrates a sustained expression of the delivered transgene, greater than that seen with

FIGURE 4 Delivery of transgenes using AAV9 with neuraminidase results in sustained expression in an increased number of Leydig cells. A, Schematic diagrams of adeno-associated viral vectors used. (bGHpA = bovine growth hormone polyA). B and C, Representative images of freshly dissected C57BL6J testis under brightfield and GFP filters at 7 dpi (both serotypes) and 50 dpi (B – AAV Serotype 8 (AAV8)) or 30 dpi (C – AAV9). Later time points (30/50 dpi) also include testis injected with AAV8/9 alongside neuraminidase (NA). D, GFP and HSD3β expression in WT testis 7 dpi with vehicle or AAV8-GFP and at 50 dpi with vehicle, vehicle with NA, AAV8-GFP or AAV8-GFP with NA. E, GFP and HSD3β expression in WT testis 7 dpi with vehicle or AAV9-GFP and at 30 dpi with vehicle, vehicle with NA, AAV9-GFP or AAV9-GFP with NA. Both D and E demonstrate delivery of transgenes to HSD3β expressing cells (Leydig cells) using AAV8 and AAV9 alone and with the addition of neuraminidase. Scale bars = 50 μm. F, Percentage of cells co-expressing delivered fluorescent protein and Leydig cell marker; HSD3β gives an estimation as to the number of cells being targeted with AAV vectors at 7 dpi (AAV8 (n = 5) and AAV9 (n = 5)), 30 dpi (AAV9 (n = 4) AAV9 + NA (n = 5)) and 50 dpi (AAV8 (n = 5), AAV8 + NA (n = 5)). (Unpaired t test; P = *). Bars represent average ± SEM


(A)
AAV8-GFP
5' ITR
CMV
eGFP
bGHpA
5' ITR
AAV9-GFP
5' ITR
CMV
eGFP
bGHpA
5' ITR
(B)
AAV8
Brightfield
GFP
Vehicle
AAV8-GFP
7dpi
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Vehicle + NA
AAV8-GFP+ NA
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AAV8-GFP + NA
50dpi (+ NA)
(C)
AAV9
Brightfield
GFP
Vehicle
AAV9-GFP
7dpi
30dpi
Vehicle + NA
AAV9-GFP + NA
30dpi (+ NA)
(D)
AAV8
Vehicle
AAV8-GFP
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DNA GFP HSD3ß
(E)
AAV9
Vehicle
AAV9-GFP
7dpi
30dpi
30dpi (+ NA)
DNA GFP HSD3ß
(F)
% LC Targeted
0
20
40
60
80
100
AAV8 7dpi
AAV8 50dpi
AAV8+NA 50dpi
AAV9 7dpi
AAV9 30dpi
AAV9+NA 30dpi

both lentiviral and adenoviral vectors, despite adeno-associated viral vectors lacking the capacity to integrate delivered transgenes into the host cell genome.

3.3.2 | Leydig cells survive targeting with adeno-associated viral vectors

Viral titre of adeno-associated viral particles delivered to the adult testes was considerably higher than that of lentiviral and adenoviral vectors. To ensure there was no toxicity as a result of viral load and that adeno-associated viral particles were not impacting Leydig cell survival, we performed co-localization studies with GFP and Cl.Casp3 on AAV8-GFP and AAV9-GFP-injected testes sections (including those co-injected with neuraminidase) from each time point (Figure 5A). In all sections observed, there was no evidence of Cl.Casp3 revealing that targeted Leydig cells did not undergo apoptosis. This indicated that Leydig cell survival was not impacted by targeting with adeno-associated viral particles despite their high titre. Additionally, we did not observe the significant immune cell infiltration in AAV8-GFP and AAV9-GFP-injected testes, seen in our adenoviral-injected testes and in previous publication.[16] This is consistent with the reduced immunogenicity characteristic of adeno-associated viral vectors.

3.3.3 | Adeno-associated virus targeting of Leydig cells is increased when using serotype 9 alongside neuraminidase

To determine the targeting efficiency of adeno-associated viral vectors, the number of GFP-positive Leydig cells vs GFP-negative Leydig cells were counted in AAV8-GFP, AAV8-GFP + NA, AAV9-GFP and AAV9-GFP + NA-injected testes. Quantification of targeted Leydig cells using these stereological techniques revealed that AAV8 had a low targeting efficiency of 6.33% of Leydig cells (less than that of lentiviral vectors) and that AAV9 targeted a significantly greater proportion of the Leydig cell population (targeting 30.49%) at 30 dpi (Figure 4F). Neuraminidase did not significantly increase targeting of AAV8-GFP at 50 dpi with the average number of targeted LC being as low as 0.05% in AAV9-GFP + NA-injected testis. The addition of neuraminidase at the point of injection with AAV9 significantly increased targeting efficiencies of AAV9 with an average of 59.63%, and an observed maximum of 84.95% of Leydig cells

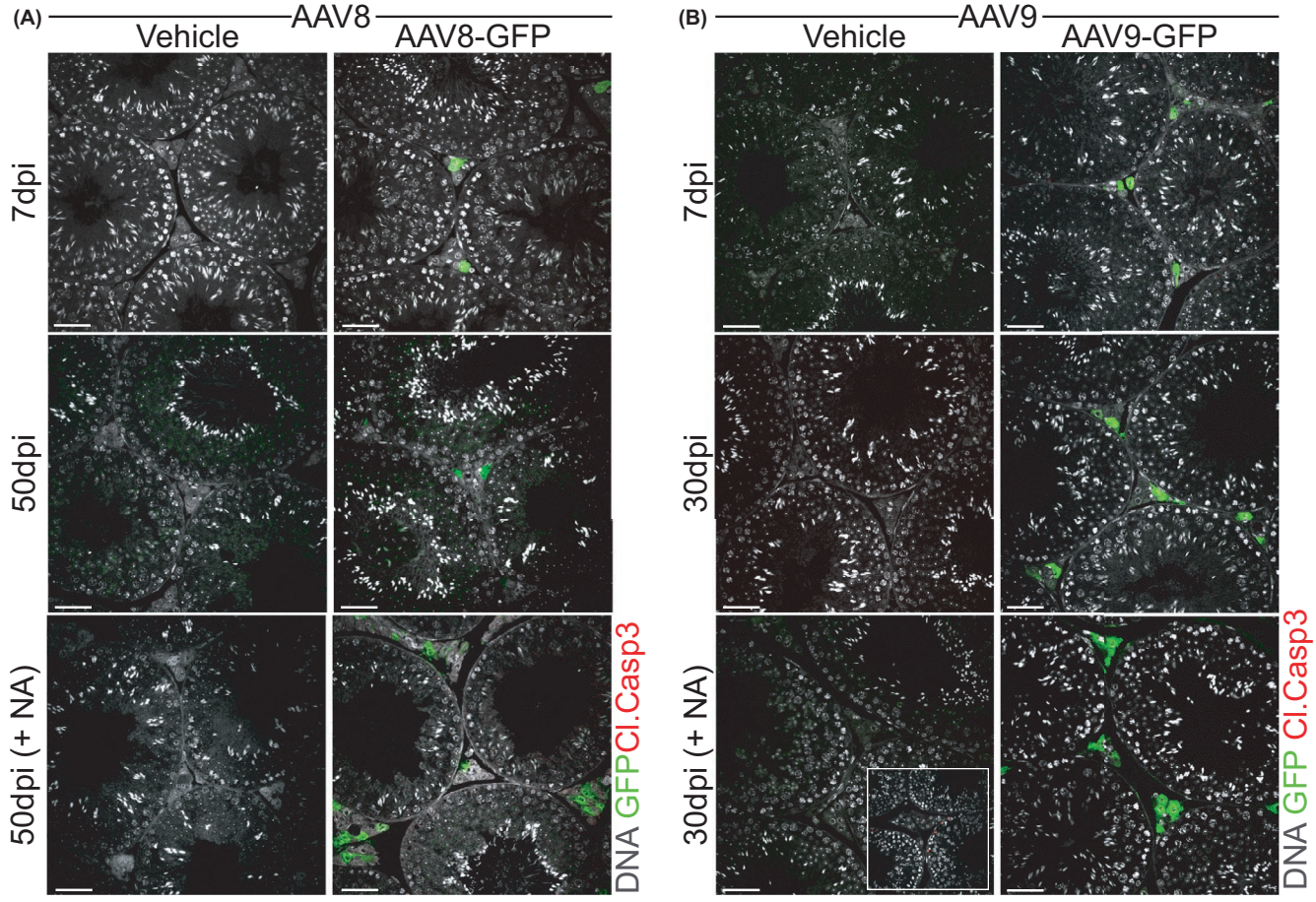

FIGURE 5 Delivery of transgenes using AAV does not result in apoptosis of the targeted Leydig cells. A, GFP and cleaved caspase 3 (Cl.Casp3) expression in Vehicle or AAV8-GFP and AAV9-GFP-injected testis with and without neuraminidase (NA) at 7 and 50 or 30 dpi. Scale bars = 50 μm

being targeted in AAV9-GFP + NA-injected testis when observed at 30 dpi (Figure 4F). A summary of the targeting characteristics of AAVs and the other viral vectors described in this study can be found in Table 1.

Together, these data identify AAV9 as an effective viral vector for the delivery of transgenes requiring long-term gene expression to the majority of the adult Leydig cell population. Furthermore, with the delivery of neuraminidase alongside AAV9, it is possible to significantly increase Leydig cell targeting. This will be of significant interest to researchers requiring transgene expression to persist in a large percentage of the Leydig cell population.

4 | DISCUSSION

The ability to target and manipulate-specific cell types in vivo is a powerful means of investigating development, maintenance, function as well as aging and disease within a living organism. Investigating the genetics behind the proper functioning of Leydig cells in adulthood has often been confounded due to difficulties distinguishing impacts during development from impacts in adulthood. An alternative, though under-utilized method for generating genetic modification specifically in the testes, is through the use of deliverable transgenics. Viral and non-viral vectors permit the rapid generation and subsequent investigation of genetic mutants at key time points. However, before utilizing such powerful tools of gene delivery and modification, vector systems must first be evaluated in terms of targeting abilities and longevity of delivered transgene expression to ensure chosen vectors are fit for purpose.

We have compared key characteristics of three commonly used viral delivery systems, namely their targeting efficiency when delivered to the interstitial compartment of the testes, the longevity of gene expression in the context of testicular function and the impact of viral infection on the targeted cell type. In doing so, we have identified methods for targeting the adult Leydig cell population. These methods will prove useful for the generation of a genetically modified Leydig cell population, for gene rescue studies or for the development of novel therapeutics. Regardless of purpose, using these methods to interrogate the genetics underlying endocrine and reproductive disorders will have significant impacts on cost, time and the numbers of animals required to obtain an appropriate experimental cohort, providing additional ethical benefits over traditional transgenic mouse studies.

Of the viral vectors examined in this study, all could target and deliver a transgene to the adult Leydig cell population when injected directly into the interstitial compartment of the testis, with all delivered reporter expression co-localizing specifically and exclusively with Leydig cell-specific markers. Where the viral vectors differ was in the longevity of delivered transgene expression, the impact on Leydig cell survival and in the percentage of the Leydig cell population being targeted.

Presence of the lentiviral-delivered reporter gene, GFP, was found in an average 8.63% of Leydig cells at 3 dpi and 7 dpi but was absent from injected testis after 10 days as a result of targeted cells undergoing apoptosis. Previous studies utilizing lentiviral vectors to target the Leydig cells have collected tissues prior to ten days post-injection and observed that reporter gene expression begins to decrease after nine days,[27,28] affording some explanation as to why lentiviral-targeted Leydig cell apoptosis has not previously been described. Increased apoptosis of cells exposed to Lentivirus has also recently been reported in human haematopoietic stem and progenitor cells. This was shown to be a result of lentiviral vectors triggering signalling through tumour protein 53 (p53), a protein encoded by a tumour suppression gene, known to have a role in stem cell maintenance and in the initiation of apoptosis.[40] Whether this is the mechanism through which lentiviral-targeted Leydig cells are undergoing apoptosis requires further investigation.

Delivery of adenoviral vectors to the seminiferous tubules of the testis has previously been shown to disrupt the seminiferous epithelium as a result of the adenovirus interacting with the junctional complexes found within the blood-testis-barrier.[41] Though impractical for the targeting of Sertoli cells, adenoviral vectors have been utilized in the literature for the delivery of transgenes to the Leydig cells.[16] Cre recombinase expressing adenoviral particles have been utilized for the study of GATA factor function in Leydig cells of the testes by injecting the particles directly into the interstitial compartment.[16] However, this previous study reported that transgene reporter expression was only detectable for up to 12 dpi, the loss of which coinciding with an increased immune response, due to the increased immunogenicity

TABLE 1 Summary of Leydig cell targeting abilities of Viral Vector Systems

Virus type	Episomal/ Integrating	Immunogenic response	Suitability	Mean % Leydig cells targeted	Maximum % Leydig cells targeted
Lentivirus	Integrating	Low	Short-term transgene expression with clearance of targeted LC	8.39	9.31
Adenovirus	Episomal	High	Delivery of gene modification technology	19.31	36.68
AAV8	Episomal	Low	Delivery of transgene for sustained expression	6.33	10.98
AAV8 + NA	Episomal	Low	Delivery of transgene for sustained expression	0.05	0.3
AAV9	Episomal	Low	Delivery of transgene for sustained expression	30.49	79.92
AAV9 + NA	Episomal	Low	Delivery of transgene for sustained expression	59.63	84.95

of adenoviral vectors.[16] We observed GFP/reporter expression in an average of 19.31% of Leydig cells and found that targeted Leydig cells were still visible at 10 days post-injection and were not undergoing apoptosis like those targeted with lentiviral vectors. Considering the short-lived expression described in the literature,[16] it would suggest that adenoviral vectors are suitable for the delivery of transgenes demanding short-term expression. An example of this is provided in the present study with the delivery of Cre-recombinase using an adenoviral vector. Following successful recombination of LoxP sites, transgene expression is no longer required. Circumstances in which the use of adenoviral vectors would also prove beneficial is for the delivery of guide RNAs for use with CRISPR/Cas9 technologies, where persistent expression of a guide RNA could result in unwanted repeated cleavage of the host cell DNA. However, careful consideration must be given to the potential for 'off-target' expression in 'non-target' organs.

More recently, adeno-associated viral vectors have been described as capable of targeting Leydig cells in the testes.[17,19] Though attempting to target the spermatogonial stem cell population, 'off-target' detection of viral reporter genes within the Leydig cell population alongside targeting of the spermatogonial stem cells was noted.[19] Our studies have confirmed selective targeting of the Leydig cells with adeno-associated viruses, in particular serotype 9 alongside neuraminidase which was shown to be capable of increasing targeting to the majority of the Leydig cell population. In all adeno-associated virus injected testis, no evidence of targeting of spermatogonial populations was observed, differing from observations made in previous studies[19] and highlighting our improved methodology for the targeting of the Leydig cell population. Increasing cell targeting efficiency with the inclusion of neuraminidase in viral delivery has been shown to be due to a significant reduction in cell surface sialic acid, effectively increasing the abundance of the adeno-associated virus's target receptor on the target cell surface.[39] Our study provides the first instance in which this increase in cell targeting using neuraminidase is seen in Leydig cells, with previous studies suggesting increases in adeno-associated viral targeting of germ cell populations.[19] Previous studies utilizing adeno-associated viral vectors in the testes collected injected testis tissue no later than 8 days post-injection, limiting the ability to determine whether adeno-associated viral particles are capable of long-term expression of the delivered transgene.[17,19] For our study, we have included later time points, relevant to the time taken to complete at least one round of spermatogenesis in mice. By confirming transgene expression is detectable for at least this time period and by confirming the lack of Leydig cell toxicity as a result of adeno-associated viral vector infection, we identify adeno-associated viral vector serotype 9 with neuraminidase as a gene delivery system with strong efficacy for the investigation of Leydig cell function. However, when using a constitutive promoter to drive transgene expression, some consideration must be given to the minimal targeting observed within the liver.

Delivery of adenoviral and adeno-associated viral vectors to the testis resulted in 'off-target' transgene expression, particularly within the liver. The use of the CMV promoter to drive lentiviral transgene expression was therefore of particular importance. Identifying that vectors are only targeting Leydig cells in the testis is a valuable piece of information when initially selecting the most appropriate tools for Leydig cell targeting. The next step of investigation into the potential of this technology for the targeting of Leydig cells, particularly within a clinical context, would be to introduce Leydig cell-specific promoters into viral constructs. This would begin to address any concern of complications due to 'off-target' homing of vectors to organs such as the liver.

In conclusion, all viral vectors examined in this study specifically targeted the Leydig cells when delivered to the interstitial compartment, with varying characteristics of targeting that may be beneficial or problematic depending upon the question being asked. We also acknowledge that this study examined just a small number of the many variations of these and other viral vector systems, however, suggest that our findings have significant efficacy for future study of Leydig cell function.

The simplicity of viral delivery to the interstitial compartment, the wide availability of off the shelf and custom viral vectors, and characterization of viral vectors for selective delivery of transgenes to Leydig cells in the adult mouse provides a significant step forward in possibilities for genetic dissection of Leydig cell function. The approach is simple, quick, highly efficient and cheap. It requires no great expertise in genetics or molecular biology and negates the need to breed, cross and maintain transgenic mouse lines in order to produce an experimental cohort. The ready availability of AAV9 + neuraminidase approach will be of significant advantage to researchers investigating the genetics underpinning Leydig cell function and holds promise to inform the development of novel therapeutics for the treatment of male reproductive disorders.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHORS CONTRIBUTIONS

LBS, AD and DR designed the study; AD, DR, MC, KK, NJ, NR, LM and CR performed the experimental work; AD, DR and LBS analysed results; PB provided expertise and viral vectors for the study; AD and LBS wrote the manuscript. All authors were provided the opportunity to review and edit the manuscript prior to submission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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