Probing GATA factor function in mouse Leydig cells via testicular injection of adeno viral vectors

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Abstract

Testicular Leydig cells produce androgens essential for proper male reproductive development and fertility. Here, we describe a new Leydig cell ablation model based on Cre/Lox recombination of mouse Gata4 and Gata6, two genes implicated in the transcriptional regulation of steroidogenesis. The testicular interstitium of adult Gata4Δlox/Δlox; Gata6Δlox/Δlox mice was injected with adenoviral vectors encoding Cre + GFP (Ad-Cre-IRES-GFP) or GFP alone (Ad-GFP). The vectors efficiently and selectively transduced Leydig cells, as evidenced by GFP reporter expression. Three days after Ad-Cre-IRES-GFP injection, expression of androgen biosynthetic genes (Hsd3b1, Cyp17a1 and Hsd17b3) was reduced, whereas expression of another Leydig cell marker, Ins3, was unchanged. Six days after Ad-Cre-IRES-GFP treatment, the testicular interstitium was devoid of Leydig cells, and there was a concomitant loss of all Leydig cell markers. Chromatin condensation, nuclear fragmentation, mitochondrial swelling, and other ultrastructural changes were evident in the degenerating Leydig cells. Liquid chromatography-tandem mass spectrometry demonstrated reduced levels of androstenedione and testosterone in testes from mice injected with Ad-Cre-IRES-GFP. Late effects of treatment included testicular atrophy, infertility and the accumulation of lymphoid cells in the testicular interstitium. We conclude that adenoviral-mediated gene delivery is an expedient way to probe Leydig cell function in vivo. Our findings reinforce the notion that GATA factors are key regulators of steroidogenesis and testicular somatic cell survival.

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Introduction

Testicular Leydig cells are essential for proper male phenotypic differentiation and fertility (Teerds & Huhtaniemi 2015). Two distinct populations of Leydig cells arise sequentially during mammalian development (Virtanen & Toppari 2014). Fetal Leydig cells, which appear soon after testicular organogenesis (E12.5 in the mouse), produce androgens required for masculinization of the fetus (O’Shaughnessy & Fowler 2011, Wen et al. 2016, Shima & Morohashi 2017). Adult Leydig cells, which arise in the prepubertal period, secrete androgens essential for sexual maturation and spermatogenesis (Teerds & Huhtaniemi 2015). Both populations of Leydig cells possess the enzymes required for conversion of cholesterol into androstenedione (cholesterol side chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B1) and cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1)) (O’Shaughnessy et al. 2002). Adult Leydig cells, but not their fetal counterparts, possess HSD17B3, an enzyme that metabolizes androstenedione into testosterone (O’Shaughnessy et al. 2000, Shima et al. 2013).

Cell-specific ablation models have provided insight into the development and function of Leydig cells (Smith et al. 2015). The most widely used of these models entails administration of ethane dimethane sulfonate (EDS) to adult rats, which triggers the rapid destruction of Leydig cells via apoptosis (Teerds et al. 1989). Three to six weeks after EDS treatment, the adult Leydig cell population regenerates (Kerr et al. 1985, Molenaar et al. 1986). This model has allowed investigators to identify factors that regulate Leydig cell differentiation...
(Molenaar et al. 1986, Yan et al. 2000, Sriraman et al. 2003, Salva et al. 2004, O’Shaughnessy et al. 2008, Zhang et al. 2013, O’Shaughnessy et al. 2014, Lobo et al. 2015, Zhang et al. 2015). Additionally, the EDS model has shed light on stem Leydig cells present in peritubular and perivascular locations within the testicular interstitium (Kilcayn et al. 2014, Chen et al. 2017). One limitation of EDS is that it does not cause Leydig cell destruction in mice except at high doses that may be associated with additional off-target effects (Smith et al. 2015).

Here, we describe a new Leydig cell ablation model based on delivery of Cre recombinase into the testes of mice harboring floxed alleles of Gata4 and Gata6, two key regulators of steroidogenic cell differentiation and function (Tevosian 2014, Röhrig et al. 2015, Tremblay 2015). Gata4 and Gata6 are expressed in fetal/adult Leydig cells (Ketola et al. 1999, 2002, Mazaud-Guittot et al. 2014) and have been shown to activate the promoters of several steroidogenic genes, including Cyp11a1 and Cyp17a1 (Tremblay & Viger 2001, Jimenez et al. 2003, Rahman et al. 2004, Sber et al. 2007). Conditional targeting of Gata4 in the adenogonadal primordium and fetal/adult Leydig cells using Sf1-Cre produces undervirilized mice with small testes that lack mature sperm (Manuylov et al. 2011). Simultaneous deletion of both Gata4 and Gata6 using Sf1-cre results in a more severe testicular phenotype marked by a paucity of Leydig cells, reduced testosterone production and the accumulation of adrenal-like cells in the interstitium (Padua et al. 2015). To focus on the function of GATA factors in Leydig cells of the adult mouse, we devised a conditional gene deletion strategy that relies on intratesticular injection of an adenoviral vector encoding Cre. We show that deletion of Gata4 + Gata6 in this manner leads to attenuated steroidogenesis followed by destruction of adult Leydig cells. More broadly, our results show that adenoviral-mediated gene delivery is an expedient and selective means of probing Leydig cell function in vivo.

Materials and methods

Experimental animals

Procedures involving mice were approved by the institutional committee for laboratory animal care and were conducted in accordance with the National Research Council’s (NRC) publication Guide for Care and Use of Laboratory Animals. Gata4<sup>flox/flox</sup> mice (also termed Gata4<sup>tm2.1Sad/J</sup>), Gata6<sup>flox/flox</sup> mice (also termed Gata6<sup>tm2.1Sad/J</sup>) and Sf1-Cre mice (also termed FVB-Tg(Nr5a1-cre)2Lowl/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and genotyped as described (Watt et al. 2004, Dhillon et al. 2006, Oka et al. 2006, Sodhi et al. 2006). Gata4<sup>flox/flox</sup> mice were crossed with Gata6<sup>flox/flox</sup> mice to produce Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice. Male Sf1-Crel; Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice were generated using an established breeding scheme (Padua et al. 2015, Tevosian et al. 2015). All mice had free access to water and standard rodent chow and were exposed to 12-h light/12-h darkness photoperiods. At specified times, mice were killed by CO<sub>2</sub> asphyxiation.

Intratesticular injection

We obtained recombinant human adenovirus (serotype 5, dE1/ E3) expressing green fluorescent protein (GFP) alone (Ad-GFP) or in combination with Cre (Ad-Cre-RES-GFP) from Vector Biolabs (Philadelphia, PA, USA). Male Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice (2 months old) were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) ip. The intra-and post-operative analgesic regimen included buprenorphine (0.05 mg/kg sc) and carprofen (5 mg/kg sc). We employed two different surgical techniques to inject adenovirus. In initial experiments, an abdominal incision (Qamar et al. 2015) was made to expose the testes for injection. To avoid the potentially confounding variable of surgically induced cryptorchidism, a scrotal incision (Kojima et al. 2003) was made in the subsequent experiments. These alternative methods yielded comparable results, particularly at early time points (<7 days) post injection, indicating that surgical approach was not a major determinant of experimental outcome. Adenovirus (20 µL, 1 × 10<sup>10</sup> plaque formation units (pfu) per µL in Dulbecco’s Modified Eagle’s medium (DMEM) containing 2% BSA and 2.5% glycerol (v/v)) was injected slowly into the interstitial space of each testes using a 30-gauge needle. Sham-operated mice underwent skin incision and testes visualization without intratesticular injection.

Histological analyses

Whole testes or other organs were fixed by overnight immersion in Bouin’s solution (Sigma) or 4% paraformaldehyde (PFA) in PBS. Paraform-embedded tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) or subjected to immunostaining (Anttonen et al. 2003, Krachulec et al. 2012). The type of fixation and the primary/secondary antibodies used for each antigen are listed in Table 1. Bound antibody was visualized using the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) and diaminobenzidine. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on paraform-embedded tissue sections using the ApopTag Peroxidase In Situ Apoptosis kit (EMD Millipore). For direct visualization of GFP, cryosections (10 µm) were prepared after embedding unfixed testes in O.C.T. compound (Thomas Scientific, Swedesboro, NJ, USA). These sections were mounted in Immu-Mount containing DAPI (ThermoFisher Scientific) and photographed using an Olympus BX61 fluorescence microscope.

Quantitative RT-PCR (RT-qPCR)

RNA was isolated from whole testes (Kyrölähti et al. 2011) and subjected to RT-qPCR analysis as described (Dörner et al. 2017).
using the primers listed in Table 2. Expression was normalized to the housekeeping genes Gapdh and Actb.

**Electron microscopy**

Mice were anesthetized and perfused for 20 min with modified Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer) via a needle inserted into the left ventricle (Pihlajoki et al. 2013). Testes were harvested, incubated overnight at 4°C in the same fixative, rinsed and then postfixed in 2% OsO₄ for 1 h. The samples were dehydrated and embedded in epoxy resin. Thick sections (1 μm) were stained with toluidine blue and examined by light microscopy to determine which blocks were to be thin-sectioned. Thin sections were stained with uranyl acetate and lead citrate and photographed using a JEOL 1400 transmission electron microscope.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS)**

Testicular biopsies (~30 mg each; n = 4 per group) were harvested and frozen at −80°C. Testis tissue was homogenized in 200 µL of 0.9% saline using Precellys beads (KT 03961-1-003.2, Bertin Technologies, France). Tissue homogenates were spiked with isotope-labeled steroids as internal standards. Samples were then extracted with 1 mL of toluene (Chromasolv plus for HPLC, Sigma), dried and reconstituted in 30% acetonitrile. Steroids were measured using an Agilent 1290 Series HPLC system connected to an Agilent 6495 QQQ mass spectrometer.

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**Table 1** Tissue fixation methods and antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Marker of</th>
<th>Fixative</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA4</td>
<td>Leydig cells, Sertoli cells, perivascular cells, some peritubular cells</td>
<td>4% PFA in PBS</td>
<td>sc-1237, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200</td>
<td>Donkey anti-goat biotinylated IgG, Jackson Immunoresearch, West Grove, PA; 1:1000</td>
</tr>
<tr>
<td>GATA6</td>
<td>Leydig cells, Sertoli cells</td>
<td>4% PFA in PBS</td>
<td>sc-1225, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200</td>
<td>Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>Apoptosis</td>
<td>4% PFA in PBS</td>
<td>sc-66850, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200</td>
<td>Donkey anti-goat biotinylated IgG, Jackson Immunoresearch, West Grove, PA; 1:1000</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>Leydig cells</td>
<td>Bouin’s</td>
<td>sc-66850, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200</td>
<td>Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000</td>
</tr>
<tr>
<td>HSD3B1</td>
<td>Leydig cells</td>
<td>Bouin’s</td>
<td>sc-30820, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200</td>
<td>Donkey anti-goat biotinylated IgG, Jackson Immunoresearch, West Grove, PA; 1:1000</td>
</tr>
</tbody>
</table>

**Table 2** Primers for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence (5′ → 3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>F: GGCTGACATCAAGAAGAAGC&lt;br&gt;R: AAGATCCATACCCAGAAGGG</td>
<td>NM_007393.3</td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: GCTCACTGGCATGCGCTTCGGT&lt;br&gt;R: TGGAAAGCTGGGTGTGGTGA</td>
<td>NM_008084.2</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>F: AGGGGTGGACACGACCTCCA&lt;br&gt;R: TGCTGGCTTTGAGGAGTGGAACC</td>
<td>NM_017979.3</td>
</tr>
<tr>
<td>Cyp11b1</td>
<td>F: GCTTACATGTTCTGAAATTC&lt;br&gt;R: AGAAGAGAGGGACATGGTCCA</td>
<td>NM_001033229.3</td>
</tr>
<tr>
<td>Cyp17a1</td>
<td>F: CCAGATGTGCTGTCGTCCTGTC&lt;br&gt;R: GGTCTGTATGGTAGTCAGTATCG</td>
<td>NM_007809.3</td>
</tr>
<tr>
<td>Ins3</td>
<td>F: CAGGGACGGTGAGGACCC&lt;br&gt;R: CGCTGGCCGCTGAAAGCCT</td>
<td>NM_013564.7</td>
</tr>
<tr>
<td>Hsd1b1</td>
<td>F: TGGACAAATGATCTGACGAC&lt;br&gt;R: GCCACACTTTGTGGAACGAG</td>
<td>NM_008293.3</td>
</tr>
<tr>
<td>Hsd1b3</td>
<td>F: GAGTGTGGCCAGAAAYGACT&lt;br&gt;R: AGCCTCAGGCTGCTTCTCA</td>
<td>NM_0082891.3</td>
</tr>
<tr>
<td>Sox9</td>
<td>F: AGGAAGCGGAGAAGAAGGG&lt;br&gt;R: GGACCCGAGAAGGAGGAGA</td>
<td>NM_011448.4</td>
</tr>
</tbody>
</table>
Triple Quadrupole mass spectrometer. Standard compounds were obtained from Sigma, Steraloids (Newport, RI, USA), Fluka (Bucharest, Romania) and Riedel-de Haën (Seelze, Germany). A publication detailing the analytical method is in preparation.

Assessment of reproductive function

To assess fertility, male mice that had undergone intratesticular injection with Ad-GFP or Ad-Cre-IRES-GFP were housed continuously with fertility-proven female \( \text{Gata}^4 \text{flox/flox}; \text{Gata}^6 \text{flox/flox} \) mice and the number of offspring documented. Serum luteinizing hormone (LH) levels were measured by ELISA (ERK R7017, Endocrine Technologies; Newark, CA, USA).

Statistical methods

Steroid, mRNA and LH levels in different groups of mice were compared using the Student's t test. Fertility was compared via two population proportions testing. Statistical significance was set at the following: \( *P < 0.05 \) and \( **P < 0.01 \).

Results

Overview of the adenoviral injection strategy

The testes of adult \( \text{Gata}^4 \text{flox/flox}; \text{Gata}^6 \text{flox/flox} \) mice were injected with replication incompetent adenoviral vectors encoding Cre+GFP (Ad-Cre-IRES-GFP) or GFP alone (Ad-GFP) (Fig. 1A). We opted to delete \( \text{Gata}^4 \) and \( \text{Gata}^6 \) simultaneously to decrease the chance of a compensatory response and increase the likelihood of a robust phenotype. The dose of virus introduced into each testis was \( 2 \times 10^8 \text{pfu} \). Based on an estimate of \( 3 \times 10^6 \) Leydig cells per testis (Hu et al. 2010), this dose corresponded to a multiplicity of infection (MOI) of \( ~70 \) virions per target cell, similar to the MOI of 100 used previously to transduce primary cultures of mouse Leydig cells (Schrade et al. 2015).

Leydig cells were efficiently transduced by the adenoviral vectors, as evidenced by GFP reporter expression 2–3 days post injection (dpi) (Fig. 1B and C). Moreover, there was uniform expression of the GFP reporter throughout the interstitial compartment, implying adequate dispersion of the small volume (20\( \mu L \)) of injected virus. Other interstitial cell types, such as peritubular cells and vascular cells, were not efficiently transduced (Fig. 1D). There was no GFP expression within the seminiferous tubules, indicating that neither Sertoli cells nor germ cells were infected (Fig. 1B, C and D). Immunostaining of extragonadal organs showed scattered GFP expression in hepatocytes but no appreciable expression of GFP in submaxillary glands, lungs, heart or adrenal glands (data not shown).

Leydig cell ablation in the testes of mice injected with Ad-Cre-IRES-GFP

The testes of \( \text{Gata}^4 \text{flox/flox}; \text{Gata}^6 \text{flox/flox} \) mice were injected with Ad-GFP or Ad-Cre-IRES-GFP and then harvested at varying times for light microscopic analysis. At 3 dpi, Leydig cells were evident in both the Ad-GFP- and Ad-Cre-IRES-GFP-treated mice (Fig. 2A and B). Six days after Ad-Cre-IRES-GFP injection, however, the testicular interstitium was depleted of Leydig cells (Fig. 2C and D). Cells remaining in the interstitial compartment of these mice were mainly vascular cells (endothelial cells, smooth muscle cells and pericytes) and resident macrophages (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). Leydig cell depletion persisted in the Ad-Cre-IRES-GFP-treated mice at 12 dpi (Fig. 2E and F) and at later time points (see below).

Delayed inflammatory response in the testes of mice injected with adenovirus

By 12 dpi, there was a lymphocytic infiltrate in the testicular interstitium of both the Ad-GFP- and
Ad-Cre-IRES-GFP-injected mice (Fig. 2E and F). Immunostaining with CD3ε (Fig. 3A, C and E) confirmed that the infiltrating cells were T-lymphocytes. In the testes of mice subjected to sham surgery, there was no evidence of an inflammatory response (data not shown). In mice injected with Ad-GFP, there was a loss of reporter gene expression at 12 dpi that coincided with T-cell infiltration (Fig. 3B, D and F). In addition to curtailing the survival of infected cells, immune cell infiltration may obfuscate the physiological impact of transgene expression (Yang et al. 1994, Blanchard & Boekelheide 1997). To circumvent the technical limitations imposed by lymphocyte infiltration, we focused the subsequent histological, ultrastructural and biochemical analyses on early time points post infection (days 3–7), when the effects of Cre-Lox recombination on Leydig cell function/survival could be assessed in the absence of overt T-cell infiltration.

**Expeditious Cre-mediated recombination in Leydig cells**

To confirm Cre-mediated recombination, testes sections were immunostained for GATA4 and GATA6. Ordinarily, nuclear GATA4 immunoreactivity is evident in Leydig cells, Sertoli cells and some peritubular cells of the adult mouse (Supplementary Fig. 2A) (Viger et al. 1998, Ketola et al. 1999, Bielinska et al. 2007). At 3 dpi, GATA4 was present in Leydig cells of Gata4floxed/floxed; Gata6floxed/floxed mice treated with Ad-GFP but was absent from Leydig cells of mice treated with Ad-Cre-IRES-GFP (Fig. 4A and B), verifying efficient Cre-mediated gene recombination in this cell type. GATA4 staining was preserved in Sertoli cells of mice treated with Ad-Cre-IRES-GFP, underscoring the notion that adenovirus injected into the testicular interstitium selectively infects Leydig cells. At 6 dpi, GATA4+ Leydig cells were evident in Ad-GFP-treated testes but not in Ad-Cre-IRES-GFP-treated testes (Fig. 4C and D). Scattered GATA4+ pericytes were observed in the interstitium of the Ad-Cre-IRES-GFP-treated testes (Fig. 4D and Supplementary Fig. 2B). Quantitative analysis of random microscopic fields confirmed a marked reduction in GATA4-immunoreactive interstitial cells in the Ad-Cre-IRES-GFP-treated animals at 3 and 6 dpi (P < 0.01) (Supplementary Fig. 3). As with GATA4, staining for GATA6 demonstrated a loss of Leydig cell immunoreactivity in mice injected with Ad-Cre-IRES-GFP (Supplementary Fig. 4).
Changes in gene expression following Cre-mediated recombination

We used RT-qPCR to compare the expression of Leydig and Sertoli cell markers in the testes of Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice injected with Ad-GFP or Ad-Cre-IRES-GFP (Fig. 5A and B). At 3 dpi, expression of the Leydig cell marker Ins13 (Anand-Ivell et al. 2009) was comparable in the two groups, confirming the presence of intact Leydig cells. In contrast, at this time point, there was an acute downregulation of androgen biosynthetic genes (Hsd3b1, Cyp17a1, Hsd17b3) in mice treated with Ad-Cre-IRES-GFP (<i>P</i> < 0.01). By 7 dpi, all Leydig cell markers examined (Ins13, Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b3) were markedly attenuated (<i>P</i> < 0.01) in the mice injected with Ad-Cre-IRES-GFP, consistent with ablation of this cell type. Expression of Sox9, a Sertoli cell marker, was unchanged at 3 and 7 dpi, reinforcing the premise that Ad-Cre-IRES-GFP injection selectively affects the interstitial compartment.

To validate the RT-qPCR findings, we performed immunohistochemistry for HSD3B1, CYP17A1 and HSD17B3 (Fig. 6). Six days after injection of Ad-Cre-IRES-GFP, immunoreactivity for these steroidogenic enzymes was reduced, consistent with a loss of Leydig cells.

Figure 4 GATA4 immunostaining in the testes of Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice injected with Ad-GFP or Ad-Cre-IRES-GFP. The testes of 2-month-old mice were injected with Ad-GFP (A and C) or Ad-Cre-IRES-GFP (B and D). Tissue was harvested at the indicated times, fixed in PFA and subjected to GATA4 immunoperoxidase staining. (A and B) At 3 days post injection, GATA4 immunoreactivity was evident in Leydig cells of mice injected with Ad-GFP (black arrows) but absent from Leydig cells of mice injected with Ad-Cre-IRES-GFP (red arrows), confirming efficient Cre-mediated recombination in this cell type. In contrast, GATA4 immunoreactivity was preserved in Sertoli cells (arrowheads). (C and D) At 6 days post injection, GATA4<sup>+</sup> Leydig cells were evident in Ad-GFP-treated testes (black arrows) but not in Ad-Cre-IRES-GFP-treated testes. GATA4<sup>+</sup> pericytes were observed in the interstitium of the Ad-Cre-IRES-GFP-treated testes (blue arrows). Scale bars = 50 µm.

Figure 5 RT-qPCR analysis of testes from Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice injected with Ad-GFP or Ad-Cre-IRES-GFP. The testes of 2-month-old mice were injected with Ad-GFP or Ad-Cre-IRES-GFP. At 3 days (A) or 7 days (B) post-infection, RNA was extracted from whole testis and subjected to RT-qPCR. Results were normalized to expression of the housekeeping gene Gapdh. Normalization to Actb expression yielded similar results. Values are expressed as the mean ± s.d. (<i>n</i> = 4 per group; **<i>P</i> < 0.01).

Ultrastructural and biochemical analysis of cell death in mice injected with Ad-Cre-IRES-GFP

To gain insights into the process of Leydig cell death in the adenovirus injection model, resin-embedded tissue was processed for light microscopy (Supplementary Fig. 5) and transmission electron microscopy (Fig. 7A, B, C and D and Supplementary Fig. 6). Vacuole-laden activated macrophages, harbingers of Leydig cell death, were prominent in the testicular interstitium of mice treated 4 days earlier with Ad-Cre-IRES-GFP. Leydig cells exposed to Ad-Cre-IRES-GFP, but not Ad-GFP, contained clumps of condensed chromatin beneath the nuclear membrane. Nuclear fragmentation was evident in some of these Leydig cells. Cytoplasmic organelles in Ad-Cre-IRES-GFP-treated Leydig cells had changes not seen in their Ad-GFP-treated counterparts.
such as mitochondrial swelling, loss of definition of mitochondrial cristae and enlargement of lipid droplets. Some of these degenerative changes were suggestive of apoptosis (Jackson et al. 1986, Gao et al. 2002), but other forms of cell death (e.g., necroptosis, ferroptosis) could not be excluded based on ultrastructural analysis alone.

Looking for biochemical evidence of Leydig cell apoptosis, we performed immunostaining for cleaved (activated) caspase-3 and TUNEL staining. Compared to sham-operated mice, there was no increase in cleaved caspase-3 immunoreactivity in the Ad-GFP or Ad-Cre-IRES-GFP mice 3 dpi (data not shown). Similarly, there was no evidence of increased TUNEL staining in either the Ad-GFP or the Ad-Cre-IRES-GFP mice 3 or 6 dpi (data not shown). Thus, the mechanistic basis of Leydig cell death in the Ad-Cre-IRES-GFP mice remains uncertain.

**Reduced androgen levels in the testes of mice injected with Ad-Cre-IRES-GFP**

We used LC-MS/MS to quantify steroids in testis homogenates from Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice treated 7 days earlier with Ad-GFP or Ad-Cre-IRES-GFP (Fig. 8). Testes from mice injected with Ad-Cre-IRES-GFP had reduced levels of androstenedione (P < 0.01) and testosterone (P < 0.05). On the other hand, there was no difference in the levels of intratesticular corticosterone in mice injected with Ad-GFP vs Ad-Cre-IRES-GFP (41 ± 14 pmol/g vs 31 ± 17 pmol/g, P = 0.4).

**Late effects of adenovirus-mediated gene targeting**

In contrast to EDS-induced ablation/regeneration in the rat, there was slow and variable Leydig cell regeneration in the testes of Gata4<sup>flox/flox</sup>; Gata6<sup>flox/flox</sup> mice treated with Ad-Cre-IRES-GFP. By ~30 dpi, much of the testicular interstitium remained devoid of Leydig cells (Fig. 9A). Residual Leydig cells clustered in small patches, and
all were GATA4+ suggesting that these cells or their stem cell progenitors escaped adenovirus-mediated Cre inactivation (Fig. 9B). Some of these Leydig cells appeared cytomegalic (Fig. 9B), perhaps reflecting a compensatory response to impaired regeneration. Between ~30 and ~120 dpi, the number of Leydig cells (all of which were GATA4+) increased (Fig. 9C, D, E and F), although there was mouse-to-mouse variability in the extent of Leydig cell regeneration. Underscoring the inconsistency in Leydig cell recovery, serum LH values at 90–92 dpi ranged from 0.6 to 32 ng/mL (mean = 11 ng/mL, n = 4), whereas serum LH values for age-matched sham controls were consistently low (mean = 0.5 ± 0.2 ng/mL, n = 5). By ~90 dpi, patches of lymphoid cells were evident in the testicular interstitium of mice treated with Ad-Cre-IRES-GFP (Fig. 9C, D, E and F). The void created by Leydig cell ablation may have permitted the accumulation of these lymphoid patches, which were as large as 1 mm in diameter. Neither the variable Leydig cell regeneration nor the accumulation of lymphoid aggregates was attributable to impaired clearance of virus, as there was no evidence of persistent GFP immunoreactivity between ~30 and ~90 dpi (data not shown). In the months following Ad-Cre-IRES-GFP injection, changes were seen in the seminiferous epithelium (e.g., thinning, vacuolization, absence of mature sperm), consistent with inadequate androgen production (Fig. 9C, D, E and F and data not shown). Testicular atrophy was evident in some of the older Ad-Cre-IRES-GFP-injected mice, likely reflecting impaired spermatogenesis (Fig. 9G). When Cata4<sup>lox/lox</sup>; Gata6<sup>lox/lox</sup> mice treated with Ad-Cre-IRES-GFP (n = 5) were housed for 3 months with fertility-proven wild-type females, no progeny were observed. By comparison, 3 of 4 Cata4<sup>lox/lox</sup>; Gata6<sup>lox/lox</sup> mice injected with Ad-GFP-sired pups in the months following injection (P < 0.05).

Tevosian and coworkers have shown that male Sf1<sup>-</sup>cre; Cata4<sup>lox/lox</sup>; Gata6<sup>lox/lox</sup> mice survive congenital adrenocortical aplasia due to production of corticoids by adrenal-like cells that accumulate in the testes postnatally (Padua <sup>et al.</sup> 2015, Tevosian <sup>et al.</sup> 2015). These heterotopic cells express adrenocortical markers (Cyp21a1, Cyp11b1, Cyp11b2 and Mc2r). To test whether adrenal-like cells arise in the testes of...
to drive the expression of not only Cre but also small hairpin RNAs (shRNAs) (Machitani et al. 2013). Such adenoviral shRNA constructs could be used to silence genes in the Leydig cells of mice lacking floxed alleles. Thus, our adenoviral delivery method could be adapted to study the roles of other genes implicated in Leydig cell function.

The principal shortcoming of adenoviral gene delivery is that it triggers an immune response in the testis and other organs (Blanchard & Boekelheide 1997, Hendrickx et al. 2014, Tsuzuki et al. 2016). In the liver, where this response has been characterized in detail, adenoviral transduction stimulates a lymphocytic infiltrate that leads to destruction of genetically modified cells and repopulation with hepatocytes lacking the transgene (Yang et al. 1994). This immune response is directed against de novo-synthesized viral components and is independent of MOI (Yang et al. 1994). In the liver and other cell types, the lymphocytic infiltrate typically appears ~10 days post infection and resolves ~30 days post infection, coinciding with a loss of transgene expression (Yang et al. 1994, Blanchard & Boekelheide 1997). We circumvented this limitation of the adenoviral delivery system by focusing on changes in Leydig cell gene expression, function and survival at early time points (<7 days) post infection, before the appearance of a pronounced T-lymphocytic infiltrate. Another limitation of the adenoviral injection method is its relative irreversibility. The method apparently kills a significant fraction of Leydig cell precursors, although some escape Cre-mediated recombination and partially repopulate the interstitium after several weeks. Inflammation may accentuate the adverse effects of adenoviral-mediated gene targeting.

The interstitial injection technique described here complements other methods for delivering transgenes or inhibitory RNAs to specific testicular cell populations in rodents. Retrograde instillation of adenoviral vectors into efferent ductules can transduce cells within the seminiferous tubules (Blanchard & Boekelheide 1997, Kanatsu-Shinohara et al. 2002, Kojima et al. 2003, Hooley et al. 2009). Injection of retrovirus (Kanatsu-Shinohara et al. 2004) or lentivirus (Ikawa et al. 2002, Kim et al. 2010) into the testis results in infection of multiple cell types including germ cells, Sertoli cells and/or Leydig cells. Direct testicular injection of a buffered salt solution can be used to introduce shRNA into germ cells, albeit with low transfection efficiency (~5%) (Ho et al. 2017). Early gonadal development can be disrupted by cultivating organs in hanging droplets of media supplemented with vivo-morpholinos (Rudigier et al. 2017). Cre can be targeted to mouse Leydig cells by germline transmission of transgenes, such as Sfi-Cre (Bingham et al. 2006), Cyp11a1-iCre (Wu et al. 2007) and Cyp17a1-iCre (Bridges et al. 2008). Although germline Cre transgenes avoid the problem of virus-induced host immunity, generation

**Discussion**

Cell-specific ablation models have proven invaluable for the analysis of Leydig cell differentiation and function (Smith et al. 2015). We have developed a new Leydig cell ablation model, the features of which are summarized in Fig. 10. Our approach relies on adenoviral-mediated delivery of Cre recombinase into the testicular interstitium of mice harboring floxed alleles of Gata4 and Gata6. This method of gene delivery has inherent advantages for the study of Leydig cells. As shown here and elsewhere (Blanchard & Boekelheide 1997, Hall et al. 2000, Kojima et al. 2003, LeCouter et al. 2003, Shiraishi & Ascoli 2007, Qamar et al. 2009, Qamar et al. 2015, Schrade et al. 2015), adenovirus efficiently transduces Leydig cells in vitro and in vivo. Moreover, adenovirus injected into the testicular interstitium selectively infects Leydig cells, thereby minimizing off-target effects. Adenoviral vectors rarely integrate into host chromosomes (0.001–1% of infected cells), so Leydig cell gene expression is not subject to viral DNA integration effects (Mitani & Kubo 2002). Recombinant adenovirus has been used...
of such mouse mutants is time consuming, and interpretation of the resultant testicular phenotypes may be challenging because of context-dependent effects, variable degrees of Cre-mediated recombination, compensatory responses, alternative pathways of differentiation and functional redundancy (Smith 2011, Tevosian 2014). Adenoviral delivery of Cre or shRNA affords a means of probing the in vivo function of a gene in Leydig cells before committing to germine transmission of a Leydig cell-specific Cre transgene. Intratesticular injection of adenovirus also can be used to study the short-term impact of overexpression of a gene in Leydig cells. For instance, LeCouter et al. (2003) used interstitial injection of adenviral vectors to drive the expression of angiogenic peptides in mouse testis; histological changes characteristic of angiogenesis were evident 7 dpf.

In vitro studies have provided genetic evidence that GATA factors regulate steroidogenesis in Leydig cells. For example, silencing of Gata4 in Leydig tumor cell lines (MA-10, mLTC-1) and primary adult Leydig cells has been shown to impair the expression of androgen biosynthetic genes (Cypl1a1, Hsd3b1, Cyp17a1) (Bergeron et al. 2015, Schrade et al. 2015). In keeping with these prior reports, we found that adenovirus-mediated deletion of Gata4/6 in adult Leydig cells caused the acute downregulation of genes involved in sex steroid production, whereas expression of another Leydig cell marker, Ins13, was preserved at early time points. An advantage of our in vivo model over cell culture-based systems is that the functional changes observed in response to conditional gene targeting occur in a more physiologic milieu of endocrine and paracrine factors (e.g., LH).

In our model of adenviral-mediated targeting of Gata4/6, we found that the acute decline in testicular steroidogenesis was followed days later by Leydig cell death. This finding is reminiscent of a study in which treatment of MA-10 cells with an apoptosis-inducing agent caused a decrease in steroid production in advance to cell death (King et al. 1998). GATA4 has been shown to enhance cell survival and/or decrease apoptosis in Leydig cell lines and other steroidogenic cell types (Bennett et al. 2013, Anttonen et al. 2014, Schrade et al. 2015, Pihlajoki et al. 2016). The precise mechanism of cell death in our model remains unclear. Ultrastructural analysis of Ad-Cre-IRE-GFP-treated Leydig cells demonstrated organelle changes compatible with apoptosis and other forms of cell death. In the rat EDS model, Leydig cells undergo apoptosis. This process is mediated by caspase-3 activation (Kim et al. 2000) but does not involve Bcl-2 family members (Taylor et al. 1998); instead, EDS appears to act through activation of Fas (Taylor et al. 1999) and other pro-apoptotic factors (Li et al. 2012). We found no immunohistological evidence of caspase-3 activation in our model, and TUNEL staining was negative. In cultured gonadal somatic cell lines, silencing of Gata4 impairs glycolysis and causes other metabolic derangements (Schrade et al. 2015, Schrade et al. 2016). Thus, it is conceivable that altered metabolism contributes to Leydig cell death in our adenoviral injection model.

Gata4 is expressed in not only fetal/adult Leydig cells but also in putative stem Leydig cells (Kilcoyne et al. 2014). Circumstantial evidence supports a role for GATA4 in the differentiation of Leydig stem/progenitor cells. For example, enforced expression of Gata4 plus two other transcription factors (Sfi and Dmrt1) can reprogram mouse fibroblasts into Leydig-like cells (Yang et al. 2017). GATA6, like GATA4, has been implicated in mesenchymal stem cell function (Almalki & Agrawal 2016). The incomplete regeneration of Leydig cells that typifies our adenoviral injection model could reflect, at least partly, impaired Leydig stem cell function, and future experiments will explore this possibility.

### Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0311.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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