Probing GATA factor function in mouse Leydig cells via testicular injection of adenoviral 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 *Gata4*^{flox/flox}; *Gata6*^{flox/flox} 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, *Ins13*, 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 expeditious 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 pre-pubertal 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), 3 β -hydroxysteroid dehydrogenase (HSD3B1) and cytochrome P450 17 α -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 (Kilcoyne *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, Sher *et al.* 2007). Conditional targeting of *Gata4* in the adrenogonadal 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 expeditious 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*^{flox/flox} mice (also termed *Gata4*^{tm1.1Sad/J}), *Gata6*^{flox/flox} mice (also termed *Gata6*^{tm2.1Sad/J}) 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*^{flox/flox} mice were crossed with *Gata6*^{flox/flox} mice to produce *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice. Male *Sf1-Cre*; *Gata4*^{flox/flox}; *Gata6*^{flox/flox} 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 CO₂ 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-IRES-GFP) from Vector Biolabs (Philadelphia, PA, USA). Male *Gata4*^{flox/flox}; *Gata6*^{flox/flox} 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 × 10⁷ 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. Paraffin-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 paraffin-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 BX60 fluorescence microscope.

Quantitative RT-PCR (RT-qPCR)

RNA was isolated from whole testes (Kyrölahti *et al.* 2011) and subjected to RT-qPCR analysis as described (Dörner *et al.* 2017),

Table 1 Tissue fixation methods and antibodies used for immunohistochemistry.

Antigen	Marker of	Fixative	Primary antibody	Secondary antibody
GFP	Adenoviral infection	Bouin's	ab290, rabbit polyclonal, Abcam, Cambridge, MA; 1:500	Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000
GATA4	Leydig cells, Sertoli cells, perivascular cells, some peritubular cells	4% PFA in PBS	sc-1237, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200	Donkey anti-goat biotinylated IgG, Jackson ImmunoResearch, West Grove, PA; 1:1000
GATA6	Leydig cells, Sertoli cells	4% PFA in PBS	AF1700, goat polyclonal, R&D Systems; 1:100	Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000
Cleaved caspase-3	Apoptosis	4% PFA in PBS	sc-1225, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200	Donkey anti-goat biotinylated IgG, Jackson ImmunoResearch, West Grove, PA; 1:1000
CD3e	T-lymphocytes	4% PFA in PBS	145-2C11, hamster monoclonal, Abcam, Cambridge, MA; 1:500	Goat anti-hamster biotinylated IgG, BA-9100, Vector Labs, Burlingame, CA; 1:800
CYP17A1	Leydig cells	Bouin's	sc-66850, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200	Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000
F4/80	Macrophages	4% PFA in PBS	ab16911, rat monoclonal, Abcam, Cambridge, MA; 1:500	Goat anti-rat biotinylated IgG, JGR066003, Accurate Chemical and Scientific Corp., Westbury, NY; 1:800
HSD17B3	Leydig cells	Bouin's	sc-135044, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200	Goat anti-rabbit biotinylated IgG, NEF-813, NEN Life Science, Boston, MA; 1:2000
HSD3B1	Leydig cells	Bouin's	sc-30820, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200	Donkey anti-goat biotinylated IgG, Jackson ImmunoResearch, West Grove, PA; 1:1000

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

Table 2 Primers for RT-qPCR.

Gene	Oligonucleotide sequence (5' → 3')	Reference
<i>Actb</i>	F: GCGTGACATCAAAGAGAAGC R: AGGATTCCATACCAAGAAGG	NM_007393.3
<i>Gapdh</i>	F: GCTCACTGGCATGGCCTTCCGTG R: TGGAAGAGTGGGAGTTGCTGTTGA	NM_008084.2
<i>Cyp11a1</i>	F: AGGGGTGGACACGACCTCCA R: TGCTGGCTTTGAGGAGTGGAACC	NM_019779.3
<i>Cyp11b1</i>	F: GCTTCACCATGTGCTGAAATCC R: AGAAGAGAGGGCAATGTGTCA	NM_001033229.3
<i>Cyp17a1</i>	F: CCAGATGGTGACTCTAGGCCTCTTGTC R: GGTCTGTATGGTAGTCAGTATCG	NM_007809.3
<i>Ins3</i>	F: CACGCAGCCTGTGGAGACCC R: CGCTGGCGCTGAGAAGCCT	NM_013564.7
<i>Hsd3b1</i>	F: TGGACAAAGTATCCGACCAG R: GGCACACTTGCTTGAACACAG	NM_008293.3
<i>Hsd17b3</i>	F: GAGTTGGCCAGACAYGGACT R: AGCTTCCAGTGGTCTCTCA	NM_008291.3
<i>Sox9</i>	F: AGGAAGTCGGTGAAGAACGG R: GGACCCTGAGATTGCCACAGA	NM_011448.4

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 *Gata4^{fllox/fllox}; Gata6^{fllox/fllox}* 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.

Figure 1 Intratesticular injection of adenovirus results in the selective and efficient transduction of Leydig cells. (A) Adenoviral injection strategy. (B) Two days after injection of Ad-Cre-IRES-GFP, testes were cryosectioned, counterstained with DAPI and examined by fluorescence microscopy. Seminiferous tubules are outlined by dashed lines. White arrows highlight GFP⁺ cells in the testicular interstitium. (C and D) Three days after injection of Ad-Cre-IRES-GFP, testes were harvested, fixed in Bouin's solution and subjected to immunoperoxidase staining for GFP. Black arrows in panels C & D highlight GFP⁺ Leydig cells. Note that Leydig cells were transduced efficiently, whereas other cell types (endothelial, peritubular, Sertoli and germ cells) were not. Prepared using image vectors from Servier Medical Art (<http://smart.servier.com/>), licensed under the Creative Commons Attribution 3.0 Unported License (<http://creativecommons.org/licenses/by/3.0/>). Ad, adenovirus; ec, endothelial cell; gc, germ cell; GFP, green fluorescent protein; IHC, immunohistochemistry; IRES, internal ribosomal entry site; pt, peritubular cell; sc, Sertoli cell; st, seminiferous tubule. Scale bars = 50 µm.

Results

Overview of the adenoviral injection strategy

The testes of adult *Gata4^{fllox/fllox}; Gata6^{fllox/fllox}* 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 *Gata4* and *Gata6* 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 2×10^8 pfu. Based on an estimate of 3×10^6 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 µL) 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 *Gata4^{fllox/fllox}; Gata6^{fllox/fllox}* 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

Figure 2 Morphological changes in the testes of *Gata4*^{flox/flox}, *Gata6*^{flox/flox} mice injected with adenoviral vectors. Mouse testes were injected with Ad-GFP (A, C and E) or Ad-Cre-IRES-GFP (B, D and F). Tissue was harvested at the indicated times, fixed in Bouin's solution and stained with H&E. (A and B) After 3 days, Leydig cells were visible in the testes of mice injected with either Ad-GFP or Ad-Cre-IRES-GFP (arrows). (C and D) By 6 days, however, the Ad-Cre-IRES-GFP-treated testes were devoid of Leydig cells. (E and F) After 12 days, lymphocytes (arrowheads) were evident in the interstitium of both the Ad-GFP- and Ad-Cre-IRES-GFP-injected testes. Scale bars = 50 µm.

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 into the testicular interstitium (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.

Expedient Cre-mediated recombination in Leydig cells

To confirm Cre-mediated recombination, testes sections were immunostained for GATA4 and GATA6. Ordinarily,

Figure 3 Adenoviral injection triggers the delayed appearance of a T-lymphocytic infiltrate in the testicular interstitium and a concomitant loss of reporter gene expression. The testes of 2-month-old mice were injected with Ad-GFP. Tissue was harvested at the indicated times, fixed in PFA (A, C and E) or Bouin's solution (B, D and F) and immunostained for CD3ε (A, C and E) or GFP (B, D and F). Note the loss of reporter gene expression at day 12, coinciding with T-cell infiltration. Scale bars = 50 µm.

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 *Gata4*^{flox/flox}; *Gata6*^{flox/flox} 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).

Figure 4 GATA4 immunostaining in the testes of *Gata4*^{fllox/fllox}; *Gata6*^{fllox/fllox} 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⁺ Leydig cells were evident in Ad-GFP-treated testes (black arrows) but not in Ad-Cre-IRES-GFP-treated testes. GATA4⁺ pericytes were observed in the interstitium of the Ad-Cre-IRES-GFP-treated testes (blue arrows). Scale bars = 50 μm.

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*^{fllox/fllox}; *Gata6*^{fllox/fllox} 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 ($P < 0.01$). By 7 dpi, all Leydig cell markers examined (*Ins13*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*) were markedly attenuated ($P < 0.01$) 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 5 RT-qPCR analysis of testes from *Gata4*^{fllox/fllox}; *Gata6*^{fllox/fllox} 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 $\pm$ S.D. ($n = 4$ per group; $**P < 0.01$).

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,

Figure 6 Immunostaining for steroidogenic enzymes in the testes of *Gata4^{flox/flox}; Gata6^{flox/flox}* mice injected with Ad-GFP or Ad-Cre-IRES-GFP. The testes of 2-month-old mice were injected with Ad-GFP (A, C and E) or Ad-Cre-IRES-GFP (B, D and F). Tissue was harvested 6 days later, fixed in Bouin's solution and subjected to immunostaining for HSD3B1 (A and B), CYP17A1 (C and D) or HSD17B3 (E and F). Bars = 50 μm.

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^{flox/flox}; Gata6^{flox/flox}* 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^{flox/flox}; Gata6^{flox/flox}* 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

Figure 7 Degenerative changes in Leydig cells of *Gata4^{flox/flox}; Gata6^{flox/flox}* mice injected with Ad-Cre-IRES-GFP. The testes of 2-month-old mice were injected with Ad-GFP (A) or Ad-Cre-IRES-GFP (B-D). Tissue was harvested 4 days later and analyzed by transmission electron microscopy. (A) Normal Leydig cell with a thin rim of heterochromatin beneath the nuclear membrane. (B) Leydig cell with patches of condensed chromatin; adjacent is an activated macrophage with filopodia and large vacuoles containing cellular debris. (C) Leydig cell with prominent chromatin condensation. (D) Leydig cell exhibiting nuclear fragmentation. cl, capillary lumen; lc, Leydig cell; mp, macrophage; pt, peritubular cell. Scale bars = 1 μm.

Figure 8 Steroid hormone levels in testis homogenates from *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice injected with Ad-GFP or Ad-Cre-IRES-GFP. Ad-GFP ('GFP') or Ad-Cre-IRES-GFP ('Cre') was injected into the testes of 2-month-old *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice. Tissue was harvested 7 days later. Testis homogenates were extracted and subjected to LC-MS/MS to determine the concentrations of (A) androstenedione and (B) testosterone. Values are expressed as pmol steroid per g of testis (mean $\pm$ s.d.; * P < 0.05; ** P < 0.01; n = 4 per group).

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 $\pm$ 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 *Gata4*^{flox/flox}; *Gata6*^{flox/flox} 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 *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice injected with Ad-GFP-sired pups in the months following injection (P < 0.05).

Figure 9 Late changes in testicular histology and weight following injection of Ad-Cre-IRES-GFP into *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice. (A and B) GATA4 immunostaining of PFA-fixed testis from a mouse injected 1 month earlier with Ad-Cre-IRES-GFP. As shown in panel A, most of the testicular interstitial space lacked Leydig cells. Panel B shows a rare patch of Leydig cells that are GATA4 immunoreactive, suggesting that they or their progenitors escaped Cre inactivation. Some of these Leydig cells exhibited cytomegaly (black arrows). Other GATA4⁺ cell types seen are Sertoli cells (black arrowheads), pericytes (green arrowhead) and peritubular cells (red arrowhead). The asterisk indicates a seminiferous tubule with thin epithelium. (C, D, E and F) H&E staining and HSD3B1 immunostaining of Bouin's-fixed testis from mice injected 3 months earlier with Ad-Cre-IRES-GFP. Arrowheads highlight Leydig cells. Arrows indicate seminiferous tubule degeneration. Asterisks highlight lymphoid aggregates. (G) The testes of 2-month-old mice were injected with Ad-GFP (open circle) or Ad-Cre-IRES-GFP (closed circle). At the indicated times, testes were harvested and weighed. Data points represent the average of two testes per mouse. There was no statistical difference between the slopes of the curves (P = 0.4). Scale bars = 25 μ m.

Tevosian and coworkers have shown that male *Sf1*-cre; *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice survive congenital adrenocortical aplasia due to production of corticoids by adrenal-like cells that accumulate in the testes postnatally (Padua et al. 2015, Tevosian et al. 2015). These heterotopic cells express adrenocortical markers (*Cyp21a1*, *Cyp11b1*, *Cyp11b2* and *Mc2r*). To test whether adrenal-like cells arise in the testes of

Figure 10 Salient features of the adult Leydig cell ablation model. Injection of Ad-Cre-IRES-GFP into the testicular interstitium of adult mice resulted in the selective transduction of Leydig cells. At 3 days post infection, there was decreased transcription of steroidogenic enzyme genes, but expression of *Ins13*, another Leydig cell marker, was unchanged. By 6 days post infection, there was Leydig cell destruction. Prepared using image vectors from Servier Medical Art (<http://smart.servier.com/>), licensed under the Creative Commons Attribution 3.0 Unported License (<http://creativecommons.org/licenses/by/3.0/>).

Gata4^{flox/flox}; *Gata6*^{flox/flox} mice injected with Ad-Cre-IRES-GFP, we performed RT-qPCR analysis for adrenal markers. In contrast to the testes of *Sf1-cre; Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice (which were included in the analysis as positive controls), there was no upregulation of adrenal markers in the testes of *Gata4*^{flox/flox}; *Gata6*^{flox/flox} mice that had been injected 90 days earlier with Ad-Cre-IRES-GFP vs Ad-GFP (data not shown).

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

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 *Sf1-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

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 germline 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 adenoviral vectors to drive the expression of angiogenic peptides in mouse testis; histological changes characteristic of angiogenesis were evident 7 dpi.

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 (*Cyp11a1*, *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, *Ins3*, 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 adenoviral-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-IRES-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 (*Sf1* 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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References

- Almalki SG & Agrawal DK 2016 Key transcription factors in the differentiation of mesenchymal stem cells. *Differentiation* **92** 41–51. (doi:10.1016/j.diff.2016.02.005)
- Anand-Ivell R, Heng K, Hafen B, Setchell B & Ivell R 2009 Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction* **81** 480–487. (doi:10.1095/biolreprod.109.077552)
- Anttonen M, Ketola I, Parviainen H, Pusa AK & Heikinheimo M 2003 FOG-2 and GATA-4 are coexpressed in the mouse ovary and can modulate Müllerian-inhibiting substance expression. *Biology of Reproduction* **68** 1333–1340. (doi:10.1095/biolreprod.102.008599)
- Anttonen M, Pihlajoki M, Andersson N, Georges A, L'Hôte D, Vattulainen S, Färkkilä A, Unkila-Kallio L, Veitia RA & Heikinheimo M 2014 FOXL2,

- GATA4, and SMAD3 co-operatively modulate gene expression, cell viability and apoptosis in ovarian granulosa cell tumor cells. *PLoS ONE* 9 e85545. (doi:10.1371/journal.pone.0085545)
- Bennett J, Baumgarten SC & Stocco C 2013 GATA4 and GATA6 silencing in ovarian granulosa cells affects levels of mRNAs involved in steroidogenesis, extracellular structure organization, IGF-I activity, and apoptosis. *Endocrinology* 154 4845–4858. (doi:10.1210/en.2013-1410)
- Bergeron F, Nadeau G & Viger RS 2015 GATA4 knockdown in MA-10 Leydig cells identifies multiple target genes in the steroidogenic pathway. *Reproduction* 149 245–257. (doi:10.1530/REP-14-0369)
- Bielinska M, Seehra A, Toppari J, Heikinheimo M & Wilson DB 2007 GATA-4 is required for sex steroidogenic cell development in the fetal mouse. *Developmental Dynamics* 236 203–213. (doi:10.1002/dvdy.21004)
- Bingham NC, Verma-Kurvari S, Parada LF & Parker KL 2006 Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44 419–424. (doi:10.1002/dvg.20231)
- Blanchard KT & Boekelheide K 1997 Adenovirus-mediated gene transfer to rat testis in vivo. *Biology of Reproduction* 56 495–500. (doi:10.1095/biolreprod56.2.495)
- Bridges PJ, Koo Y, Kang DW, Hudgins-Spivey S, Lan ZJ, Xu X, DeMayo F, Cooney A & Ko C 2008 Generation of Cyp17iCre transgenic mice and their application to conditionally delete estrogen receptor alpha (Esr1) from the ovary and testis. *Genesis* 46 499–505. (doi:10.1002/dvg.20428)
- Chen H, Wang Y, Ge R & Zirkin BR 2017 Leydig cell stem cells: identification, proliferation and differentiation. *Molecular and Cellular Endocrinology* 445 65–73. (doi:10.1016/j.mce.2016.10.010)
- Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, Kenny CD, Christiansen LM, White RD, Edelstein EA *et al.* 2006 Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* 49 191–203. (doi:10.1016/j.neuron.2005.12.021)
- Dörner J, Martinez Rodriguez V, Ziegler R, Rohrig T, Cochran RS, Götz RM, Levin MD, Pihlajoki M, Heikinheimo M & Wilson DB 2017 GLI1⁺ progenitor cells in the adrenal capsule of the adult mouse give rise to heterotopic gonadal-like tissue. *Molecular and Cellular Endocrinology* 441 164–175. (doi:10.1016/j.mce.2016.08.043)
- Gao HB, Tong MH, Hu YQ, Guo QS, Ge R & Hardy MP 2002 Glucocorticoid induces apoptosis in rat leydig cells. *Endocrinology* 143 130–138. (doi:10.1210/endo.143.1.8604)
- Hall SJ, Bar-Chama N, Ta S & Gordon JW 2000 Direct exposure of mouse spermatogenic cells to high doses of adenovirus gene therapy vector does not result in germ cell transduction. *Human Gene Therapy* 11 1705–1712. (doi:10.1089/10430340050111359)
- Hendrickx R, Stichling N, Koelen J, Kuryk L, Lipiec A & Greber UF 2014 Innate immunity to adenovirus. *Human Gene Therapy* 25 265–284. (doi:10.1089/hum.2014.001)
- Ho NR, Usmani AR, Yin Y, Ma L & Conrad DF 2017 Multiplex shRNA screening of germ cell development by in vivo transfection of mouse testis. *G3* 7 247–255. (doi:10.1534/g3.116.036087)
- Hooley RP, Paterson M, Brown P, Kerr K & Saunders PT 2009 Intra-testicular injection of adenoviral constructs results in Sertoli cell-specific gene expression and disruption of the seminiferous epithelium. *Reproduction* 137 361–370. (doi:10.1530/REP-08-0247)
- Hu GX, Lin H, Chen GR, Chen BB, Lian QQ, Hardy DO, Zirkin BR & Ge RS 2010 Deletion of the Igf1 gene: suppressive effects on adult Leydig cell development. *Journal of Andrology* 31 379–387. (doi:10.2164/jandrol.109.008680)
- Ikawa M, Tergaonkar V, Ogura A, Ogonuki N, Inoue K & Verma IM 2002 Restoration of spermatogenesis by lentiviral gene transfer: offspring from infertile mice. *PNAS* 99 7524–7529. (doi:10.1073/pnas.072207299)
- Jackson NC, Jackson H, Shanks JH, Dixon JS & Lendon RG 1986 Study using in-vivo binding of 125I-labelled hCG, light and electron microscopy of the repopulation of rat Leydig cells after destruction due to administration of ethylene-1,2-dimethanesulphonate. *Journal of Reproduction and Fertility* 76 1–10. (doi:10.1530/jrf.0.0760001)
- Jimenez P, Saner K, Mayhew B & Rainey WE 2003 GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. *Endocrinology* 144 4285–4288. (doi:10.1210/en.2003-0472)
- Kanatsu-Shinohara M, Ogura A, Ikegawa M, Inoue K, Ogonuki N, Tashiro K, Toyokuni S, Honjo T & Shinohara T 2002 Adenovirus-mediated gene delivery and in vitro microinsemination produce offspring from infertile male mice. *PNAS* 99 1383–1388. (doi:10.1073/pnas.022646399)
- Kanatsu-Shinohara M, Toyokuni S & Shinohara T 2004 Transgenic mice produced by retroviral transduction of male germ line stem cells in vivo. *Biology of Reproduction* 71 1202–1207. (doi:10.1095/biolreprod.104.031294)
- Kerr JB, Donachie K & Rommerts FF 1985 Selective destruction and regeneration of rat Leydig cells in vivo. A new method for the study of seminiferous tubular-interstitial tissue interaction. *Cell and Tissue Research* 242 145–156. (doi:10.1007/BF00225571)
- Ketola I, Rahman N, Toppari J, Bielinska M, Porter-Tinge SB, Tapanainen JS, Huhtaniemi IT, Wilson DB & Heikinheimo M 1999 Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. *Endocrinology* 140 1470–1480. (doi:10.1210/endo.140.3.6587)
- Ketola I, Anttonen M, Vaskivuo T, Tapanainen JS, Toppari J & Heikinheimo M 2002 Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. *European Journal of Endocrinology* 147 397–406. (doi:10.1530/eje.0.1470397)
- Kilcoyne KR, Smith LB, Atanassova N, Macpherson S, McKinnell C, van den Driesche S, Jobling MS, Chambers TJ, De Gendt K, Verhoeven G *et al.* 2014 Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. *PNAS* 111 E1924–E1932. (doi:10.1073/pnas.1320735111)
- Kim JM, Luo L & Zirkin BR 2000 Caspase-3 activation is required for Leydig cell apoptosis induced by ethane dimethanesulfonate. *Endocrinology* 141 1846–1853. (doi:10.1210/endo.141.5.7444)
- Kim TS, Choi HS, Ryu BY, Gang GT, Kim SU, Koo DB, Kim JM, Han JH, Park CK, Her S & Lee DS 2010 Real-time in vivo bioluminescence imaging of lentiviral vector-mediated gene transfer in mouse testis. *Theriogenology* 73 129–138. (doi:10.1016/j.theriogenology.2009.07.028)
- King SR, Rommerts FF, Ford SL, Hutson JC, Orly J & Stocco DM 1998 Ethane dimethane sulfonate and NNN'-N'-tetrakis-(2-pyridylmethyl) ethylenediamine inhibit steroidogenic acute regulatory (StAR) protein expression in MA-10 Leydig cells and rat Sertoli cells. *Endocrine Research* 24 469–478. (doi:10.3109/07435809809032635)
- Kojima Y, Sasaki S, Umemoto Y, Hashimoto Y, Hayashi Y & Kohri K 2003 Effects of adenovirus mediated gene transfer to mouse testis in vivo on spermatogenesis and next generation. *Journal of Urology* 170 2109–2114. (doi:10.1097/01.ju.0000092898.91658.08)
- Krachulec J, Vetter M, Schrade A, Löbs AK, Bielinska M, Cochran R, Kyrölähti A, Pihlajoki M, Parviainen H, Jay PY *et al.* 2012 GATA4 is a critical regulator of gonadectomy-induced adrenocortical tumorigenesis in mice. *Endocrinology* 153 2599–2611. (doi:10.1210/en.2011-2135)
- Kyrölähti A, Euler R, Bielinska M, Schoeller EL, Moley KH, Toppari J, Heikinheimo M & Wilson DB 2011 GATA4 regulates Sertoli cell function and fertility in adult male mice. *Molecular and Cellular Endocrinology* 333 85–95. (doi:10.1016/j.mce.2010.12.019)
- LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ & Ferrara N 2003 The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: localization of Bv8 receptors to endothelial cells. *PNAS* 100 2685–2690. (doi:10.1073/pnas.0337667100)
- Li T, Hu J, He GH, Li Y, Zhu CC, Hou WG, Zhang S, Li W, Zhang JS, Wang Z *et al.* 2012 Up-regulation of NDRG2 through nuclear factor-kappa B is required for Leydig cell apoptosis in both human and murine infertile testes. *Biochimica et Biophysica Acta* 1822 301–313. (doi:10.1016/j.bbdis.2011.11.013)
- Lobo MV, Arenas MI, Huerta L, Sacristan S, Perez-Crespo M, Gutierrez-Adan A, Diaz-Gil JJ, Lasuncion MA & Martin-Hidalgo A 2015 Liver growth factor induces testicular regeneration in EDS-treated rats and increases protein levels of class B scavenger receptors. *American Journal of Physiology – Endocrinology and Metabolism* 308 E111–E121. (doi:10.1152/ajpendo.00329.2014)
- Machitani M, Sakurai F, Katayama K, Tachibana M, Suzuki T, Matsui H, Yamaguchi T & Mizuguchi H 2013 Improving adenovirus vector-mediated RNAi efficiency by lacking the expression of virus-associated RNAs. *Virus Research* 178 357–363. (doi:10.1016/j.virusres.2013.09.021)
- Manuylov NL, Zhou B, Ma Q, Fox SC, Pu WT & Tevosian SG 2011 Conditional ablation of Gata4 and Fog2 genes in mice reveals their

- distinct roles in mammalian sexual differentiation. *Developmental Biology* **353** 229–241. (doi:10.1016/j.ydbio.2011.02.032)
- Mazaud-Guittot S, Prud'homme B, Bouchard MF, Bergeron F, Daems C, Tevosian SG & Viger RS 2014 GATA4 autoregulates its own expression in mouse gonadal cells via its distal 1b promoter. *Biology of Reproduction* **90** 25.
- Mitani K & Kubo S 2002 Adenovirus as an integrating vector. *Current Gene Therapy* **2** 135–144. (doi:10.2174/1566523024605591)
- Molenaar R, de Rooij DG, Rommerts FF & van der Molen HJ 1986 Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with ethylene dimethane sulfonate is dependent on luteinizing hormone and not follicle-stimulating hormone. *Endocrinology* **118** 2546–2554. (doi:10.1210/endo-118-6-2546)
- O'Shaughnessy PJ & Fowler PA 2011 Endocrinology of the mammalian fetal testis. *Reproduction* **141** 37–46.
- O'Shaughnessy PJ, Baker PJ, Heikkilä M, Vainio S & McMahon AP 2000 Localization of 17 β -hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis—arostenedione is the major androgen secreted by fetal/neonatal leydig cells. *Endocrinology* **141** 2631–2637. (doi:10.1210/en.141.7.2631)
- O'Shaughnessy PJ, Willerton L & Baker PJ 2002 Changes in Leydig cell gene expression during development in the mouse. *Biology of Reproduction* **66** 966–975. (doi:10.1095/biolreprod66.4.966)
- O'Shaughnessy PJ, Morris ID & Baker PJ 2008 Leydig cell re-generation and expression of cell signaling molecules in the germ cell-free testis. *Reproduction* **135** 851–858. (doi:10.1530/rep-07-0529)
- O'Shaughnessy PJ, Monteiro A, Fowler PA & Morris ID 2014 Identification of Leydig cell-specific mRNA transcripts in the adult rat testis. *Reproduction* **147** 671–682. (doi:10.1530/rep-13-0603)
- Oka T, Maillet M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA & Molkentin JD 2006 Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. *Circulation Research* **98** 837–845. (doi:10.1161/01.RES.0000215985.18538.c4)
- Padua MB, Jiang T, Morse DA, Fox SC, Hatch HM & Tevosian SG 2015 Combined loss of the GATA4 and GATA6 transcription factors in male mice disrupts testicular development and confers adrenal-like function in the testes. *Endocrinology* **156** 1873–1886. (doi:10.1210/en.2014-1907)
- Pihlajoki M, Gretzinger E, Cochran R, Kyrönlähti A, Schrade A, Hiller T, Sullivan L, Shoykhet M, Schoeller EL, Brooks MD *et al.* 2013 Conditional mutagenesis of Gata6 in SF1-positive cells causes gonadal-like differentiation in the adrenal cortex of mice. *Endocrinology* **154** 1754–1767. (doi:10.1210/en.2012-1892)
- Pihlajoki M, Färkkilä A, Soini T, Heikinheimo M & Wilson DB 2016 GATA factors in endocrine neoplasia. *Molecular and Cellular Endocrinology* **421** 2–17. (doi:10.1016/j.mce.2015.05.027)
- Qamar I, Park E, Gong EY, Lee HJ & Lee K 2009 ARR19 (androgen receptor corepressor of 19 kDa), an antisteroidogenic factor, is regulated by GATA-1 in testicular Leydig cells. *Journal of Biological Chemistry* **284** 18021–18032. (doi:10.1074/jbc.M900896200)
- Qamar I, Ahmad MF & Narayanasamy A 2015 A time-course study of long term over-expression of ARR19 in mice. *Science Reports* **5** 13014. (doi:10.1038/srep13014)
- Rahman NA, Kiiveri S, Rivero-Muller A, Levallet J, Vierre S, Kero J, Wilson DB, Heikinheimo M & Huhtaniemi I 2004 Adrenocortical tumorigenesis in transgenic mice expressing the inhibin α -subunit promoter/SV40 virus T-antigen transgene: relationship between ectopic expression of luteinizing hormone receptor and transcription factor GATA-4. *Molecular Endocrinology* **18** 2553–2569. (doi:10.1210/me.2002-0282)
- Röhrig T, Pihlajoki M, Ziegler R, Cochran RS, Schrade A, Schillebeekx M, Mitra RD, Heikinheimo M & Wilson DB 2015 Tying with fate: redirecting the differentiation of adrenocortical progenitor cells into gonadal-like tissue. *Molecular and Cellular Endocrinology* **408** 165–177. (doi:10.1016/j.mce.2014.12.003)
- Rudiger LJ, Dame C, Scholz H & Kirschner KM 2017 Ex vivo cultures combined with vivo-morpholino induced gene knockdown provide a system to assess the role of WT1 and GATA4 during gonad differentiation. *PLoS ONE* **12** e0176296. (doi:10.1371/journal.pone.0176296)
- Salva A, Hardy MP, Wu XF, Sottas CM, MacLaughlin DT, Donahoe PK & Lee MM 2004 Müllerian-inhibiting substance inhibits rat Leydig cell regeneration after ethylene dimethanesulphonate ablation. *Biology of Reproduction* **70** 600–607. (doi:10.1095/biolreprod.103.021550)
- Schrade A, Kyrönlähti A, Akinrinade O, Pihlajoki M, Fischer S, Rodriguez VM, Otte K, Velagapudi V, Toppari J, Wilson DB & Heikinheimo M 2016 GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells. *Endocrinology* **157** 2416–2431. (doi:10.1210/en.2015-1927)
- Schrade A, Kyrönlähti A, Akinrinade O, Pihlajoki M, Häkkinen M, Fischer S, Alastalo TP, Velagapudi V, Toppari J, Wilson DB & Heikinheimo M 2015 GATA4 is a key regulator of steroidogenesis and glycolysis in mouse Leydig cells. *Endocrinology* **156** 1860–1872. (doi:10.1210/en.2014-1931)
- Sher N, Yivgi-Ohana N & Orly J 2007 Transcriptional regulation of the cholesterol side chain cleavage cytochrome P450 gene (CYP11A1) revisited: binding of GATA, cyclic adenosine 3',5'-monophosphate response element-binding protein and activating protein (AP)-1 proteins to a distal novel cluster of cis-regulatory elements potentiates AP-2 and steroidogenic factor-1-dependent gene expression in the rodent placenta and ovary. *Molecular Endocrinology* **21** 948–962. (doi:10.1210/me.2006-0226)
- Shima Y & Morohashi KI 2017 Leydig progenitor cells in fetal testis. *Molecular and Cellular Endocrinology* **445** 55–64. (doi:10.1016/j.mce.2016.12.006)
- Shima Y, Miyabayashi K, Haraguchi S, Arakawa T, Otake H, Baba T, Matsuzaki S, Shishido Y, Akiyama H, Tachibana T *et al.* 2013 Contribution of Leydig and Sertoli cells to testosterone production in mouse fetal testes. *Molecular Endocrinology* **27** 63–73. (doi:10.1210/me.2012-1256)
- Shiraishi K & Ascoli M 2007 Lutropin/choriogonadotropin stimulate the proliferation of primary cultures of rat Leydig cells through a pathway that involves activation of the extracellularly regulated kinase 1/2 cascade. *Endocrinology* **148** 3214–3225. (doi:10.1210/en.2007-0160)
- Smith L 2011 Good planning and serendipity: exploiting the Cre/Lox system in the testis. *Reproduction* **141** 151–161. (doi:10.1530/REP-10-0404)
- Smith LB, O'Shaughnessy PJ & Rebourcet D 2015 Cell-specific ablation in the testis: what have we learned? *Andrology* **3** 1035–1049. (doi:10.1111/andr.12107)
- Sodhi CP, Li J & Duncan SA 2006 Generation of mice harbouring a conditional loss-of-function allele of Gata6. *BMC Developmental Biology* **6** 19. (doi:10.1186/1471-213X-6-19)
- Sriraman V, Sairam MR & Rao AJ 2003 Evaluation of relative roles of LH and FSH in regulation of differentiation of Leydig cells using an ethane 1,2-dimethylsulfonate-treated adult rat model. *Journal of Endocrinology* **176** 151–161. (doi:10.1677/joe.0.1760151)
- Taylor MF, Woolveridge I, Metcalfe AD, Streuli CH, Hickman JA & Morris ID 1998 Leydig cell apoptosis in the rat testes after administration of the cytotoxin ethane dimethanesulphonate: role of the Bcl-2 family members. *Journal of Endocrinology* **157** 317–326. (doi:10.1677/joe.0.1570317)
- Taylor MF, de Boer-Brouwer M, Woolveridge I, Teerds KJ & Morris ID 1999 Leydig cell apoptosis after the administration of ethane dimethanesulphonate to the adult male rat is a Fas-mediated process. *Endocrinology* **140** 3797–3804. (doi:10.1210/endo.140.8.6919)
- Teerds KJ & Huhtaniemi IT 2015 Morphological and functional maturation of Leydig cells: from rodent models to primates. *Human Reproduction Update* **21** 310–328. (doi:10.1093/humupd/dmv008)
- Teerds KJ, de Rooij DG, Rommerts FF, van den Hurk R & Wensing CJ 1989 Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethyl sulphonate: the role of LH/human chorionic gonadotrophin. *Journal of Endocrinology* **122** 689–696. (doi:10.1677/joe.0.1220689)
- Tevosian S 2014 Transgenic mouse models in the study of reproduction: insight into GATA protein function. *Reproduction* **148** R1–R14. (doi:10.1530/REP-14-0086)
- Tevosian SG, Jimenez E, Hatch HM, Jiang T, Morse DA, Fox SC & Padua MB 2015 Adrenal development in mice requires GATA4 and GATA6 transcription factors. *Endocrinology* **156** 2503–2517. (doi:10.1210/en.2014-1815)
- Tremblay JJ 2015 Molecular regulation of steroidogenesis in endocrine Leydig cells. *Steroids* **103** 3–10. (doi:10.1016/j.steroids.2015.08.001)
- Tremblay JJ & Viger RS 2001 GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. *Endocrinology* **142** 977–986. (doi:10.1210/endo.142.3.7995)

- Tsuzuki S, Tachibana M, Hemmi M, Yamaguchi T, Shoji M, Sakurai F, Kobiyama K, Kawabata K, Ishii KJ, Akira S et al.** 2016 TANK-binding kinase 1-dependent or -independent signaling elicits the cell-type-specific innate immune responses induced by the adenovirus vector. *International Immunology* **28** 105–115. (doi:10.1093/intimm/dxv058)
- Viger RS, Mertineit C, Trasler JM & Nemer M** 1998 Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter. *Development* **125** 2665–2755.
- Virtanen HE & Toppari J** 2014 Embryology and physiology of testicular development and descent. *Pediatric Endocrinology Reviews* **11** (Supplement 2) 206–213.
- Watt AJ, Battle MA, Li J & Duncan SA** 2004 GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *PNAS* **101** 12573–12578. (doi:10.1073/pnas.0400752101)
- Wen Q, Cheng CY & Liu YX** 2016 Development, function and fate of fetal Leydig cells. *Seminars in Cell and Developmental Biology* **59** 89–98. (doi:10.1016/j.semcdb.2016.03.003)
- Wu HS, Lin HT, Wang CK, Chiang YF, Chu HP & Hu MC** 2007 Human CYP11A1 promoter drives Cre recombinase expression in the brain in addition to adrenals and gonads. *Genesis* **45** 59–65. (doi:10.1002/dvg.20266)
- Yan W, Kero J, Huhtaniemi I & Toppari J** 2000 Stem cell factor functions as a survival factor for mature Leydig cells and a growth factor for precursor Leydig cells after ethylene dimethane sulfonate treatment: implication of a role of the stem cell factor/c-kit system in Leydig cell development. *Developmental Biology* **227** 169–182. (doi:10.1006/dbio.2000.9885)
- Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E & Wilson JM** 1994 Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *PNAS* **91** 4407–4411. (doi:10.1073/pnas.91.10.4407)
- Yang Y, Li Z, Wu X, Chen H, Xu W, Xiang Q, Zhang Q, Chen J, Ge RS, Su Z et al.** 2017 Direct reprogramming of mouse fibroblasts toward Leydig-like cells by defined factors. *Stem Cell Reports* **8** 39–53. (doi:10.1016/j.stemcr.2016.11.010)
- Zhang L, Wang H, Yang Y, Liu H, Zhang Q, Xiang Q, Ge R, Su Z & Huang Y** 2013 NGF induces adult stem Leydig cells to proliferate and differentiate during Leydig cell regeneration. *Biochemical and Biophysical Research Communications* **436** 300–305. (doi:10.1016/j.bbrc.2013.05.098)
- Zhang YF, Yuan KM, Liang Y, Chu YH, Lian QQ, Ge YF, Zhen W, Sottas CM, Su ZJ & Ge RS** 2015 Alterations of gene profiles in Leydig-cell-regenerating adult rat testis after ethane dimethane sulfonate-treatment. *Asian Journal of Andrology* **17** 253–260. (doi:10.4103/1008-682X.136447)

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