THE UNIVERSITY
of EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Targeting and Repair of Adult Testicular Somatic Cells through Viral Gene Therapy

Annalucia Leigh Darbey

MSc by Research in Reproductive Science, University of Edinburgh
BSc (Hons) Biology, Liverpool John Moores University

Thesis submitted to the University of Edinburgh for the Degree of Doctor of Philosophy
July 2018

Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The literature review presented in Chapter 1 (Introduction) was previously published in Cellular and Molecular Endocrinology as “Deliverable transgenics & gene therapy possibilities for the testes” by Annalucia Darbey (student and author of declaration) and Lee B. Smith (supervisor). This review was conceived by both authors. I carried out literature searches, writing and generation of figures.

Annalucia Leigh Darbey

July 2018

Acknowledgements

Firstly, I would like to thank my supervisor; Professor Lee Smith, for your guidance and support through a project I have thoroughly enjoyed working on and for the opportunity to study within a successful and enthusiastic research group over the past four years. Your motivation and passion for science as well as your ability to put a 'Smith spin' on results that may not initially have gone the way that was expected has helped me progress as a scientist and has taught me some valuable lessons that will stay with me for the rest of my career.

I would also like to thank my second supervisor, Professor Philippa Saunders for her input and advice throughout my PhD and to the Tissue Repair programme for providing me with this excellent opportunity.

To the Smith Group members, past and present; thank you for putting up with me for all these years and for providing a solid support system through my whole PhD and during my mini projects for my Master's degree. I was very lucky to have so much technical help and advice from many people over the years including Dr Laura Milne, Nathan Jeffery, Lyndsey Wilson, Sarah Smith, Hazel Stewart, Mike Dodds, Will Mungall and Forbes Howie particularly with genotyping, hormone assays and animal work and to Dr Pamela Brown and Linda Ferguson for passing on their lentiviral and cloning expertise – Thank you!

I would like to express my sincere gratitude to Dr Laura O'Hara, Dr Karen Kilcoyne, Dr Michael Curley and Dr Anne Gannon, my office pal; Melissa Tharmalingam and to everyone else at the CRH and beyond (particularly Jil, Claire, Maria Elena, my Liverpool lot, Faye, Cat and my flatmates Sara and Matina) for all of their motivation and for all the fun times outside of work (from what I can remember of them anyway). In particular, would like to wholeheartedly thank Dr Diane Rebourcet for helping me take my first steps into research, for always answering my blonde questions and for providing me with all your support both professionally and personally over the past five years – not a bad assignment after all!

Finally, I would like to thank my family; Mum, Dad, Annastacia, Elizabeth, Erin, Harrison, John and Lisa for all of their love and support not only for the past 4 years but throughout all of my time at university, I promise this will be my last degree! Here's to all the adventures to come - Diolch yn fawr pawb!

Abstract

Androgens are essential for the maintenance of male health and wellbeing. A disturbance in androgen signalling has been associated with a number of clinically relevant disorders such as cardiovascular disease, diabetes and metabolic disorders as well as infertility. Primarily produced in the testis in males, the actions of androgens are mediated through binding to androgen receptor (AR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. The somatic cells of the testis are known to have a number of key roles in both testis function and development and the Sertoli, Leydig and Peritubular Myoid cells are known to express AR in adulthood. It is through AR that some testicular functions are mediated; for example, the Sertoli cells support of complete spermatogenesis with Sertoli cell androgen receptor knockout (SCARKO) testis demonstrating a halt of spermatogenesis before meiosis. However, how androgen signalling is impacting testicular function through each of the somatic cell types is not yet fully understood.

Currently, treatments for male reproductive disorders such as hypogonadism (low androgens) and infertility are limited to treatment of the symptoms; using androgen replacement therapy and in vitro fertilisation techniques. This has been, up until recently, a result of a lack of understanding of the causes of these conditions and a lack of resources able to treat them, with research suggesting that a genetic component may be responsible in a number of cases. However, due to the limited genetic investigation diagnosis of men with male reproductive disorders, the wider understanding of the genetics underpinning male hypogonadism and infertility is incomplete.

Developments in technology for the investigation and editing of the genetic code are triggering a surge in the exploration of genetic disorders and, in parallel, into the fields of gene delivery vectors and editing technologies. These technologies will allow an expansion into the knowledge and understanding of genetic disorders whilst simultaneously affording the opportunity to exploit this understanding for the development of therapeutics.

There have been a small handful of previous studies using technologies such as viral vectors to target the testicular somatic cells and deliver exogenous transgenes with the purpose of both gene editing and repair, all with varying degrees of success.

Here, techniques to introduce and target the Leydig and Sertoli cells were investigated to determine the most appropriate methodology for gene delivery to and manipulation of the

testis. Refinement of injections into the interstitial compartment were carried out before introducing lentiviral vectors and targeting of Leydig cells was validated and optimised. Lentiviral vectors are able to permanently integrate into the host cell. Surprisingly, analysis of testis post lentiviral injection determined that the lentiviral targeted Leydig cells began to undergo apoptosis one week post injection and were subsequently cleared from the testis after ten days. Contrastingly, this was not the case when adenoviral vectors were introduced into the interstitial compartment, with Leydig cells continuing to express the delivered reporter transgene and, importantly, not expressing markers of apoptosis, ten days post injection. This would suggest that using adenoviral vectors to target the Leydig cell population in the adult testis would be more appropriate than using lentiviral vectors.

Previous studies have successfully used lentiviral vectors to target the Sertoli cells in the adult testis via the introduction of the particles through the efferent duct. However, this can result in damage to efferent duct, resulting in blockages and subsequently the seminiferous tubules. To circumvent this, introduction of the lentiviral particles through the rete compartment of the testis at a range of lower injection pressures was examined and injecting at a lower pressure through the rete testis was found to reduce the likelihood of introducing negative impacts on testicular histology when targeting the seminiferous tubules. Using these refined methods of introducing lentiviral vectors, targeted Sertoli cells stably expressed the delivered transgene for up to one year post injection.

Using viral vector delivered transgenes for both the investigation of testicular genetic disorders and for the development of therapeutics has great potential. To explore this potential, we first generated a mouse model in which AR was ablated from both the Leydig and Sertoli cells using Cre/LoxP technology, termed the SC-LC-ARKO. Alongside providing a potential model to 'repair' with viral vectors, the SC-LC-ARKO model also provided an additional model for comparison with other models exhibiting ablation of AR from both single somatic cell types and double somatic cell types. This further enabled a characterisation of the roles of AR in adult testicular function, with results suggesting that loss of AR from more than one cell type results in an additive phenotype when compared to single cell knock outs. Despite providing further insight into the roles of AR in the testis, further analysis of the Cre line used to generate the SC-LC-ARKO model indicated that a small number of Leydig cells were expressing the Cre recombinase, resulting in only a small population of Leydig cells with ablated AR.

Considering this, to explore the potential of rescuing Sertoli cell AR using lentiviral vectors, we then utilised an already well characterised Sertoli Cell AR knockout (SCARKO) model. Lentiviral vectors expressing mouse AR and monomeric GFP (moeGFP) downstream of a CMV promoter were generated and injected into the rete testis of WT and SCARKO adult (day 100) males at low pressure. The contralateral testis was injected with a lentiviral vector expressing moeGFP alone (also downstream of a CMV promoter) using the same technique. Analysis of testis sections revealed a reintroduction of AR to Sertoli cells in 100% of SCARKO testis injected with lentivirus expressing mouse AR. As a result of this re-expression of AR in Sertoli cells, 66% of the testis injected with lentivirus expressing mouse AR had evidence of morphologically mature elongated spermatids, indicative of ongoing spermatogenesis. These results suggest that a rescue of the infertility phenotype reported in previous studies of SCARKO testis. Also demonstrated is the reversal of the SCARKO testicular phenotype in tubules targeted by the mAR expressing lentiviral vector. This suggests that absence Sertoli cell AR throughout development does not have a permanent impact on the Sertoli cells capacity to support spermatogenesis in adulthood following rescue of SC AR expression in adulthood.

In summary, the results of these studies have provided a refinement in the methodologies for targeting the Sertoli and Leydig cells of the adult testis with viral vectors as well as demonstrating successful rescue of a previously reported mouse model exhibiting infertility through reintroduction of a functional gene. Alongside this, comparisons of AR knockout models have afforded insight into maintenance of testis function through AR.

Lay Summary

Currently, cutting edge techniques are being developed to investigate and edit the genome; which is ultimately responsible for the formation and function of the body and its processes. These techniques have the potential to correct genetic disorders in a number of organ systems; known as gene therapy. To access the affected organ systems, researchers have also developed systems to deliver the gene therapy to the specific cells in the affected organ using viruses. These systems exploit the virus's ability to enter the body, infect a cell and deliver viral DNA. By incorporating the gene therapy into the viral DNA, the virus is, therefore, able to deliver gene therapy to a number of different cell types in the body; these viruses are known as viral vectors.

A number of studies have demonstrated the use of viral vectors to target cells in the testicles. These studies have demonstrated targeting of the Sertoli cells (the cells responsible for supporting sperm production; found in tube structures in the testicle) and the Leydig cells (the cells responsible for making the hormone Testosterone, found in the compartment outside the tubes known as the interstitial compartment) with the purpose of both gene editing and repair, with varying degrees of success.

Once the best route of injection into the interstitial compartment was determined, we then investigated targeting Leydig cells in the testicle using a lentiviral vector; a viral vector from the same family as (but not causing) HIV as it is able to permanently infect a cell. It was found that after 10 days, the cells infected by the lentiviral vector had undergone cell death and were subsequently cleared from the testis, indicating that it may not be a suitable viral vector for targeting Sertoli cells. A similar experiment was then conducted using an adenoviral vector, a viral vector from the same family as (but not causing) common colds, which only temporarily infects cells. Results suggested that using an adenoviral vector to target Leydig cells was more appropriate than using lentiviral vectors as cells infected with the adenoviral vector did not undergo cell death 10 days post injection.

To target the Sertoli cells, injections were first optimised to reduce the risks of damage to the tube structures in the testicle and found that using injections at a low pressure were the most effective. Following injection of lentiviral vectors using the optimised method we were able to demonstrate long lasting infection of the Sertoli cells for up to one year with no major

negative side effects. This suggested that lentiviral vectors are an ideal delivery system to target gene therapy in the Sertoli cells.

To examine if this was the case, we first generated lentiviral vectors carrying a molecule known as Androgen Receptor (AR). AR is a structure located on a cell that responds to hormones known as androgens, such as testosterone, acting as a messenger for processes such as sperm production and development of the male sexual characteristics. When the body is lacking AR it is then unable to respond to testosterone, resulting in infertility, among other characteristics. By injecting the lentiviral vector carrying AR into the tube structures of a testicle lacking AR, we were able to reintroduce AR to the Sertoli cells and restore the production of sperm in an adult infertile mouse.

In summary, the results of these experiments demonstrate the most appropriate viral vectors to target different cell types in the testicles and have used these techniques to introduce gene therapy into the testicle of an infertile mouse. In doing so, we were able to restore sperm production, demonstrating the potential of the viral vectors for the use of gene therapy in the testicles. In doing so, this demonstrates a technique which could be used in men suffering from male reproductive disorders resulting in dysfunction testicular cell types.

Presentations Relating to this Thesis

Tissue Repair in the Testis through Viral Gene Therapy

European Testis Workshop (St. Malo, France) – Poster/Oral Presentation, June 2016

Developing Techniques to Target the Adult Testicular Somatic Cells with Deliverable Transgenics.

European Testis Workshop (Óbidos, Portugal) – Poster/Oral Presentation, May 2018

Publications Relating to this Thesis

Darbey A. and Smith L. B.

Deliverable transgenics & gene therapy possibilities for the testes.

Molecular and Cellular Endocrinology 2017, 468; 81-94

Bound in this thesis with permission of co-authors.

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

1.1 Testis, Testicular Function & Impacts on Male Health

1.1.1 Structure & Function of the Adult Testis

The testis in adulthood has two key functions; production of sperm and production of steroid hormones, primarily androgens. The cell types responsible for these functions are the Leydig cells (LC), for steroidogenesis and the Sertoli cells (SC), for support of spermatogenesis. The Leydig and Sertoli cells can be found in two separate compartments of the testis, the former being located in the interstitial compartment, and the latter in the tubular compartment of the testis (Figure 1.1). Despite their spatial separation within the testis, these cell types, as well as the other cell types found in the testis (namely the Peritubular Myoid (PTM) cells and the Germ cells), conserve closely regulated relationships in order to maintain both steroidogenic and spermatogenic functionality of the testis. Disruption to testicular functions can result in male infertility and/or hypogonadism, both of which are clinically prevalent conditions with a high socioeconomic burden. Infertility affects about 15-20% of couples [1] and thus is as prevalent as diabetes, and yet for male factor infertility, 60% of all cases (and even 75% in the case of oligozoospermia) remain idiopathic [1]. In addition, up to 25% of middle-aged and elderly men have subnormal levels of testosterone [2, 3]. As such there is an urgent need to better understand the causes and develop new treatments for these conditions that affect millions of men [4].

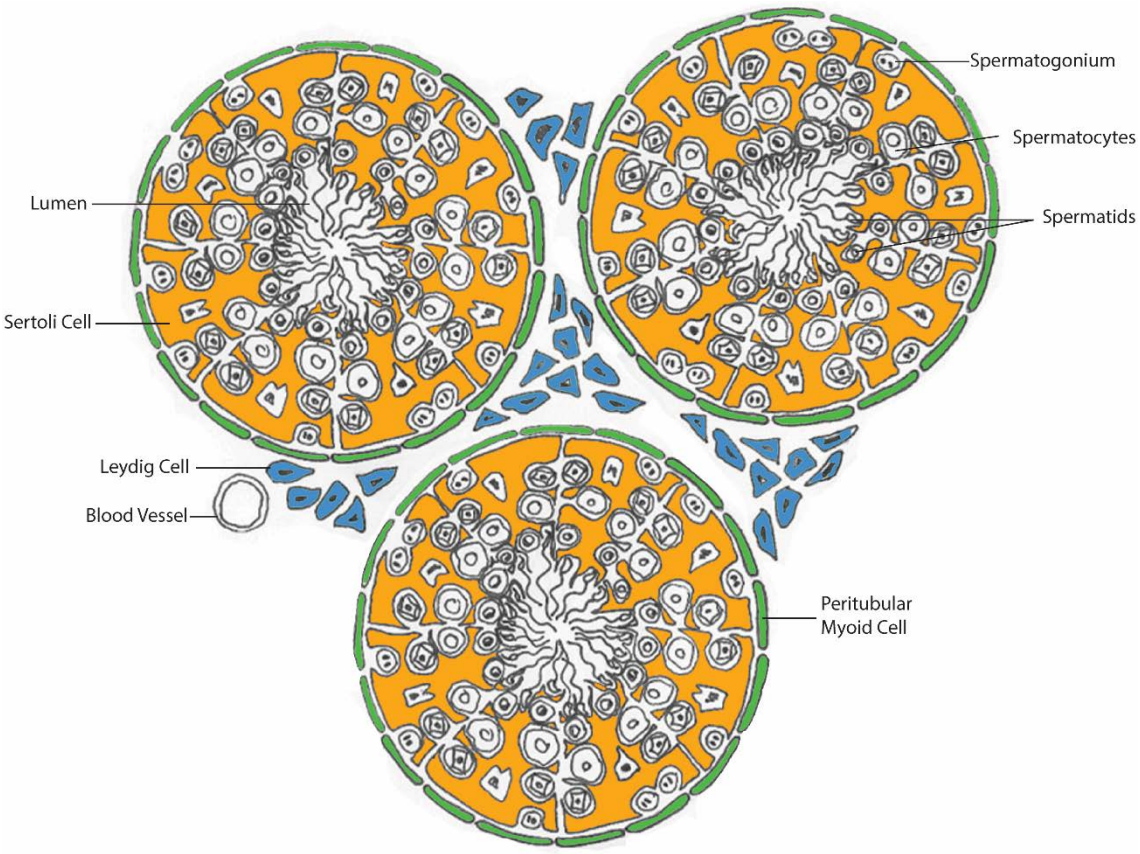


Figure 1.1 Schematic of a cross section of an Adult Testis. Testicular components are depicted in a schematic diagram of the testis. Within the tubular compartment, delimitated by the peritubular myoid cells (Green), is the differentiating germ cell populations (white) and the Sertoli cells (orange). Within the interstitial compartment, the steroidogenic Adult Leydig cells can be found alongside blood vessels.

1.1.1.1 Sertoli cells

Sertoli cells are somatic cells of the testis that are located within the tubular compartment of the testis, specifically at the basement membrane of the seminiferous tubules (Figure 1.1). They are known to be key regulators of testis formation and to be essential for the support of germ cell progression through spermatogenesis [5, 6]. Alongside this, Sertoli cells have also recently been recognised to have a number of additional roles in testis function, including the maintenance of the adult Leydig cell population, retention of peritubular myoid cell differentiation and regulation of the testicular microvasculature, confirming the importance of this cell type not only for formation of the testis throughout development, but also for retaining testicular functions in adulthood [5, 7-9].

1.1.1.1.1 Sertoli cell Development

The presence of Sertoli cells in the testis is crucial not only for their ability to support spermatogenesis, but also for the proper formation of the testis during development [5]. At 10.5 days post coitum (dpc) (in the mouse), activation of the Y chromosome linked testis determining gene; *Sry* in the bipotential gonad initiates the differentiation of Sertoli cells from multipotent precursor cells derived from ingressing coelomic epithelium cells [10]. Following this, expression of *Sox9* by these immature Sertoli cells initiates a cascade of events ultimately leading to testis cord formation, differentiation of other testicular cell types including the androgen producing foetal Leydig cells and regression of the Mullerian ducts through the secretion of anti-Mullerian hormone (AMH) [11].

In the adult testis, the number of Sertoli cells dictates the finite number of Germ cells that the testis can produce. This is because each Sertoli cell has a fixed capacity for the numbers of germ cells it can support in adulthood as evident from both a Sertoli cell depleted rat model [12] and more recently in a model of partial Sertoli cell ablation [9]. Due to the lack of proliferation seen in adult Sertoli cells, the final, maximum number of Sertoli cells must be reached before adulthood in order to ensure spermatogenic output is abundant in later life. In the rodent, this proliferation occurs during the late embryonic and pre-pubertal stages of life with final Sertoli cell number being achieved by postnatal day (pnd) 15-20 in the mouse [13, 14]. This proliferation has been to shown to be directly stimulated by a number of different factors. In particular, by the gonadotrophins; Follicle Stimulating Hormone (FSH); in experiments utilising hypogonadal [14] and transgenic knock outs of FSH receptor (FSHR) and the FSH β-subunit (FSHβ), final Sertoli cell number was significantly reduced [15]. An even greater reduction in Sertoli cell number was reported in mice with a Sertoli cell specific knockout of the Insulin receptor (*Insr*) and IGF1 receptor (*Igf1r*) that was not improved by hemicastration (resulting in increased FSH signalling to the remaining testis) [16] revealing that the actions of FSH on Sertoli cell proliferation may be mediated by or associated with Insulin/IGF1 signalling in Sertoli cells.

Increased thyroid hormone signalling (or hyperthyroidism) has been shown to have a negative impacts on Sertoli numbers in experimental models [17, 18] and the contrary in models of hypothyroidism; with an increase in Sertoli cell numbers, germ cell numbers and subsequently, testis weight being reported [19-21]. The effects of thyroid hormones on Sertoli cell number is believed to be due to thyroid hormones impacting the duration in which

Sertoli cells proliferate and the time of maturation in pre-pubertal testis. This was demonstrated by Pitetti *et al* (2013), who revealed that thyroid hormones act independently of insulin/IGFs, modulating the proliferation of Sertoli cells through different pathways, following observation of an increase of testis weight and Sertoli cell number recorded in Sertoli cell *Insr*/*Igf1r* knockout mice with induced transient hypothyroidism [16]. A recent study has suggested that the effects of thyroid hormones on Sertoli cell proliferation may be associated with inducible Nitric Oxide (iNOS) or at least functioning through similar mechanisms [22]

A reduction in Sertoli cell number was also observed in testicular feminised mice (*Tfm)*; which lack a functional androgen receptor (AR) due to a single base pair deletion in the AR gene [15], but not in mice with a Sertoli cell specific knockout of AR (SC-ARKO). This, along with increasing expression of AR being indicative of Sertoli cell maturation (and is therefore not expressed on Sertoli cell during the extent of Sertoli cell proliferation), would suggest that androgens are influencing Sertoli cell proliferation, but are doing so indirectly of Sertoli cell AR signalling [23, 24].

1.1.1.1.2 Sertoli Cell Functions

1.1.1.1.2.1 Formation of the Blood Testis Barrier

Coinciding with the terminal differentiation of Sertoli cells, the formation of the blood-testis-barrier (BTB) begins with the increased expression of junctional proteins in Sertoli cells, known to be dependent upon stimulation from gonadotrophins and testosterone [25, 26]. The role of the BTB is to restrict entry of molecules and specific cell types into the seminiferous tubule compartment in order to maintain an appropriate microenvironment for the germ cell development and maturation – contributing to the immune privileged status of the seminiferous tubules. To do so, Sertoli cell to Sertoli cell tight junctions; composed of occludins, claudins and junctional adhesion molecules, form a physical barrier between Sertoli cells between the basal compartment/ basal lamina (where the spermatogonia and early spermatocytes can be found) and the adluminal compartment (where auto-antigen expressing haploid germ cells reside). Absence of a mature BTB can result in degeneration of the testicular histology in aged animals (as a result of loss of Occludin [27]) and even sterility as seen in Claudin-11 null testis resulting in epithelial disorganisation and detachment of Sertoli cell from the basement membrane and adult differentiation in the Sertoli cell as well

as a spermatogenic defect in in neighbouring germ cells [28]. The BTB is a dynamic structure that undergoes reorganisation during spermatogenesis to allow maturing early spermatocytes into the adluminal compartment [29]. The functioning of the BTB is regulated by a number of factors including FSH [30] , androgens [31, 32] and a number of cytokines [33, 34]. Additionally, it has also been suggested that peritubular myoid cells may play a role in the functioning of the BTB. This is unsurprising given their location and previously described associated functions with Sertoli cells [35, 36], however, the role peritubular myoid cells may play in the functioning of this structure is yet to be described.

1.1.1.1.2.2 Spermatogenesis

Alongside the development of the BTB, maturation and terminal differentiation of Sertoli cells marks the expression of proteins required to support germ cell development in the testis. Adult Sertoli cells are well known for their supportive roles throughout spermatogenesis, a process that has been shown to be mediated by androgens, FSH and a number of other factors (reviewed in [37, 38]).

Studies utilising either androgen depleted or AR depleted models highlight the importance of androgens for complete spermatogenesis, with models exhibiting a disruption in spermatogenesis [39, 40] despite there being no functional AR in germ cells [40-56]. In particular, these studies have highlighted the requirement of androgen signalling through Sertoli cells for the progression of spermatogenesis past meiosis with a halt in spermatogenesis being observed in SC-ARKO testis [48, 50]. However, it is not known whether this crucial role of Sertoli cell in spermatogenesis is specific to adulthood or whether the function of Sertoli cell have been impacted as a result of a reduction in androgen signalling through Sertoli cell AR throughout development. Studies attempting to determine the specific Sertoli cell roles of AR in adulthood and the post meiotic effects of androgens first utilised a tamoxifen inducible ARKO model (iARKO) [44]. However, given the estrogenic activity of tamoxifen, the endocrine system of both controls and iARKO animals was significantly impacted, making it difficult to elucidate which effects are solely due to the loss of AR in the adult. Furthermore, as there is currently no Sertoli cell specific tamoxifen inducible Cre available, the ubiquitous inducible Cre resulted in complete loss of AR, making it difficult to dissect out the specific roles of Sertoli cell AR. To circumvent this, another study revealed that, by delivering lentiviral vectors carrying Cre recombinase to the Sertoli cell in the adult testis, a loss of Sertoli cell AR resulted in the same block at meiosis during

spermatogenesis concluding that Sertoli cell AR expression is essential in the adult for continuous spermatogenesis [45]. Mouse models with increased levels of Sertoli cell AR reveal a reduction in total Sertoli cell numbers and an increase germ cell load for the Sertoli cell that remain, resulting in an increases in apoptotic germ cells, indicating that a balance of AR signalling is required for male fertility [57].

Sertoli cells are direct targets of FSH signalling in the testis given their expression of FSHR. Mice lacking gonadotrophins, specifically FSH [53, 58, 59] and FSHR knockouts [23, 47, 60] each have significant reductions in germ cell numbers, but do not exhibit complete infertility, demonstrating the FSH signalling it not required for in the process of spermatogenesis. Given the reductions in germ cell numbers during spermatogenesis, it was determined that FSH signalling may be crucial for acquiring the maximum numbers of germ cells during, but not for progression of, spermatogenesis.

The renewal of spermatogonial stem cell in the adult testis has previously been reported to be under the regulation of the gonadotropins, in particular, FSH [61, 62]. It was also determined that this occurred through gonadotropin stimulation of glial cell line-derived neurotrophic factor (GDNF) in the Sertoli cells. However, more recently, conflicting studies have reported that gonadotrophins may not be responsible for the stimulation of GDNF production in Sertoli cell, supporting another publication describing the negative impacts of *Gdnf* deletion from the peritubular myoid cells on spermatogonial stem cell development [63, 64]. This report demonstrated that there were no differences in spermatogonial stem cell self-renewal in *Fshb* knock out mice and has suggested that Sertoli cells may be stimulating spermatogonial stem cell renewal through WNT5A signalling and that this was negatively regulated by LH through suppressive actions of testosterone [64]. However, the mechanisms introduced do not explain the reduction in germ cell numbers seen in testis with disrupted FSH signalling.

1.1.1.2 Leydig cells

Located in the interstitial compartment of the testis (Figure 1.1), Leydig cells are primarily responsible for the production of testosterone in the adult male.

1.1.1.2.1 Leydig Cell Development

The development of Leydig cell populations in the testis occurs in two separate phases; one in foetal life and the other developing during puberty and into adulthood, in the latter, four Leydig cell subtypes of development can be observed; Stem Leydig cells, Progenitor Leydig cells, Immature Leydig cells and Adult Leydig cells.

1.1.1.2.1.1 Foetal Leydig Cells

The foetal population of Leydig cells support the masculinisation of the male foetus through the production of androgens, that are converted to testosterone by the Sertoli cells (through 17β-hydroxysteroid dehydrogenase 3 expression) and the production of Insulin like 3 (Insl3) (believed to be responsible for the descent of the foetal testis from the intra-abdominal position to the scrotum [65, 66]) [67, 68]. The induction of these cells to produce hormones (at around 13dpc in the rodent) begins with expression of Dhh, from developing Sertoli cells in the foetal testis, interacting with its receptors on foetal Leydig cells and resulting in a both a down regulation of chicken ovalbumin upstream promoter II (COUP-TFII) and up regulation of steroidogenic factor 1 (Sf1) dependent steroidogenic enzymes [68, 69]. The regulation of androgen production in the foetal rodent testis is yet to be fully described, but appears to eventually attain but not depend upon LH responsiveness [70]. Further study into the factors responsible for stimulation of foetal Leydig cell provided further evidence into the promiscuity of foetal Leydig cell s, though it appears an intact pituitary is still required, other factors include adrenocorticotrophic hormone (ACTH), corticotrophin releasing hormone (CRH), Platelet derived growth factor subunit A (PDGF-A) and Dhh among others [70, 71].

1.1.1.2.1.2 Stem Leydig Cells

It is accepted that the adult Leydig cell progenitor or stem cell is present in the testis in foetal life [72]. However, whether the foetal Leydig cell and adult Leydig cell share a common stem cell ontogeny is still debated. The processes of post-natal regression of the foetal Leydig cell population and replacement with the developing adult Leydig cell populations have previously been debated, with evidence available for both the atrophy and regression of the foetal Leydig cell population [73, 74] and for the retention of a small sub-population of the

foetal Leydig cell in the adult testis [75-78]. However, the latter is now widely accepted to be the case.

The adult Leydig cell population begins to develop around puberty from stem Leydig cells present in the postnatal testis. Studies utilising Leydig cell ablation/regeneration with ethane dimethanesulfonate (EDS) have indicated that stem Leydig cells; morphologically spindle shaped cells, can be located close to outer surface of the seminiferous tubules and the vasculature [79-81], Though they do not produce androgens and don't express Leydig cell lineage markers (such as the steroidogenic enzymes and LH/chorionic gonadotrophin receptor (LHCGR)) as demonstrated by Ge *et al* (2006) [72], Kilcoyne *et al* (2014) has also shown that these stem Leydig cell express AR and can be impacted by reduced AR signalling in foetal life (subsequently impacting the final number of adult Leydig cell) [82]. Ge *et al* (2006) also revealed that proliferation of this cell type *ex vivo* was stimulated by the growth factors; leukaemia inhibitory factor (LIF), PDGF and stem cell factor, all factors typically associated with stem cell renewal in other systems [72, 83].

Differentiation of the stem Leydig cells into progenitor Leydig cells during puberty is dependent upon LH, IGF-1 and Thyroid hormone signalling. Treatment with exogenous LH resulted in an increase in progenitor Leydig cell in both WT animals and in IGF-1 knock out mice (which have a decreased number of Leydig cell [84] due to impacted progenitor Leydig cell development [85]) , though full recovery of progenitor Leydig cell numbers was still reduced in these animals [86]. Indeed, signalling of LH and its receptor; LHCGR, is critical for the development of the of the adult Leydig cell population, with LHCGR knock out animals exhibiting a dramatic reduction in final adult Leydig cell numbers and in the expression of fundamental genes for endocrine function [87-90].

Daily administration of thyroid hormones in rats led to an advancement in the formation of Leydig cell progenitors and as a result an increase in final Leydig cell numbers in the testis. On the contrary, induced hypothyroidism results in eventual decreased numbers of Leydig cells as a result of a delay but not a complete arrest in the development of the adult Leydig cell population [80, 91, 92].

1.1.1.2.1.3 Progenitor Leydig Cells

The commitment of the stem cells into progenitor Leydig cells at around pnd10-14 in the rodent coinciding with the expression of Leydig cell lineage markers such as HSD3β and

LHCGR. As a result, they are now capable of producing androgens, albeit at relatively low levels due to steroidogenic enzyme expression levels also being lower than at later developmental stages [76, 93-95]. Along with LH, the intense proliferation of these cells (with a significant increase in interstitial cells being observed during this time point [76, 96, 97]) is dependent on Sf-1, and upon Sertoli cell produced factors such as TGF-β as supported by Sertoli cell ablation models demonstrating reduction in Leydig cell numbers following Sertoli cell ablation at pnd2 and pnd18 – key stages of stem Leydig cell differentiation and progenitor Leydig cell proliferation in the pre-pubertal testis [5, 98-100]. A key role of Sertoli cell AR has also been implicated in this proliferation, with SC-ARKO testis having significant reductions in Leydig cell number in adulthood as well as significant reductions in transcript expression of growth factors known to be implicated in progenitor Leydig cell proliferation [101]. The constitutive activity of FSHR has also been suggested to have a role in the development of final Leydig cell number [102]. This was demonstrated with a reduction in Leydig cell number being observed in mice deficient of FSHR but not in mice deficient of it ligand; FSHβ. This would suggest that, both FSHR and LH (but not FSH) are required for proper attainment of Leydig cell number in the developing testis.

1.1.1.2.1.4 Immature Leydig Cells

The proliferation and transition of progenitor Leydig cell to immature Leydig cell occurs at around pnd21 in the rodent and, in Mullerian inhibiting substance (MIS) or AMH knock out mice, Leydig cell number is increased, but Leydig cell are phenotypically and functionally immature, suggesting a role of MIS/AMH in this transition from progenitor to immature Leydig cells [11, 103-105]. At this age point, Amh expression in Sertoli cells is reducing, further suggesting an influence of Sertoli cell maturation on Leydig cell development. The transcription factor COUP-TFII is crucial for the transition of progenitor Leydig cell to immature Leydig cell. Coup-tfII ablation at puberty resulted in retention of spindle shaped Leydig cell in the interstitial compartment of the testis comparable to the progenitor Leydig cell seen in the pre-pubertal testis [106], confirming the importance of Coup-tfII in this progenitor to immature Leydig cell differentiation.

Morphologically, the transition of progenitor to immature Leydig cell signifies the loss of this spindle shaped characteristic seen in stem and progenitor Leydig cell. The immature Leydig cells become more oval with a large nuclei containing condensed chromatin. A typical feature of immature Leydig cells in the rodent is the presence of numerous large lipid droplets,

indicative of the increasing capacity for steroidogenesis as production gradually shifts from that of androstanediol (in immature Leydig cell due to the presence of testosterone metabolising enzymes) to testosterone in the adult Leydig cell [96, 107].

The proliferative activity of immature Leydig cell is significantly less of that than the progenitor Leydig cell highlighted by a reduction in proliferative markers such as PCNA and cyclin D3 [97]. In fact, immature Leydig cells have been shown to divide only once before finally maturing to adult Leydig cells [107]. Though limited, this proliferation cycle is important as it is effectively a final doubling of the immature Leydig cell population. Consequently, interference of this proliferation cycle in testis with knock out of IGF-1 has been shown to significantly reduce final adult Leydig cell numbers, evidently demonstrating the importance of IGF-1 in this final proliferation step (as well as for the proliferation of the progenitor Leydig cell) [85, 86].

AR has been demonstrated to be crucial for the maturation of immature Leydig cell [108]. In model in which AR is ablated in Leydig cell from foetal life, Leydig cell maturation and regulation of steroidogenic enzymes was disrupted, revealing that autocrine roles of AR in Leydig cell. In this model, differences in Leydig cells are noted in adulthood, with AR negative Leydig cell expressing immature Leydig cell markers such as Insl3 and HSD3β1, confirming that Leydig cell maturation was indeed being impacted confirming suggestions from earlier studies [109].

1.1.1.2.1.5 Adult Leydig Cells

By pnd50, the adult Leydig cells populate the interstitial compartment of the rodent testis and are capable of producing testosterone from cholesterol synthesised *de novo* [110, 111]. The proliferative activity of adult Leydig cell is low under normal conditions, though it can be stimulated following ablation of the Leydig cell population with EDS. This indicates that the adult Leydig cell population does retain the potential to proliferate, but only upon stimulation from autocrine and paracrine factors such as DHH and PDGF-A [112-116] as a result of stem Leydig cell survival within the adult testis.

Functioning and retention of the adult Leydig cells has also been shown to be dependent upon paracrine signalling from other testicular cell populations. For example, in adult Sertoli cell ablated testis, Leydig cell number was significantly reduced suggesting a crucial role/factor secreted by Sertoli cells for Leydig cell retention, though whether this is a direct

effect or an indirect one signalling through peritubular myoid cells is not yet certain given the impact of adult Sertoli cell ablation on peritubular myoid cell function also observed in this study [7]. Lack of Sertoli cell proliferation seen in this model also suggests that the signalling required for Leydig cell regeneration, as seen in pre-pubertal Leydig cell development and in EDS models of Leydig cell ablation, may originate from the Sertoli cells, peritubular myoid cells and resident macrophages found in the adult testis, an idea that is supported by other studies [116, 117].

One of these Sertoli cell expressed factors may be Dhh, known to be essential for proper development of adult Leydig cells (who express its receptor Patched 1 (Ptch1)), it is reduced in EDS models of Leydig cell ablation, and is thought to be involved in the commitment of stem Leydig cell to progenitor Leydig cell [113, 116, 118]. Another could be PDGFA and LIF, given the transient increase in expression of both through the regeneration of Leydig cell in EDS models of ablation [116].

1.1.1.3 Peritubular Myoid Cells

1.1.1.3.1 Peritubular Myoid Cell Function

The peritubular myoid cells are located in the outer periphery basal lamina of the seminiferous tubules (Figure 1.1) and are smooth muscle-like cells known to be involved in tubule contractility as first noted by Clement (1958) [119]. Along with the Sertoli cells, peritubular myoid cells are also responsible for contributing to the basement membrane of the seminiferous tubules through the production of extracellular matrix components such as fibronectin, laminin and collagens [120, 121]. Depending on the species, the organisation of the peritubular myoid cells can differ. In larger mammals such as humans, the peritubular myoid cells are arranged around the seminiferous tubules in more than one layer, whereas in rodents such as rats and mice, there is only one layer of peritubular myoid cells surrounding the seminiferous tubules [122].

Interestingly, multilayering of the peritubular myoid cells in the mouse testis has been observed following ubiquitous loss of AR but not in peritubular myoid cell specific (PTM-ARKO) and SC-ARKO testis [48, 52], though the reason for this and whether it is associated directly with the loss of AR in a cell type or as a by-product of the intra-abdominal location of the ARKO testis is not yet clear.

The peritubular myoid cells are the first to display expression of AR protein in the testis in foetal life and knock out of AR from these cells results in a reduction in germ cells and subsequent impact on fertility [123]. This was suggested to be a consequence of the androgen mediated influence of peritubular myoid cells on Sertoli cell function being disrupted, confirming the importance of cell to cell interactions within the testis for its function. Alongside interacting with the Sertoli cell in the adult testis, the same authors also described the impacts of disrupted peritubular myoid AR signalling on the regulation of normal Leydig cell development, structure and function, with a population of immature Leydig cell being identified in the PTM-ARKO testis that persists into adulthood [124].

1.1.1.3.1.1 Contraction of Seminiferous Tubules

The smooth muscle like morphology of the peritubular myoid cells is suggestive of their contractile functions in the adult testis with peritubular myoid cells expressing markers such as smooth muscle actin (SMA), myosin and desmin [125]. The purpose of this contractile ability is to aid the transport of spermatozoa in the tubular lumen into the rete testis and subsequently out to the epididymis. The contraction of the peritubular myoid cells has been shown to be stimulated to a number of triggers including prostaglandins, cyclic guanosine monophosphate (cGMP), testosterone, transforming growth factor beta (TGF-β), Angiotensin II and endothelin [126-129].

1.1.1.3.1.2 Support of Spermatogonial Stem Cell Population

The spermatogonial stem cell and spermatogonia population are located within the seminiferous tubules of the adult testis; on the basal lamina of the seminiferous tubules, in direct contact with the Sertoli cell and with peritubular myoid cells underlying the basal lamina. Therefore, peritubular myoid cells may play an important role in maintaining and supporting the spermatogonial stem cell population and niche in the testis.

This is further supported with the loss of germ cells at all stages of the spermatogenic cycle in the adult PTM-ARKO testis suggesting an impact early in the cycle [52].

Transgenic knockouts of components implicated in GDNF signalling result in phenotypically infertile males as a result of disruptions early in spermatogenesis [130-133]. Though previously thought to originate from Sertoli cells, the increased expression of GDNF from peritubular myoid cells at key stages of the spermatogenic cycle indicate that it is peritubular myoid cell derived GDNF that is implicated in spermatogonial stem cell maintenance.

Furthermore, in cultured peritubular myoid cells, GDNF expression was found to be stimulated in the presence of testosterone, providing further support to the observations seen in the PTM-ARKO testis [63, 134].

A cytokine involved in spermatogonial stem cell renewal; colony stimulating factor 1 (CSF1), has also been found to be produced by peritubular myoid cells, along with Leydig cells [135] and testicular macrophages. De Falco *et al* have implicated peritubular myoid cells in contributing to CSF1 signalling with its receptor on spermatogonial stem cells , further supporting the notion that peritubular myoid cell communication within the testis is crucial for testicular function [136].

Finally, a study into the inhibition of tyrosine kinases PDGFR and c-Kit in the postnatal testis resulted in a decrease in proliferating peritubular myoid cells in postnatally testis [137] and a reduction in the pool of spermatogonial stem cells [138]. This implies that there may be a role for the peritubular myoid cells in the early establishment of the spermatogonial stem cell pool in the postnatal testis.

1.1.1.4 Germ cells

Arrival of the primordial germ cells within the genital ridges of a 10dpc embryo and the embryonic Sertoli cells at 12.5dpc permits association of these two testicular cell types and eventual formation of the seminiferous cords which will go on to form the seminiferous tubules. It is within the cords that the primordial germ cells first undergo a period of mitotic proliferation before entering a quiescent period until birth, a step that is regulated by transforming growth factor beta (TGF-β) [139] . It is through factors secreted by Sertoli cells that germ cells are prevented from entering meiosis until adulthood. In the embryonic female gonad, primordial germ cells enter meiosis at 13.5dpc through the stimulation of Stra8 activation as a result of retinoic acid (the biologically active form of vitamin A/retinol) expression [140]. In the male, the retinoic acid is metabolised following secretion of CYP26B1 from Sertoli cells, preventing expression of Stra8 and subsequently germ cell entry into meiosis at 13.5dpc in the developing testis [140]. This inhibition of Stra8 is maintained in the days following through Nanos2 signalling. Nanos2-null testis revealed an upregulation of Stra8 expression, blocking male germ cell entry into meiosis after 13.5dpc [141]. Induction of Stra8 expression through retinoid acid signalling is required in the male during puberty for

the meiotic entry of the germ cells initiating the first wave of spermatogenesis at around pnd40-45 in the rodent, though it is not required for future meiotic divisions of germ cells [142-146]. Another factor implicated in the regulation of male germ cell development in both mouse and human foetal testis is FGF9, with a number of studies demonstrating its expression in the foetal testis, its role in meiosis suppression and promotion of male germ cell fate [140, 147-149]

The first wave of spermatogenesis is highly regulated process that differs greatly from subsequent waves of spermatogenesis occurring in the testis and is known to be dependent on FSH and LH mediated stimulation of testosterone production. For instance, during the first wave of spermatogenesis there is an increase in apoptosis specifically in the pachytene spermatocytes as a result in an increase in apoptosis-promoting BAX protein in the germinal cells [150]. The expression of BAX protein at this time point is believed to maintain the proper Sertoli cell: germ cell ratio in the seminiferous tubules and perturbation of this protein and the subsequent wave of apoptosis results in marked alterations of spermatogenesis later in life [151]. In mice treated with a GnRH antagonist, there was a significant increase in apoptotic cells associated with a reduction in FSH levels during this time period, suggesting that FSH may be limiting or regulating the levels of spermatocyte apoptosis [152].

FSH signalling, however, is not required in adulthood for the completion of quantitative spermatogenesis [153]. This has been demonstrated using *hpg* animals expressing transgenic FSH with and without testosterone treatment. Researchers demonstrated that transgenic expression of FSH stimulated maturation and proliferation of Sertoli cell and established a normal Sertoli: germ cell ratio. However, numbers of post meiotic haploid cell, though absent in *hpg* mouse (minus transgenic FSH expression), were limited. Testosterone treatment in *hpg* mouse had no impact on the proliferation of spermatogonia in the postnatal testis, but that progression into meiosis was greatly enhanced. Combination of transgenic FSH expression and testosterone treatment in the *hpg* mouse revealed both an additive and synergistic effect on the establishment of normal meiotic Sertoli cell : germ cell ratio and on the total number of spermatids in the testis [154].

FSH can induce Leydig cell function indirectly through stimulation of Sertoli cells [155]. Therefore, some observations on spermatogenesis seen in *hpg* mice treated with FSH may also be due to stimulation from Leydig cells presumably through AR. O'Shaughnessy *et al*

utilised *hpg* mice with loss of AR in either Sertoli cells alone (*hpg.*SC-ARKO) or ubiquitously (*hpg*.ARKO) to determine the direct effects of FSH action on spermatogenesis. Treatment with recombinant FSH was found to increase spermatogonial number and entry into meiosis but had no effect on the completion of meiosis [6]. Taking the results of both studies together this would imply that androgens are required for the completion of meiosis during spermatogenesis and that FSH, along with its influences on Sertoli cell number, can support the entry of spermatogonia into meiosis and increase proliferation of spermatogonia.

1.1.1.4.1 Stages of Spermatogenesis

Spermatogenesis is the process in which diploid germ cells develop into haploid spermatozoa in the seminiferous tubules of the adult testis. The process is continuous throughout adulthood following initiation after puberty and takes place in three stages; mitosis, meiosis and cytodifferentiation.

1.1.1.4.1.1 Mitosis

As discussed previously, the spermatogonial stem cells reside in the basal lamina of the seminiferous tubules in the adult testis. The spermatogonial stem cells are a sub-type of spermatogonia, capable of both self-renewal and of developing into differentiating spermatogonia [156]. Huckins (1971) and Oakberg (1971) were first to describe this population of early spermatogonia and subsequently proposed a model of spermatogonial self-renewal, proliferation and differentiation in the rat and mouse respectively [157-159] (Figure 1.2). In this model these early spermatogonia or undifferentiated spermatogonia can be grouped into different stages; single spermatogonia (A_{single} or A_s), pairs of spermatogonia (A_{paired} or A_{pr}), and chains of four, eight and 16 cells ($A_{aligned}$ or A_{al}). The 'spermatogonial stem cells' in this model are the A_s stage of the model and following the mitotic division of the A_s spermatogonium, the two arising daughter cells can either migrate away from one another to become two new spermatogonial stem cells or A_s, or, as a result of incomplete cytoplasmic division, can remain connected, constituting the first step of differentiation to the A_{pr} stage [157, 160]. Further mitotic divisions of these A_{pr} then results in differentiation to the A_{ai} in which the chain four, eight or 16 clones will emerge. These chain of A_{ai} spermatogonia will then undergo a limited number of mitotic divisions (depending on species) and each stage is denoted A_1 (the A_{ai} spermatogonia), A_2, A_3 and A_4 after the first three mitoses, intermediate spermatogonia after the fourth and final type B spermatogonia after the final division. Each of these type B spermatogonia then divide to form resting primary spermatocytes [156-159].

Alternative strategies have been later suggested for the maintenance of the spermatogonial stem cell pool. One model known as the "fragmentation model" describes the ability of the A_{pr} and A_{ai} chains to fragment into smaller clones from which more clones will be generated through mitotic divisions (Figure 1.2). In this model, A_s spermatogonia rarely produce two new A_s daughter cells and are more likely to form pairs [161, 162]. More recently, the 'hierarchical' model has described the heterogeneity of the A_s population, suggesting that 20% of A_s spermatogonia express high levels of the protein Inhibitor of DNA binding 4 (ID4), a protein reported to be involved in the self-renewal of spermatogonial stem cells [163, 164]. In the hierarchical model, the ID4 bright A_s spermatogonia are postulated to be the 'ultimate' spermatogonial stem cells and the A_s spermatogonia expressing lower levels of ID4 were described as transitory spermatogonial stem cells, with a reduced capacity for self-renewal (Figure 1.2).

As discussed in 1.1.1.3.1.2, the proliferation and differentiation of the spermatogonia is dependent on direct or indirect support from the Sertoli cells, peritubular myoid cells and macrophages through the secretion of factors such as GDNF and CSF1. Other Sertoli cell secreted factors that may be implicated in this support are fibroblast growth factor 2 (FGF2) and wingless-type MMTV integration site family member 5A (WNT5A) which has recently been shown to be negatively regulated by LH via testosterone secretion and interaction with Sertoli cells, suggesting a role of the Leydig cells in the regulation of the spermatogonial stem cell niche [64].

1.1.1.4.1.2 Meiosis

The proliferation of spermatogonia described in 1.1.1.4.1.1 takes place in the basal lamina of the seminiferous tubules, outside of the BTB. The resting primary spermatocytes formed must then cross the BTB to reach the adluminal compartment; a process which requires disassembly of existing junctional proteins and the simultaneous assembly of new junctions behind the entering spermatocyte. This ensures the integrity of the immune privilege in the adluminal compartment whilst permitting the continuous progression of spermatogenesis.

The expression of AR in Sertoli cells is cyclical and has been linked to specific stages of the spermatogenic cycle, with a particular increase in expression at stages VI-VII, stages at which there is a presence of resting primary spermatocytes [165, 166]. The close relationship of Sertoli cells and germ cells and the phenotype seen in models with disrupted Sertoli cell AR signalling; a halt of spermatogenesis at meiosis [48], downregulation of *Claudin 3* [31] (a

protein associated with the formation of new tight junctions in the BRB) and an influx of immune cells in the testis [167], suggests a clear role for androgens in the restructuring of the BTB following primary spermatocyte entry into the adluminal compartment . This association of androgens and the regulation of Claudin 3 expression was also further supported with a reduction in expression in *hpg* mice that was rescued following treatment with dihydrotestosterone (DHT) [26] (Figure 1.2).

Once within the adluminal compartment; benefitting from the immune privilege provided by the BTB, the resting primary spermatocytes can now enter meiosis, involving two sequential divisions (Meiosis I and Meiosis II), each divided into four stages; prophase, metaphase, anaphase and telophase. Prior to Meiosis 1, during premeiotic interphase, the centrioles and chromosomes of the spermatocyte are duplicated. The first meiotic prophase is extended, during which homologous chromosomes condense, pair and DNA exchange occurs, resulting in chromosome crossover. In metaphase and anaphase, the homologous chromosome pairs align along the equator of the spindle before migrating in opposite directions. In telophase, cytokinesis occurs, yielding two daughter cells each with half the number of chromosomes with each chromosomes consisting of two unique chromatids. In the second phase of meiosis (which has no premeiotic interphase), the chromatids are separated to yield four, genetically unique haploid round spermatids from each spermatocyte entering the process.

As discussed earlier in 1.1.1.4, entry of the spermatocytes into this process is reliant expression of *Stra8*, stimulated by the presence of retinoic acid.

1.1.1.4.1.3 Spermiogenesis & Spermiation

The haploid round spermatids must then undergo major morphological changes to differentiate into a mature spermatozoon. During this last stage of spermatogenesis, there are no further cycles of cell division and the round spermatids begin to undergo complex cytodifferentiation. This process takes places in three stages; i) the development of the acrosome, ii) the development of a flagella from an axoneme and iii) the elongation and condensation of the nucleus to form the characteristic and species dependant head of the elongated spermatid. During this the histones present in the chromatin of the early round spermatids are eventually replaced by protamines through an intermediate replacement with transition proteins (TP) to protect the DNA during this re-organisation [168]. In mice lacking the FSH receptor there was a significant decrease in the numbers of elongated spermatids [169]. This was reported to be a result of reduced nuclear compaction during

spermiogenesis suggesting that FSH signalling through it receptor may be important for the completion of qualitative spermatogenesis. This observation could also be relevant to the increased apoptosis seen in association with reduced levels of FSH as discussed earlier in section 1.1.1.4.

As the spermatid is undergoing elongation, unique intercellular junctions with the supporting Sertoli cells are formed. Suppression of intratesticular testosterone has been shown to impact this attachment due to the premature release of round spermatids from the Sertoli cells [170]. Following complete spermiogenesis the elongated spermatids are translocated to the luminal edge of the Sertoli cell, facilitated by the Sertoli cell cytoskeleton, undergo their final remodelling and are released into the seminiferous epithelium supported by the seminiferous tubule fluid, secreted by Sertoli cell as a result of androgen signalling.

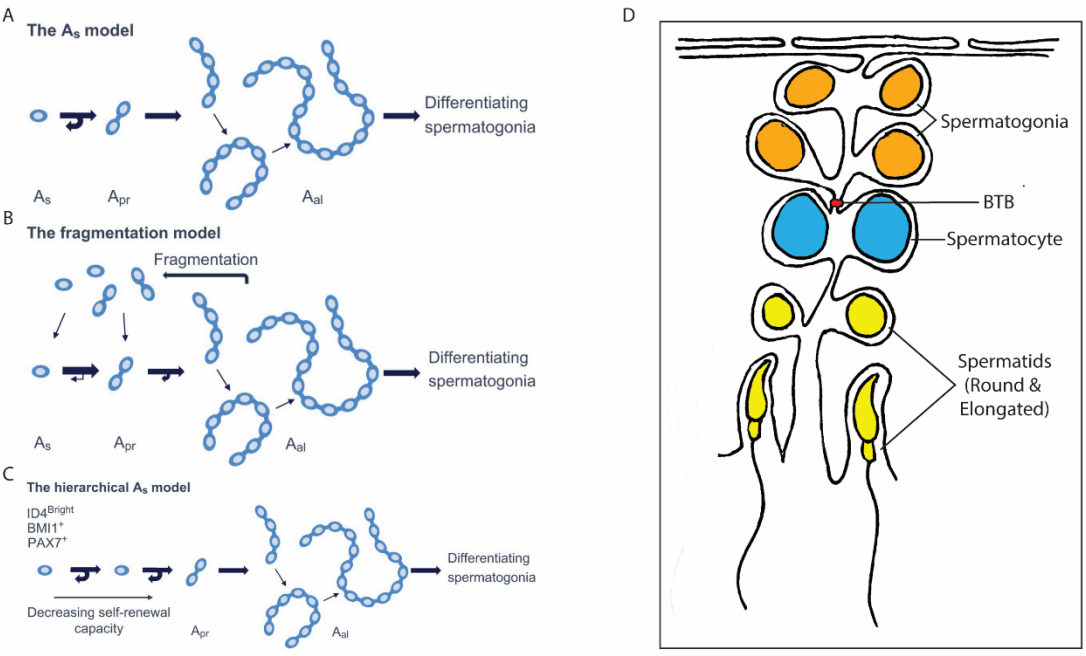


Figure 1.2 Models of Spermatogonial Stem Cell Renewal and Spermatogenesis. Proposed models of spermatogonial stem cell renewal and differentiation; (A) the As model, (B) the Fragmentation model and (C) the Hierarchal model. (Modified from de Rooij (2017) [156] (D) The mitotically dividing spermatogonia (orange) will then move from the basal lamina through the BTB (red) into the adluminal compartment begin meiosis become spermatocytes (blue) before developing into spermatids (yellow).

1.1.1.5 The Hypothalamic-Pituitary-Gonadal Axis

Both spermatogenesis and steroidogenesis are dependent on an intact hypothalamic-pituitary-gonadal (HPG) axis through a system of negative feedback mechanisms. An overview of the negative feedback mechanisms involved in the HPG axis can be found in (Figure 1.3). Starting at the hypothalamus, Gonadotrophin Releasing Hormone (GnRH) secretion binds to GnRH receptors located on pituitary gonadotrophs (found in the anterior pituitary). The production of GnRH from the hypothalamus has recently been shown to be regulated by kispeptins, signalling through the kispeptin receptor (GPR54) [171-173].

Upon GnRH binding, the pituitary gonadotrophs synthesis and secrete FSH and LH. These gonadotrophins subsequently bind to their respective receptors found on specific somatic cells in the testis; FSH to FSHR on Sertoli cells and LH to LHR on Leydig cells. Binding of the gonadotrophins to their receptors in the testis has a number of impacts on both the spermatogenic and steroidogenic functions of the testis, as described earlier (in sections 1.1.1.2.2 and 1.1.2.2).

Production of testosterone and Inhibin from the Sertoli cells and Leydig cells (in response to gonadotrophin receptor activity) respectively act to negatively regulate the synthesis and production of the gonadotrophins by supressing production at both the level of the hypothalamus and the pituitary.

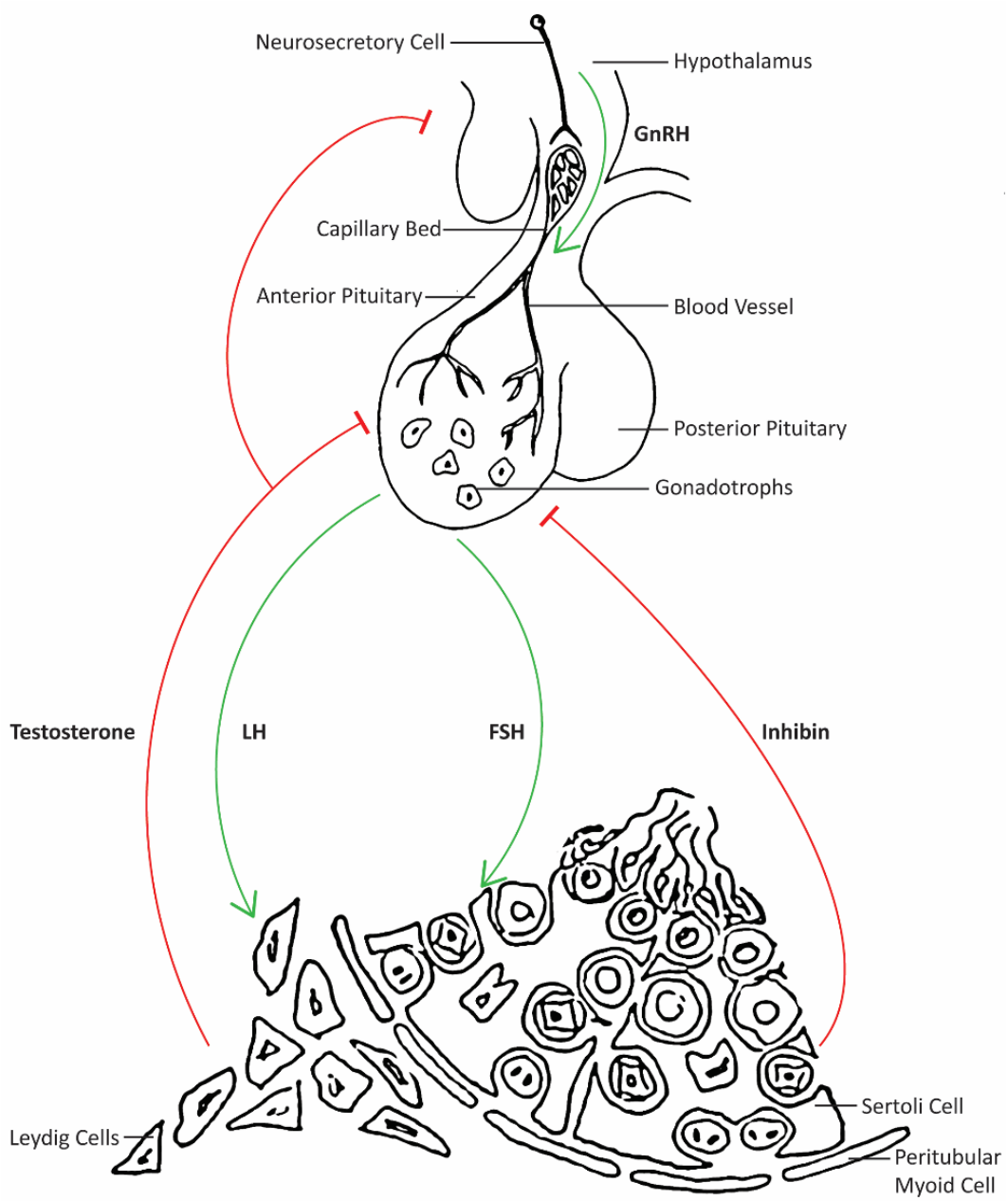


Figure 1.3 The Hypothalamic-Pituitary-Gonadal (HPG) Axis. Steroidogenesis and Spermatogenesis are under the control of the HPG axis. GnRH signalling from the hypothalamus and stimulated the production of LH and FSH from the anterior pituitary. On binding to their receptors on the Leydig cells (LH) and Sertoli cells (FSH), steroidogenesis and spermatogenesis is stimulated. Secretion of testosterone (from Leydig cells) and inhibin (from Sertoli cells) feeds back to the hypothalamus and pituitary to negatively regulate the production of GnRH. Green lines represent stimulation, red lines represent inhibition of the feedback loop.

1.1.1.6 Testosterone Biosynthesis

The main role of the adult Leydig cells is to produce testosterone through the process of steroidogenesis. As a result of LHCGR signalling, testosterone is synthesised from cholesterol through a number of transformative and enzymatic interactions (Figure 1.4).

1.1.1.6.1 Luteinising Hormone Receptor Activity

On binding of LH to LHCGR; a G protein coupled receptor, protein kinases A (PKA) and C (PKC) are activated through an increase in intracellular cyclic adenosine monophosphate (cAMP). This increase in cAMP signalling and activation of PKA and PKC results in the binding of transcription factors such as cAMP-responsive element binding protein (CREB), GATA-4 and Sf-1 [174-177]. The binding of the transcription factors then initiates the activation of key target genes, resulting in expression of steroidogenic enzymes [178]. Interaction of LH with its receptor, expressed by Leydig cells, is essential for the subsequent production of testosterone. Natural inactivating mutations in LHCGR have resulted in partial or complete loss of receptor function leading to patients presenting with Leydig cell hypoplasia as a result of failure of Leydig cell differentiation [179]. This is mirrored in mouse models in which LHCGR is knocked out, in which postnatal development of the steroid producing Leydig cell is impaired with downregulation of Leydig cell markers *HSD3β6* and *HSD17β3.* Consequently, serum testosterone levels were also reduced and LH significantly increased [88]. Exogenous testosterone delivery was sufficient to stimulate spermatogenesis but not rescue of adult Leydig cell and subsequent steroidogenesis in the LHCGR knockouts, implying that it may be androgen independent actions of LHCGR mediating Leydig cell differentiation postnatally.

1.1.1.6.2 Cholesterol Synthesis & Trafficking

All steroid hormones derive from the enzymatic metabolism of cholesterol. Cholesterol is first synthesised *de novo* from acetyl coenzyme A (acetyl-CoA) via the mevalonate pathway via the enzyme Hydroxymethylglutaryl-CoA Reductase 1 (HMGCR1) [180]. The resultant cholesterol is shuttled from the outer to the inner of Leydig cell mitochondrial membrane by steroidogenic acute regulatory protein (StAR), a rate limiting step of steroidogenesis [181, 182]. Knock out of StAR resulted in accumulation of lipid droplets in the Leydig cell and low, but detectable plasma testosterone, presumably through less efficient StAR independent steroidogenesis [183, 184].

1.1.1.6.3 Steroidogenesis

Once located in the mitochondria, active cholesterol is then converted into Pregnenolone through the action of cholesterol side chain cleavage P450 (P450cc), encoded by the gene Cytochrome P450 11A1 (*CYP11A1*). Pregnenolone is a common precursor of all steroid hormones including mineralocorticoids, glucocorticoids and sex hormones. Male *CYP11A1* null mice are feminised with a reduction in anogenital distance and un-descended testis [185]. This was echoed in 46,XY patients with frame shift causing *CYP11A1* mutations [186-188]. The subsequent enzymatic processes converting pregnenolone to testosterone can occur through either the Δ4 or Δ5 pathways, depending on enzyme preferences and substrate affinity and these processes occurs in the smooth endoplasmic reticulum. In the Δ4 pathway, HSD3β acts to convert pregnenolone to progesterone. Progesterone is then converted to 17α-hydroxyprogesterone and then androstenedione through the actions of CYP17a1. Androstenedione is then finally converted to testosterone via HSD17β3. In the Δ5 conversion of pregnenolone to testosterone, pregnenolone is converted to 17α-hydroxypregnenolone and subsequently to dehydroepiandrosterone (DHEA) by the enzyme cytochrome P450 17Aa (CYP17A1) in both instances. DHEA can then be converted to testosterone through androstanediol or androstenedione, depending on whether HSD3β or HSD17β3 respectively are first to act. Studies have suggested that the dominance of a particular pathway is dependent on the species of the animal; with the Δ4 pathway (via progesterone) being dominant in rodents and the Δ5 pathway being preferential in humans [189, 190]. Earlier studies suggested that this may be the case in the neonatal testis but also provided evidence for changes in preferential pathways through development, with an increase in Δ5 metabolites detected in the adult testis when compared to the neonate and pubertal testis, indicating a shift from Δ4 in the neonate to Δ5 in the adult testis [191]. It was suggested that this may be due to the differences in Leydig cell populations and their differential capacities for testosterone production.

Testosterone can be further metabolised into dihydrotestosterone (DHT) or estradiol through the actions of the enzymes 5α-reductase and aromatase (cytochrome 19A1 – CYP19A1) respectively. Conversion of testosterone to DHT usually occurs in the peripheral tissues where concentrations of testosterone may be much lower than that at the testis, due to the increased affinity of DHT for AR compared to testosterone.

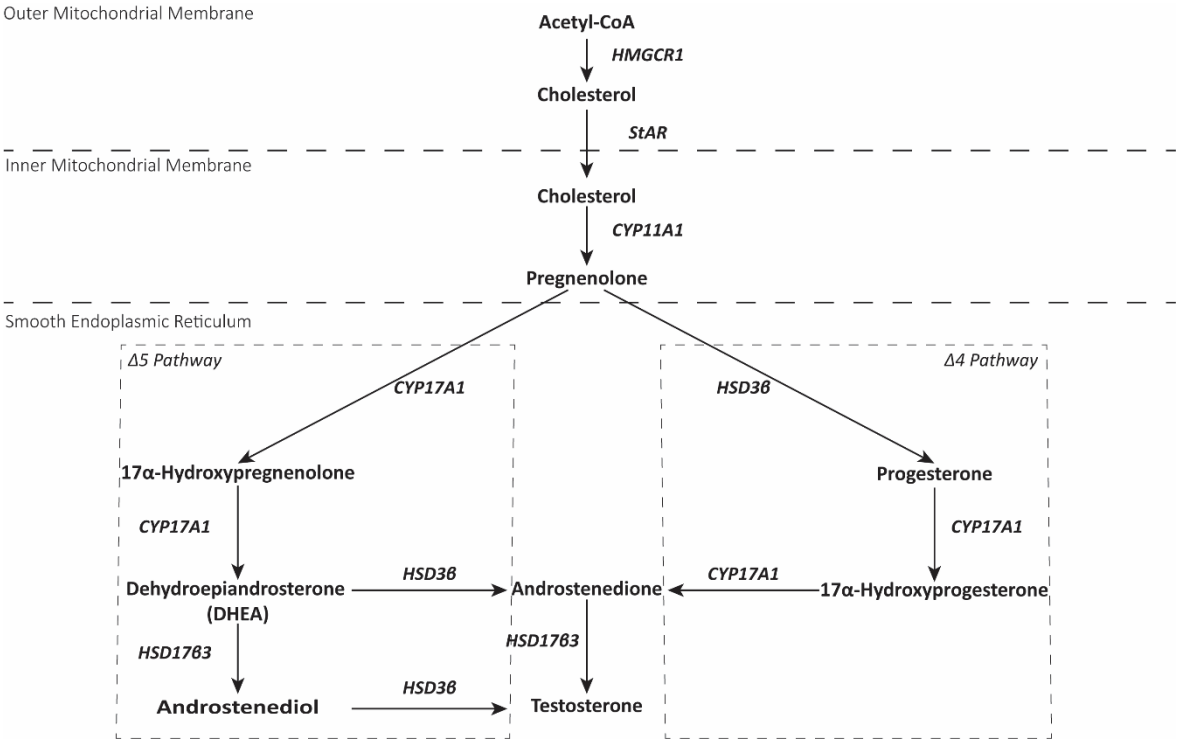


Figure 1.4 The Steroidogenic Pathway. Schematic of the production of testosterone from cholesterol through the Δ5 and Δ4 pathways.

1.1.1.7 Androgen Receptor

Following synthesis and migration to target tissue/cell types, androgens mediate their functions through binding to their receptor; AR. AR (also referred to as nuclear receptor subfamily 3 group c member 4 – NR3C4), is a type of steroid/nuclear receptor found within the cytoplasm when without ligand.

AR is a DNA binding transcription factor located on the X chromosome and is known to be expressed by a number of different tissues and cell types. AR –KO and deficiency models have reported impacts on a number of these systems including the cardiovascular system [192-194], the haematopoietic and immune system [195-197], in bone [198-202], the reproductive system [6, 23-25, 44, 48, 52, 60, 101, 108, 109, 124, 203, 204], in muscle [205, 206], the brain [207-209] and in adipose system (influencing metabolic pathways) [210-212]. This supports the notion that androgens have a number of different function roles in the body.

AR, like other steroid/nuclear receptors is comprised of an N terminal domain (NTD); which is the transcriptional regulatory region of the protein, a highly conserved DNA binding domain (DBD), a small hinge region and a ligand binding domain (LBD) [213]. These four functionally distinct regions of AR protein are encoded by 8 exons spanning around 90kb of DNA on the X chromosome [213].

The NTD domain of AR is a less conserved region in comparison to the DBD, with homology of only 15% with other steroid hormone receptors [214]. This region of AR, also known as AF-1, is considered the main activation domain of AR, with two transcription activation units having been identified; Transcription activation unit (Tau)-1 and Tau-5 [215], which can bind co-regulator proteins to increase or decrease transcription of downstream genes. The Tau-5 fragment of the NTD is capable of retaining the action potential in absence of the LBD – allowing ligand independent AR activity.

The DBD, recognises and binds to androgen responsive gene promoter and enhancer regions. This region of the nuclear receptor is highly conserved for a number of steroid receptors and is organised into two zinc finger motifs, the first makes nucleotide specific contacts and is therefore responsible for recognition of the target DNA sequence and the second functions as a DBD to DBD binding site on dimerization of nuclear receptors [216, 217].

Between the DBD and the LBD is the small hinge region, which as a characteristic globular fold made up of two perpendicular α-helices. Previously believed to just function as a flexible linker, the hinge region is now known to be involved in nuclear translocation, DNA selectivity and affinity and in the transactivational potential of the AR [218].

The LBD (containing a second transcription region; AF-2) is located at the carboxyl terminus of the AR and facilitates the binding of ligands such as testosterone and DHT as well as contributing to the dimerization interface of AR. AF-2 is also the target for several co-regulators, all of which contain a conserved leucine rich motif with the sequence; LXXLL (where L is leucine and X denotes any amino acid) and are dependent upon ligand binding for their interaction [219, 220]. The interaction of AF-2 with these co-regulators are essential for androgen responsive gene regulation, whether that be activation or repression of the androgen responsive gene. The LBD is also the site of heat shock protein (hsp) (specifically hsp90) association and binding of ligand to the LBD results in dissociation of AR from hsp90; allowing translocation of AR to the nucleus [215, 221, 222].

In the absence of a ligand, AR is bound to heat shock proteins (such as hsp90) and cofactors and is located within the cytoplasm [213]. The binding of a ligand to AR initiates a conformational change in the LBD, releasing the heat shock proteins and enabling AR to interact with co-regulators, such as importin-α, facilitating nuclear targeting of AR and consequent dimerization at the nucleus, permitting the binding of AR to genomic androgen response elements [223, 224]. This is commonly referred to as classical AR signalling, regulating gene transcriptional processes via AR nuclear translocation, binding to response elements and recruitment of transcription factors. AR also functions in a non-classical manner, in which following ligand binding at the membrane or within the cytoplasm, the intracellular release of calcium in the cytoplasm triggers the activation of protein kinase pathways such as the extracellular kinase (ERK), mitogen-activated protein kinase (MAPK) and RAC-alpha serine/threonine-protein kinase (Akt) pathways [225-227] (Figure 1.5).

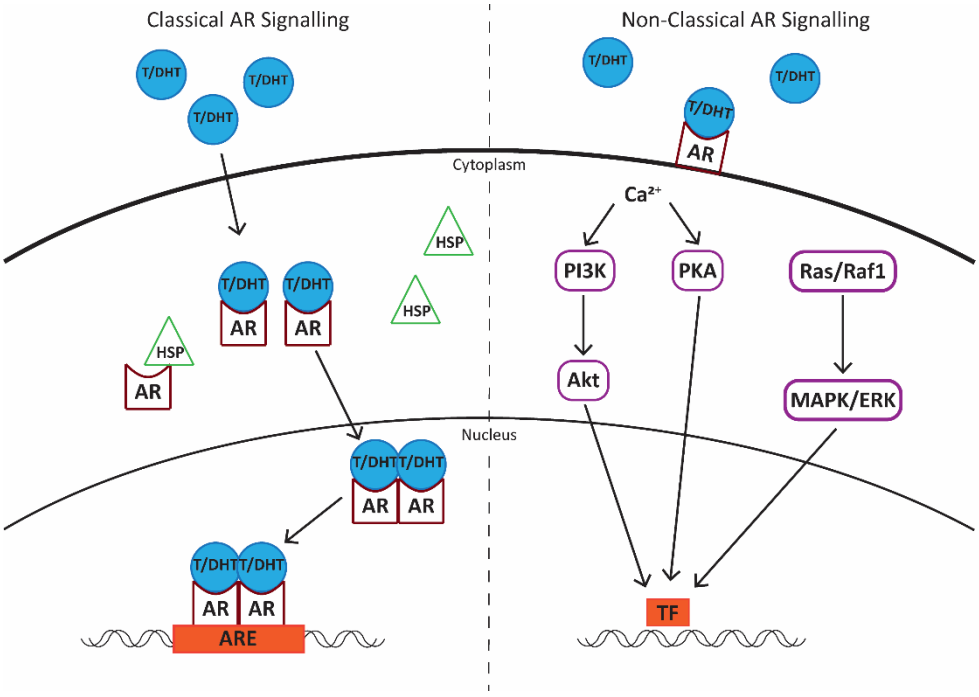


Figure 1.5 Classical and Non-Classical AR Signalling. Schematic depicting binding of testosterone or DHT to AR and the subsequent classical and non-classical pathways downstream. AR – Androgen Receptor, T/DHT – Testosterone/DHT, HSP – Heat Shock Proteins, ARE – Androgen Responsive Element, Ca^{2+} - Calcium, PI3K - Phosphoinositide 3-kinase, PKA - Protein Kinase A, Akt - serine/threonine-protein kinase (also known as Protein Kinase B), Ras – a GTP binding switch protein, Raf1 – Rapidly Accelerated Fibrosarcoma (a serine/threonine-specific protein kinase), MAPK - mitogen-activated protein kinases, ERK - extracellular signal-regulated kinases, TF – Transcription Factors.

1.1.2 Androgens, Male Health & Wellbeing

1.1.2.1 Androgen Signalling in the Healthy Male

Androgens are essential for the development and functioning of the testis as well as the masculinisation of the male during foetal and pubertal development. However, they are also crucial for male health throughout adulthood, with a number of tissues expressing AR (as described in section 1.1.1.7). Disruptions in androgen production and signalling in men is associated with a number of clinically prevalent conditions including male infertility and an impaired reproductive physiology, increased risk of cardiovascular and metabolic diseases [228-230] and earlier mortality [231, 232].

For example, the influence of androgens of body fat composition and muscle mass in the male is well described whereby a high incidence of metabolic syndrome has been associated with lower testosterone levels alongside an inverse association between men with low testosterone and men with an increase in waist circumference and obesity [233-235]. Therefore, the relationship between low testosterone and the increased risks for developing metabolic syndrome are evident. However, the causality of these factors is not clear (i.e. whether the low testosterone is responsible for the increased risk factors (such as obesity) associated with the development of metabolic syndrome or vice versa).

The inverse relationship of testosterone with body fat in men can be attributed to an increase in the aromatisation of testosterone to estradiol as result of increase aromatase enzyme (CYP19A1) found in adipose tissue [236] Furthermore, testosterone has also been shown to inhibit lipid uptake and stimulate lipolysis, particularly in visceral adipose tissue [237]. This can create a feedback loop which could promote a worsening condition in hypogonadal men (i.e. generating a situation in which low testosterone results in a reduction of lipolysis and increase in lipid uptake, resulting in an increase in adipose tissue capable of aromatising circulating testosterone and thus lowering testosterone further).

1.1.2.2 Causes & Consequences of Disrupted Androgen Signalling in the Male

1.1.2.2.1 Genetic Disorders

The causes of impaired male reproductive physiology are heterogeneous [4], but a genetic component is suspected in many cases [238, 239]. Yet despite the testis being the most specialized of human organs (by number of enriched genes expressed; indeed far more

complex even than the brain http://www.proteinatlas.org/humanproteome/testis), only karyotyping, analysis of AZF microdeletions and *CFTR* gene mutations are routinely used in genetic diagnostic work-up of infertile men [240]; our wider understanding of the genetics underpinning male fertility is sporadic at best. Despite this, significant inroads in understanding have consistently been made, primarily from the study of transgenic mouse models on an ad hoc basis. More recently, the development and roll-out of major phenotyping screens, e.g. ENU screens such as the MRC Harwell screen http://www.har.mrc.ac.uk/services/archiving-distribution/enu-dna-archive and the Reproductive Genomics screen completed at the Jackson Laboratory https://www.jax.org/research-and-faculty/tools/reproductive-genomics-resource and more recently the IMPC (http://www.mousephenotype.org), have exponentially increased the number of genes known to support male reproductive physiology [241]. However, whether this is relevant in men often remains unclear.

Historically, this is in part due to the 'outbred' gene-pool and multifactorial nature of reproductive pathophysiology in men. Mutations with severe effects cannot be traced in pedigrees, and are predicted to be rare and mostly sporadic, whereas moderate genetic risk variants exhibit variable level of penetrance and interaction with non-genic variants [238]. Until very recently, the field has essentially been working in two near-mutually exclusive silos: sporadic patient cases with no known functional mechanism, and mouse models for which no patient information has been identified. Limited mechanistic knowledge underpinning the influence of genetics hinders clinical management.

Mutations to the AR gene have been well described in the literature and can lead to partial or complete loss of AR function. AR mutations are found in most subjects with complete androgen insensitivity syndrome (AIS) (previously known as testicular feminisation syndrome [242]) which is the most common cause of 46,XY disorders of sexual development. Similar sex reversal phenotypes and reduced masculinisation is also reported in patients with disorders of testosterone biosynthesis; usually as a result in steroidogenic enzyme deficiencies.

1.1.2.2.2 Age Related Disruptions in Androgen Signalling

Alongside obesity, another risk factor associated with low testosterone is aging [243, 244]. As men age, they are likely to experience decreased muscle mass, reduced sexual function, decrease in bone mineral density and increase fat mass; all indicators that have been associated with low circulating testosterone [245-249]. A number of longitudinal studies have investigated the age related changes in serum testosterone, who reported aberrations in the HPG axis as men age, regardless of ethnicity and geographical location [243, 244, 250, 251]. Though a number of studies described a moderate reduction in total testosterone as men age, the reductions in free or bioavailable testosterone are more striking, coinciding with increases in sex hormone binding globulin (SHBG; the protein that binds to circulating testosterone), suggesting that there is a reduction in testosterone that is available to target androgen responsive tissues [243, 244, 250, 251]. Recently Travison *et al* (2017) generated a reference range of total testosterone levels in men which was found to range from 264-916ng/dL in healthy non-obese men aged 19 to 39 years [252]. Also noted in this study was the lack of age related trend seen in total testosterone levels, contrary to previously published cross-sectional studies [253]. Wu *et al* also described a similar lack of difference in total testosterone between young and aged men [243]. Whether this is a true representation of circulating testosterone levels in aging men or whether there may be a cohort effect is yet to be fully elucidated. Though Wu *et al* was unable to establish any differences between the total circulating testosterone levels in young and aged men, a significant increase in circulating LH levels was detected in older men [243]. This increase in LH hormone is indicative of dysfunction at the level of the Leydig cell, suggesting that the Leydig cell population is requiring increasing levels of LH to stimulate the generation of 'normal' levels of testosterone. This age related decline in the steroidogenic function of Leydig cell has also been cited in the literature, attributing this to alteration to a number of factors including defects in the HPG axis, changes in levels of cytokines crucial for the maintenance of LH receptor and steroidogenic enzymes and increases in the levels of reactive oxygen species in Leydig cells resulting in disrupted mitochondrial function and StAR gene expression; the rate limiting factor of steroidogenesis [254-257].

1.1.2.2.3 Androgen Replacement Therapy

In men suffering from a reduced number of Leydig cell or from a reduced capacity of the Leydig cell to synthesise androgens; as a consequence of genetic mutations or from aging [258], a strategy to maintain a healthy androgen profile is a highly desirable one. Currently,

the most common treatment to address androgen deficiency in the male, is androgen replacement therapy (ART) with prescriptions for testosterone increasing globally between 2000 and 2011 [259, 260]. Indeed, treatment with exogenous testosterone has been reported to be beneficial for some hypogonadal patients, depending on extent of comorbidities, with some improvements in symptoms associated with both aging and low testosterone [261-263]

However, a number of studies have been published indicating the potential adverse effects of ART on the cardiovascular system, with the risk of mortality and morbidity yet to be defined, particularly for individuals with pre-existing cardiovascular complications [264-268].

1.2 Gene Editing, Vector Delivery and their Uses in the Testis

The literature reviewed in section 1.2 has previously been summarised and published in the literature in 2017 by myself under the guidance of Professor Lee Smith [269] (PMID: 29191697). As author of this publication, the rights are retained to reuse within a thesis or dissertation. Therefore, the following section is a duplicate of this review with the addition of studies published after the date of submission.

1.2.1 History of Modifying Genes

1.2.1.1 Global Gene Modification

The concept of modifying genes has long been explored, with the first example of gene modification being demonstrated by the transformation of bacteria in the 1970s [270]. This was quickly followed by the successful transfer of genes into a number of other organisms including mammals, the first of which being a mouse in 1974 in an experiment which demonstrated the integration and persistence of simian virus 40 DNA in healthy adult mouse DNA following microinjection into the blastocyst cavity [271]. The introduction of gene modifying technology such as gene knock out by homologous recombination in Embryonic Stem (ES) cells, and more recently development and exploitation of the Cre/Lox system [272-274] has permitted the experimental investigation of the function of thousands of genes. These approaches have allowed researchers to manipulate genes in a multitude of different ways, in specific or multiple organ systems, and across the whole lifespan of the organisms.

For the understanding of mammalian systems, the species of choice has primarily been the mouse due to their genetic and genomic similarities to humans; allowing the modelling of human disease.

1.2.1.2 Modification of Genes in the Testis

Use of gene knockouts, and other genetic manipulations (e.g. ENU screens) in the mouse has identified many genes involved in testis function and male fertility. At the time of writing (June 2018), 87 knockout mouse lines are listed on the International Mouse Phenotyping Consortium (IMPC) as showing male infertility (www.impc.org). As information such as this grows and functional details emerge as to the roles of these genes, we have the opportunity to match this to patient data to define cause to instances of male infertility and testicular dysfunction. This is a golden time in the field, as the coincident development of cheap, high throughput sequencing technologies means we are now able to identify likely causal mutations in patients, and marry this information to functional studies in model species to understand the issues in play, and moving forward, to develop bespoke gene therapy systems to directly correct these aberrant genes to restore fertility to these men.

1.2.2 Gene Delivery Systems & their use Globally and in the Testis

A number of systems have been developed for the delivery of gene therapy and their success rates vary depending on many factors, not least the organ system being targeted and species being studied. These gene delivery and transfection technologies can be divided into two broad categories depending on the carrier or vector being used; (i) Viral and (ii) Non-viral vectors, each of which have different benefits and drawbacks, some of which have been highlighted in Table 1.1.

Theoretically, the ideal system to be used for gene therapy delivery in the testis would: (i) be easy to deliver, (ii) have a high efficacy/be robust enough to illicit an impact on a deficient system, (iii) have low immunogenicity so as to reduce the likelihood of initiating an immune response, (iv) be long lasting so as not to require repeated treatments (due to the increased chances of an immune response being raised on recurrent treatments), (v) be cell specific; to

only impact the target cell type (this is particularly important when targeting testicular cell types for human gene therapy in order to avoid impacting the spermatogonial stem cell population and thus preventing the generation of 'transgenic human offspring') and (vi) have minimal off target effects/toxicity to prevent a negative impact on the surrounding cell populations, both inside and outside of the testis. Consideration must also be given when determining the most appropriate delivery system, to the size of the genetic material to be transferred, the longevity of transgene expression required and the purpose for targeting the testis. For example, in some instances such as in an agricultural or in a research setting, targeting the germ cell population in the testis may be beneficial for generation of genetically modified offspring whereas for use in human gene therapy, permanently altering the germ cell population would be undesirable thus limiting the targeting capacity of the vector to either the somatic cells in the testis or to target the germ cells population temporarily using a transiently expressed transgene. Therefore, the success of a number of the current and previous applications of the gene delivery systems is/was dependent on the desired output or purpose of the study

Table 1.1 Table outlining the characteristics of each of the gene therapy delivery systems described.

Vector	Typical Delivery Method	Capacity of Inserted Transgene	Integration	Longevity of Transgene Expression	Capable of Infecting Both Dividing and Non-Dividing Cells	Immunogenecity	Transfection Rate	Ability to Modify for Cell/Organ Specificity?	Pathogenecity
Naked/Plasmid DNA	Local/ Intravenous Injection/Microinjection, Gene Gun, Hydrodynamic Gene Injection, Electroporation/ultrasonic wave	Unlimited?	No	Short term (days)	Dividing and Non Dividing Cells (though lower efficiency in non-dividing cells)	Low	Low	Yes	Low
Liposomes	Local/Intravenous Injection	Up to 20Kb?	No	Short term (days)	Dividing Cells Only	Low	Low	Yes	Low
Adenovirus	Local/Subcutaneous Injection	>8Kb	No	Short term (days)	Dividing and Non Dividing Cells	High (due to high exposure of humans to adenoviruses and thus have generated antibodies)	High	Yes	Immune/ inflammatory response likely due to hosts pre-existing antibodies
Adeno-Associated Virus	Local/Intramuscular Injection	~4.5 Kb	Dependent upon modifications to the AAV (Wild type - Yes)	Long term	Dividing and Non Dividing Cells	Low	Low?	Yes	Low
Retrovirus	Local Injection, Ex-vivo transduction of isolated cells	<8Kb	Yes	Long term (permanent)	Dividing Cells Only	Low	Moderate	Yes	High risk of insertional mutagenesis
Lentivirus	Local Injection, Ex-vivo transduction of isolated cells	<8Kb	Yes	Long term (permanent)	Dividing and Non Dividing Cells	Low	High	Yes	Some risk of insertional mutagenesis

1.2.2.1 Non-Viral Vectors for Gene Delivery

1.2.2.1.1 Naked/Plasmid DNA

Considered one of the simpler systems for gene modification/therapy, naked DNA was first used in vivo in 1990 , where the direct transfer of both RNA and DNA into mouse skeletal muscle was demonstrated [275]. This was quickly followed by several other studies demonstrating gene expression following injection of naked DNA in a number of tissues, including heart, thyroid, and liver [276]. Whilst seemingly basic in terms of sophistication, this is now one of the most prevalent methods used in gene therapy clinical trials; 17.2% of such clinical trials worldwide in 2016 used naked/plasmid DNA for genetic vaccines (in which DNA that codes for specific proteins/antigens for a pathogen are introduced into the body where the native cells synthesize these foreign proteins which then trigger an immune response), and a number of other genetic conditions, such as Duchenne muscular dystrophy (http://www.wiley.com/legacy/wileychi/genmed/clinical/).

Introduction of naked DNA and RNA into the target cell has proven difficult due to the hydrophilic nature of the DNA and the negative charge of both the DNA and the cell and nucleus membranes. To overcome this, physical methods, such as needle injection (mechanical force) [276-279], gene gun (use of pressure) [280-282], hydrodynamic gene injections [283, 284] and electroporation (application of an electrical field to cells in order to increase the permeability of the cell membrane) [285] have been developed to 'force' the molecules through the membrane and thus introduce them in to the cell's cytoplasm (Figure 1.6).

Hydrodynamic injection can be described as a large volume and high velocity injection (up to 2ml over a period of three to five seconds) of saline containing the plasmid DNA (pDNA) causing an increased local pressure resulting in an improved uptake of genetic material. This method of DNA delivery has been successful used in studies focused on gene therapy in the liver [286, 287] and in skin [288]. Following delivery of DNA using hydrodynamic injection, transgene expression has been detected in a number of major internal organs including the lung, heart, kidney, spleen and particularly in the liver [283]. Studies have also shown that by directing the delivery of the hydrodynamic injection in to the vasculature supplying the target organ, a higher transgene expression can be reached, for example by injecting into the renal

vein, higher transgene expression was reported in the kidney [289, 290]. However there have been a number of studies indicating a link between an increase in testicular pressure and spermatogenic damage in the seminiferous tubules suggesting that hydrodynamic injection of naked DNA in the testis vasculature may result in a damaged testis as opposed to a repaired one [291-293].

Efficient transfection of plasmid DNA using electroporation was first demonstrated in vivo in 1996 in both the rat liver; in which genes transferred encoding luciferase were detectable in up to 40% of liver cells 48h post transfer and up to 5% of this level at least 21 days following electroporation [294] and rat brain tumours; in which human monocyte chemoattractant protein-1 cDNA was transferred and even deemed functional (as evident from an increase in the presence of monocytes in the tumour tissue) three weeks post gene transfer [295]. Electroporation has since been used to aid chemotherapy treatments by increasing the permeability and thus the susceptibility of cells to the toxicity of lipophilic drugs and facilitating entry of plasmids expressing cytokines such as interleukin [296, 297]. More recently, this technique has been used for gene transfection in vivo in the mouse testis [298, 299] and in rat testis [300] with the goal of integration of DNA into the germ cells and transmission to progeny in which researchers were able to demonstrate successful generation of transgenic offspring in both mice; with an average of 57-62% of progeny being transgenic, and rats; which resulted in an average of 33.08% of transgenic progeny (following mating of treated males with wild type females). However, electroporation of the testis is reported to be less reliable/suitable for large animal transgenesis in the testis due to variations in testis size and scrotal thickness [301]. Attempts at optimising this system of gene transfer in goat testis were relatively unsuccessful. Using an isolated testis ex vivo, a GFP expressing plasmid was injected into the interstitial compartment then electroporated before the seminiferous tubules were separated and then cultured for up to four weeks. Despite expression of GFP being achieved in the transfected tubules, expression was unexpectedly short lived potentially due to lack of integration of the plasmid as a result of its use in circular form as opposed to linear and it is also not known whether it was germ cells or the Sertoli cells that were expressing GFP within the seminiferous tubules.

With the majority of current gene transfer research in the testes focusing on transfer to germ cells and the generation of transgenic animals, little is also known about the potential impacts of using electroporation on the supporting somatic cells of the testis and the effects

it could have both on spermatogenesis and steroidogenesis. One study has demonstrated disordered tubules, impacted spermatogenesis and an initial increase in apoptosis within testis following injection of gene/vehicle with electroporation and electroporation alone [302], indicating the requirement for further investigation into the impacts this technique could have on testis function before considering its use for gene therapy in men with already existing reproductive disorders.

Another consideration must also be that, by using this technique, regardless of injection site, the naked or plasmid DNA can be delivered to the germ cells of the testis. Whether this be intentional or accidental, human germline modification is currently banned in 29 countries [303], making the use of the technique for human gene therapy in the testis both an unethical and impractical one.

Additionally, one major disadvantage of using naked DNA molecules for gene therapy is their susceptibility to degradation via nucleases on entry into bloodstream and into the cell, reducing the targeting efficiency of the naked DNA and pDNA. To overcome this, a number of 'carriers' or vectors have been developed and modified to facilitate gene transfer to the target cells reducing nuclease mediated degradation [304, 305].

1.2.2.1.2 Liposomes

Nanoparticles, such as liposomes are commonly used non-viral vectors that can aid the delivery exogenous genetic material (whether this be DNA, RNA interference (RNAi), small interfering RNA (siRNA)) into the target cell of interest and to subsequently modify the signalling pathways of this cell. Nanoparticle gene delivery systems are known to have a low immunogenicity, high loading capacities and are relatively easy to produce.

First described in 1964, liposomes are membranous lipid vesicles which enclose an aqueous volume [306]. Generated by the formation of a complex between plasmid DNA and a lipid solution, DNA can be transferred into cells via the fusion of the liposome with a cell membrane. On fusion with the cell membrane, the genetic material is introduced into the cytoplasm and the nucleus of the cell where it is then transiently expressed (Figure 1.6). The idea of utilising liposomes for a therapeutic use was first proposed in 1971 by Gregoriadis and colleagues [307] for enzyme replacement treatment of genetic disorders impacting

enzyme production and function. This was later successfully demonstrated by Weissman et al in 1976 in which they were able to introduce enzymes via immunoglobulin-coated liposomes into lysosomes of deficient *Mustelus canis* phagocytes, demonstrating their potential as a mechanism for delivery of drugs and genes *in vivo* [308]. Just over a decade later the first protocol for the use of liposomes for DNA transfection was published [309].

Today there are a number of different modified liposome vectors including cationic liposomes [309-311], solid lipid nanoparticles (SLN) [312, 313] and reconstituted high density lipoproteins (rHDL) [314, 315]. Attempts to improve the targeting specificity of liposomes have been made by combining these lipid nanoparticles with antibodies directed to a receptor expressed by the target cell/tissue of interest. For example, Kuo & Wang (2016) were able to increase the efficiency of delivery of chemotherapeutic drugs across the blood-brain-barrier using antibody and tamoxifen conjugated nanoparticles [316]. Studies have utilised liposomes for the delivery of transgenes to the testis [317, 318]. These studies were carried out with the purpose of creating transgenic offspring via the transfection of the spermatogonial stem cells in the testis. Unfortunately, neither of these studies demonstrated a high integration of the exogenous DNA into the genome of the injected animals. Both studies confirmed expression of the plasmid (though in circular form in) in between 28% and 40% of the F1 generation and 0%-37% in the subsequent F2 generations from F1 matings [300]. Though successful, both studies demonstrate the reduced efficiency of liposomal transfection in vivo and the potential for lack of integration into the genome of the germ cells of the fathering males. For the purpose of generating transgenic offspring, non-integration is not ideal, however, for a gene therapy situation, a system that produces fertile offspring that are not transgenic could be a desirable one. In such a situation, it would be anticipated that function would only be temporarily restored and repeat treatments may be required.

1.2.2.2 Viral Vectors for Gene delivery

In addition to the use of liposomes, viruses that are able to naturally and specifically target different cell types, or that have been engineered to do so have been widely exploited in gene therapy. A number of different viral vector systems have been developed and utilised for gene therapy applications, and in 2016, over 67% of gene therapy clinical trials used viral vectors (http://www.wiley.com/legacy/wileychi/genmed/clinical/) highlighting the utility that these systems have for gene delivery.

1.2.2.2.1 Adenovirus

First isolated in 1953, adenovirus are non-enveloped viruses with an icosahedral protein shell surrounding an inner core enclosing a double stranded DNA genome [319]. Due to their large genome size and overall stability, members of the Adenoviridae family have been utilised in the development of viral vectors. Of the six species and over 50 infective serotypes, viruses Ad2 and Ad5 of species C have been exploited the most for creating viral vectors for use in gene therapy applications [320]. On entering the host cell via receptors such as the coxsackie adenovirus receptor (CAR) and integrins, the adenoviral vector then loses its protein coat before introducing the DNA cargo into the host nucleus where it can then be transcribed (Figure 1.6). However, as adenoviruses are not able to integrate the viral DNA into the host cell's genome, the DNA remains episomal in the nucleus of the host cell before eventually being lost as the cell progresses through the cell cycle. If the host cell is terminally differentiated, duration of transgene expression is still limited due to immune responses elicited by the viral transduction, resulting in clearance of the infected cells [321].

In most tissues treated with an adenoviral gene therapy vector, the transgene expression is transient. For the treatment of permanent genetic disorders this may not be a suitable, however, for treatment of conditions such as cancer and cardiac dysfunction and in principle for the treatment of dysfunctional germ cells, transient expression may be more appropriate. For example; one group, using adenovirus delivered shRNA, were able to reduce inflammatory responses and improve cardiac remodelling when examined seven days after delivery in a model of myocardial infarction in rats [322]. Modifications to adenoviral vectors have been shown to prolong the expression of the introduced transgene with one study demonstrating expression of a transgene for between two to ten months in mice following the initial injection depending on the vector modification, transgene being delivered and the tissue being targeted [323, 324]

Any expression, including transient expression, of the transgene in tissues other than the target tissue may produce unwanted side effects and thus safety issues. In a study evaluating the biodistribution of adenovirus following catheter mediated transfer to the aorta in the rabbit, a number of non-target tissues, such as the liver and the testis, were found to express the transgene as a result of leakage of the vector into the systemic circulation [325]. Some

investigation into the potential for modifying adenoviral vectors has resulted in a successful targeted approach for the in vivo delivery of tumour antigens to dendritic cells [326]. This demonstrates that specific targeting is possible and that further refinement of viral specificity to target tissues and subsequent analysis of distribution to other tissues would be beneficial for both therapeutic and research applications.

Despite mouse germ cells expressing CAR, to date there have been no reports of germ line transmission by adenovirus mediated gene transfer to mouse testis [327-329]. On the contrary, intratesticular and intratubular injection of adenovirus vector has been shown to result in a strong expression of the transgene in both Leydig and Sertoli cells respectively [330]. In terms of producing transgenic mouse lines this may be a drawback to using adenoviral vectors, however, with regards to gene therapy delivery to the testis, this can be considered a positive factor indicating that this method could be exploited, following some optimisation, for the treatment of male reproductive disorders as a consequence of somatic cell dysfunction. Delivery of genes to testicular somatic cells using adenovirus has already been demonstrated, ex vivo [331], in vitro (for the study of testicular germ cell cancer) [332, 333] and in vivo [175, 334, 335]. However, one study investigating the use of adenoviral vectors in the testis in vivo noted disturbances within the seminiferous epithelium and suggested that this may be due to a disturbance in the tight junctions that form part of the BTB as a result of the adenoviral vector binding to the coxsackie/adenovirus receptor [336]. Contrastingly, when delivered to the interstitial compartment of the testis, adenoviral vectors were able to successfully target Leydig cell and deliver both exogenous GFP and CRE recombinase transgenes. Due to the transient nature and increased immunogenicity of adenoviral vectors, reporter expression began to diminish 12 days post injection. However, as the investigators delivered CRE recombinase with the adenoviral vectors, which altered host DNA within 3 days of adenoviral injection, longevity of transgene expression was not required.

It has been suggested that the transduction efficiency of adenoviral vectors in vivo could be impacted by lipid levels and lipoproteins in the host system. Using mice maintained on a high fat diet, Kivelä *et al* [337], demonstrated a reduced transduction efficiency in mice. This was attributed to specific lipoproteins competing with the viral vector uptake via lipoprotein receptors indicating that adenoviral gene therapy may be less efficient in patients with high cholesterol levels. The implications this could have for adenoviral treatment in the testis, in

which both lipoproteins and cholesterol are essential for steroidogenesis, are yet to be defined.

Due to the high exposure of humans to adenoviruses in the form of common colds, respiratory illnesses such as pneumonia, gastroenteritis and conjunctivitis, the majority of adults have already generated antibodies against the virus which may also limit the effectiveness of adenoviral vector mediated gene therapy, particularly in treatments which may require a second dose. Additional studies investigating the possibility for engineering adenovirus serotypes by chemically modifying the viral capsid, generation of chimeric adenoviral serotypes and genome modification could allow the generation of tailored adenoviral vectors with distinct features specific to their intended purpose, whether that be for gene therapy or for DNA vaccines [338-340]

1.2.2.2.2 Adeno-Associated Virus

First believed to be a contaminant in adenoviral preparations, Adeno-associated viruses (AAV) were first utilised as DNA cloning vectors in 1984 in which the viral capsid genes were substituted for a neomycin resistance gene before being used to transduce this foreign DNA into human and murine tissue culture cells [341]. By binding to heparin or other primary receptors on the host cell surface and subsequently to co-receptors such as integrin and human fibroblast growth factor receptors, AAV is then internalised by endocytosis, gaining entry into the host cell (Figure 1.6). Due to low pathogenicity and replication defectiveness, AAV have been gaining popularity for use as gene therapy delivery systems.

Wild type AAV retains the ability to integrate into the host cell DNA, making AAV a more permanent strategy for combating genetic disorders. In the past decade, human clinical trials using AAV delivered gene therapy have shown promise with some improvement noted in each of the treated patients diagnosed with inherited loss of vision [342]. More recently, researchers were able to rescue bisretinoid accumulation and photoreceptor degeneration for one year following subretinal injections of AAV expressing the gene for a complement negative-regulatory protein in a mouse model of Stargardt macular degeneration [343]. The integration of the viral DNA into the host genome was able to rescue the phenotype via modulation of the complement system, potentially giving clues for treatment strategy for pathologies associated with complement dysregulation and for developing combination gene

therapies in cases where replacement gene therapy may require additional treatment to prevent further degeneration.

In the testis, AAV has been utilised in the investigation of testicular autoimmunity. In this study, AAV encoding human IL-10 was delivered via intra-muscular injection to mice immunised by unilateral testicular injury. This suppressed the incidence of orchitis and disturbance of spermatogenesis by regulating the cell-mediated immunity in the testis [344]. Expression levels of the human IL-10 peaked 3 weeks following the injection to fourfold the initial expression level.

As with the previous vectors, attempts have been made to target the germ line using AAV with conflicting results. In one study, despite detection of the AAV delivered transgene in the gonads of some injected animals, PCR of isolated sperm DNA and investigation of the progeny of these animals revealed no germ line transmission of the transgene [345]. In contrast, a later study using mouse germ cells harvested from donor testis, transduced with AAV vectors carrying a GFP transgene and then reintroduced to a germ cell depleted recipient testis, resulted in the transmission of the GFP to the germ cells. It is reported that this group favoured transduction of the germ cells in vitro due to the uptake of the virus by Sertoli cells when introduced to the testis in vivo, as reported in studies using retroviral and adenoviral vectors. This study resulted in the transgene being detected in 10% of the F1 offspring of the recipient mouse and 33% detection of the transgene in the resultant F2 offspring (when two heterozygous transgenic F1 offspring were mated together). This percentage of offspring is relatively low compared with the expected rates of transgene transmission from heterozygous parents and this was attributed to the potential episomal location of the transgene in a proportion of the transgenic F1 pups [346].

More recently, a study has suggested the potential for different adeno-associated viral serotypes to target Leydig cells [347]. It was found that at higher concentrations ($5x10^{12}$ TU/ml), adeno-associated serotypes 1 and 9 could infect Leydig cells as well as Sertoli cells, peritubular myoid cells and spermatogonia – though when spermatogonia were targeted, the AAV vectors did not stably integrate into the spermatogonial genome. The same group were also able to demonstrate rescue of an infertility phenotype and lack of germ line transmission to the resultant offspring, providing a promising argument for the use of AAV as a vector for gene therapy [348]

One disadvantage of using AAV is the limitation of the transgene size they are able to accommodate. One way in which researchers are attempting to overcome this is to use a number of AAV vectors expressing overlapping gene fragments with the hope of reconstitution upon transduction of the host cell [349]. Despite being successful, it is unknown whether this approach may be as efficient in other tissue and to deliver other genes. Indeed, a recent review concluded that the use of dual vector systems may not be a "one-fits-all system" and that the tissue being targeted could drastically impact the success of their use [350].

1.2.2.2.3 Retrovirus

Retroviruses are a family of enveloped viruses that encode a single stranded RNA alongside a gene for reverse transcriptase and thus are able to transcribe their own viral genetic material into double stranded DNA upon infection of a host cell via membrane fusion through viral attachment proteins binding to their receptors; resulting in irreversible binding of the virus to the host cell. Following delivery of the viral genome into the host cell, viral DNA is then incorporated into the host DNA, allowing the host cell machinery to produce any necessary viral component for replication [321] (Figure 1.6). As a result of this stable integration in to the host cell DNA, any modifications made by the retroviral DNA will thus be present in any daughter cells upon cell division. Members of this family include a number of pathogens such as HIV-1, murine leukaemia (MLV) and some cancer causing viruses for example *Rous sarcoma virus*.

Despite the association with these pathological conditions, the use of retroviral vectors has been key to the development of gene delivery techniques for therapeutic use in the past three decades. In fact, it was this family of vectors that were utilised in the first ex vivo gene therapy trial [351, 352]. For this trial, a subset of T cells were removed from a patient suffering from adenosine deaminase severe combined immunodeficiency (ADA SCID). These cells were then treated with a gammaretrovirus expressing the ADA gene before being reintroduced into the patient's circulation. This procedure was a relative success as the treated T cells were able to reconstitute the patient's immune system up to 4 years later however, production of ADA did not increase [351, 352]. This clinical trial involved two

patients and results varied in each of the subjects. This is potentially due to transfection rate of the retroviral vector being considerably lower in one of the patients T cells.

Gammaretroviral vectors, as demonstrated in this first clinical trial, are able to stably integrate into the genome of the host cell, which results in a long term expression of the transgene. However, a consequence of this integration is an increased risk of insertional mutagenesis. Insertional mutagenesis is caused by the insertion of additional DNA bases into the host cells genome that disrupts local gene function near the site of integration. In the most severe cases, this can accelerate tumour development due to the insertion of the transgene close to endogenous oncogenes, resulting in activation and/or overexpression of the oncogene. This was first realised following clinical trials carried out in France [353-356] and the UK [357] in X linked severe compromised immune deficiency (SCID) patients following treatments with retroviral gene therapy. Almost three years post-completion of the study, four out of nine patients presented with T cell leukaemia following treatment with the retroviral vector. Retrospectively, it is now known that gammaretroviral vectors are more likely to integrate in undesirable places within the host cell DNA and this results in a high probability of negative insertional mutagenesis outcomes, such as leukaemia. One potential strategy researchers are investigating is the use of chromatin insulators which may able to block the interaction between an integrating vector and the target cell genome, however, more research is needed to determine the impacts of this approach on viral titre and transductions efficiencies and indeed, its impact on genotoxicity, before this could be utilised in clinical trials [358, 359].

Initial studies of retroviral vector transduction in the testis detected expression of the virus in Leydig cells following intraperitoneal inoculation with murine leukaemia virus at birth [360]. Retroviral vectors have also been utilised in the testis with the objective of creating transgenic offspring. This was first demonstrated in 2003 by Nagano and colleagues [361], who achieved successful transduction of spermatogonial stem cells in vitro before injecting these transduced cells into recipient testis (that were depleted of germ cells via cryptorchidism or with busulfan) [361]. However, no mating experiments were carried out during this study. Similar results have later been achieved in the mouse using both in vitro [362] and in vivo [363] methods. In these experiments, descendants of the recipient/injected male expressed the inserted transgene demonstrating integration of the transgene by retroviral transduction. However, efficiency of the targeting in the in vivo experiments was

low; producing an average of just 2.8% of subsequent offspring carrying the transgene. Studies using larger mammals, such as sheep, have demonstrated a very low transduction rate of germ cells and a higher transduction rate of somatic cells when injecting retroviral vectors into foetal sheep in-utero indicating a risk of transducing germ cells in foetal gene therapy treatments [364]. Later studies have demonstrated, however, that this risk is reduced when treatment is performed later in gestation [365].

1.2.2.2.4 Lentivirus

Lentiviral gene therapy vectors have been developed from lentiviruses; a member of the family *Retroviridae*. First utilised as a vector in 1996, where the envelope of a HIV virus was replaced with the envelope glycoprotein of vesicular stomatitis virus (VSV), resulting in a stable, high titre virus with the ability to infect and integrate into a wide range of cellular targets [366]. Consequently, lentiviral vectors are a particularly attractive gene delivery system to both increase knowledge and understanding of biological systems and for gene therapeutics in a clinical setting. The utility of this system was initially demonstrated by stable in vivo gene transfer into terminally differentiated neurons in the rat brain [367].

To minimise the risk of generating lentiviral vectors capable of replication in the host cells, second, third and fourth generation lentiviral vectors have been engineered. The development of these subsequent generations involved the removal of accessory genes not required for integration (to reduce the likelihood of a replication competent lentivirus), elimination of the transactivator gene, splitting the viral plasmid into three to further reduce recombination potential and finally splitting the genes necessary for transgene expression over the separate plasmids to further prevent replication upon viral generation [368]. The design of such replication incompetent lentiviral vectors has subsequently allowed for an increase in the size of the carried transgene (up to 10kb) in comparison to limitations of transgene size with gammaretroviral and AAV vectors (up to 5kb).

As with gammaretroviral vectors, another concern with the use of lentiviral vectors as a vector for gene therapy is the risk of insertional mutagenesis due to its ability to integrate into the host cell DNA. However, unlike other retroviral vectors, which have been shown to preferentially integrate in or around the promoter region of a gene, lentiviral vectors integrate further along the transcribed region of the gene resulting in a lower chance of

stimulating the promoter via enhancer effects and as a consequence, reducing the oncogenic potential caused by insertional mutagenesis [369].

A number of modifications have been made to both retroviral and lentiviral vectors to enhance the expression of the delivered transgene one of which being the addition of elements known to increase gene expression post transcriptionally, one of the first being the posttranscriptional regulatory element (PRE) present in hepatitis B virus (HPRE) [370]. The posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) (a close relative of HPRE) into the 3' untranslated region of coding sequences in both gammaretroviral and lentiviral vectors was found to be a more active element than HPRE and thus has been used to even further enhance the efficiency of retroviral and lentiviral vectors [371, 372]. However, a caveat of introducing this element comes with an increased oncogenic risk due to the presence of a promoter for the woodchuck hepatitis virus X protein and the sequence for the first 60 amino acids of this X protein within the WPRE fragment that has been incorporated into both retroviral and lentiviral vectors [373]. This X protein, particularly the truncated form, has been linked to increases in cellular proliferation in human hepatocellular cancers in which there is a high frequency of hepatitis B virus sequences and has also been shown to act as a co-factor for oncogenesis [374-376]. Though speculative, this raises concerns as to the safety of the use of unmodified PREs in gene therapy vectors. To overcome this, PRE mutants have been generated that lack the X protein open reading frames without significant loss of lentivirus titre and transgene expression (as demonstrated in a number of studies [45, 377-380]) thus minimising the potential harmful effects of the PRE [381]

The first ex vivo clinical trial to utilise lentiviral vectors failed to detect any adverse events eight years post treatment in 65 patients treated with lentiviral transduced CD4 T cells with the purpose of providing viral load control on HIV-2 positive subjects [382, 383]. More recently, another clinical trial investigating a dose escalation of Prosavin; a potential lentiviral vector based in vivo gene therapy for Parkinson's disease, was found safe and well tolerated by patients alongside a significant improvement in motor behaviour symptoms usually experienced by patients with Parkinson's disease [384]. However, despite the seemingly lower risk/benefit of lentiviral vectors, the potential for insertional mutagenesis is still present, therefore genome wide analysis of integration frequency and pattern of lentiviral vector systems (among other integrating vector systems) is still required following their use.

One way in which researchers are attempting to further prevent insertional mutagenesis is with the generation of integrase deficient lentiviral vectors. These lentiviral vectors have an inserted mutation in the lentiviral integrase gene resulting in the integration of the viral DNA being driven by transient double strand sites in the host genome [385-388]. Consequently there is a lower frequency of random integration of the viral vector DNA reducing chances of any negative off-target effects such as oncogenesis. However, whether these vectors are capable of sustaining vector DNA expression in the host genome, like their integrase competent counterparts, is yet to be fully determined, with studies indicating that their use may be more suited for transduction in non-dividing cells due to the transient expression observed in cells undergoing division and proliferation [385-388].

Attempts have also been made to target the germline using lentiviral vectors. Hamra and colleagues were the first to demonstrate transduction of rat germ cells ex vivo with a lentiviral vector [389]. Following transfer of the transduced germ cells into a recipient wild-type testis and mating with a wild type female, pups expressing the lentiviral transgene were produced, with this transgene being transferred to the next generation indicating a stable, integrated expression. Similar results have also been found in mice [390] and in larger mammals, for example, in pig and bovine testicular gonocytes [391-393]. This technique, though promising for the production of transgenic animals with the purpose of research, or possibly agriculture, would be more difficult to transfer to a clinical setting, for the ethical reasons mentioned earlier.

Transduction of germ cells using lentivirus has also been attempted in vivo with varying degrees of success. The first study describing the delivery of lentiviral vectors into the testis in vivo suggested transduction of spermatogenic as well as Sertoli and Leydig cells. Following this, similar results were also demonstrated resulting in the production of transgenic offspring after injection of lentiviral vectors into either the interstitial or the tubular compartments of the testis; thus targeting the spermatogonial stem cells in vivo [394-396]. However, the expression of the transgene in offspring seems to vary depending on the promoter driving lentiviral vector gene expression. This was addressed in a study in which the ability of lentiviruses expressing GFP driven by two different promoters to target spermatogonial stem cells (and thus potentially generate transgenic offspring) were compared [394, 395]. Lentiviral delivered GFP expression under the CMV (cytomegalovirus) promoter to the testis has resulted in production of GFP expressing offspring visible under

fluorescent lamp and this was further confirmed with both immunohistochemistry and genotyping results. When injected with lentivirus with EF1 (human elongation factor 1) promoter driving eGFP expression, despite eGFP being present in offspring genomic DNA, transgene expression was not detectable by fluorescent microscopy or by RT-PCR due to the silencing of the transgene via DNA methylation [394, 395]. Using either promoter, using lentiviral injections into the testis for the production of transgenic offspring resulted in overall success rates of over 60% in both studies [394, 395]. In contrast, earlier study found that, following injection of lentivirus expressing PICK1 into the testis, overall transgenic efficiency of 1.3% but also that using this method had a high preference to generate testis specific transgenic mice [396].

There are also a number of studies, conducted with the aim of targeting the somatic cells in the testis for gene therapy, that claim there is no risk of germ line transduction using in vivo injection of lentiviral vectors. In an early study by Ikawa and colleagues, spermatogenesis of infertile mice was restored by injection of a lentiviral vector expressing the transmembrane form of the c-kit ligand, targeting the Sertoli cells [397]. Though demonstrating the potential of lentiviral vectors for the treatment of male reproductive disorders impacting fertility, this study initiated the investigation of germ line transmission of lentiviral vector DNA just days following lentiviral vector injection and, therefore, neglecting to investigate the transduction of the spermatogonial stem cells present in the testis at the time of injection. Transduction of these cells would potentially result in transduced, mature spermatozoa after one round of spermatogenesis (approximately 35 days in the mouse), indicating that germ line transmission could be possible using this technique.

Recently, a study utilising a doxycycline inducible short hairpin RNA system expressed by lentiviral vectors was utilised to demonstrate the novel target; urokinase-type plasminogen activator (uPA) for the regulation of infertility [398]. Using this system researchers were able to reduce pregnancy rate by approximately 40%. This would suggest that targeting of the lentiviral vector was not highly efficient, though researchers did not provide information on cell type being targeted and efficiency of targeting. Interestingly, the researchers were able to demonstrate recovery of infertility following withdrawal of doxycycline. The further refinement of this inducible lentiviral delivered technology could prove beneficial for the study of genes essential for male fertility as well as for methods of male contraception.

Evidence of targeting Sertoli cells using lentiviral vectors has recently been demonstrated both ex vivo [399] and in vivo [45]. These studies were both able to demonstrate, following delivery directly to the seminiferous tubules, that stable expression of lentiviral vector DNA was specifically in the Sertoli cells (and not in the Germ cells) and the resultant tubule disruption as a consequence of disruption of essential genes in targeted cells. In conjunction with this, a study has also been able to demonstrate the transduction of Leydig cells in vivo using lentiviral vectors [400]. In this study, overexpression of 50 kDa protein activating transcription factor 6 in the Leydig cells using lentiviral vectors disrupted production of key steroidogenic enzymes in the testis. This indicates the potential of this technology for targeting gene therapy in the Sertoli and Leydig cells in vivo for use in both research and also for the treatment of male reproductive disorders in the clinic, following further due diligence investigation into their potential for transmission to the germ line.

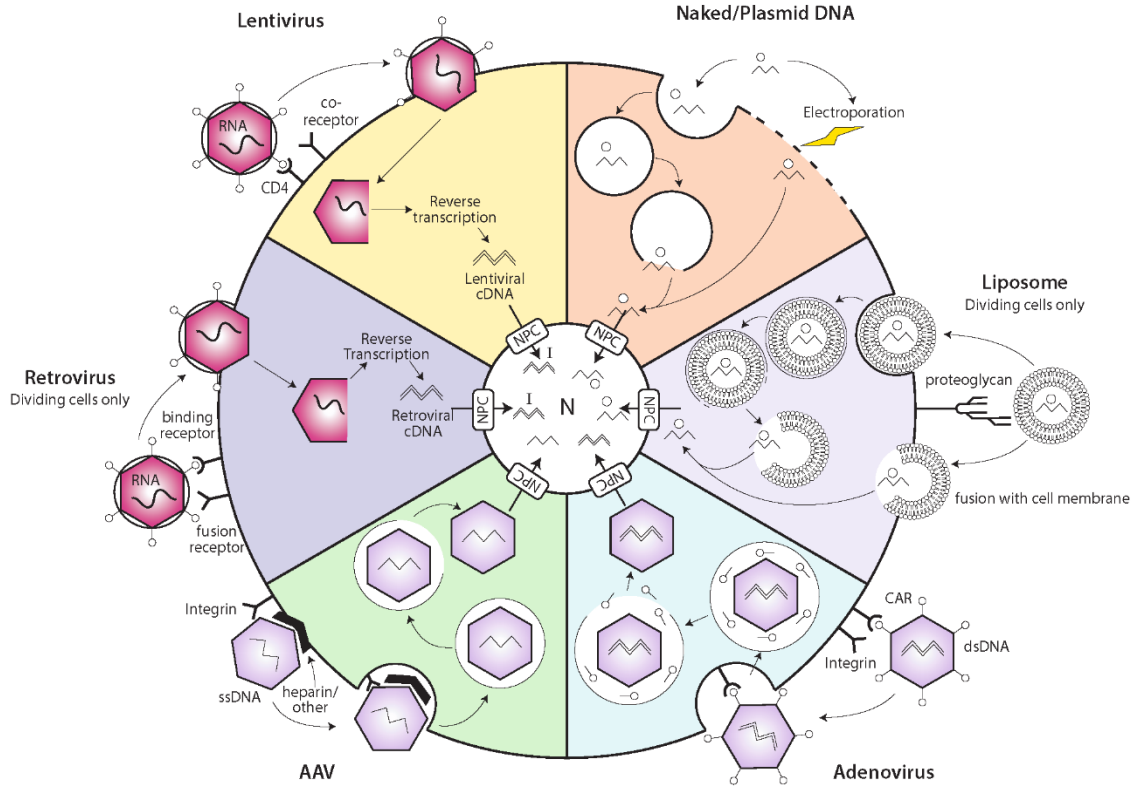


Figure 1.6 Schematic overview of the different methods of cell entry for each of the vector systems used for gene therapy delivery. **Naked/Plasmid DNA** - due to the negative charge of both the DNA and the Cell membranes, physical methods such as electroporation have been developed to introduce the DNA to the target cells cytoplasm. Naked or Plasmid DNA can also enter by endocytosis and on reaching the nuclear membrane, can be transported into the nucleus (N) via the nuclear pore complex (NPC) where it is then transcribed. **Liposome** – Liposomes, following anchorage to the cell via proteoglycans, can also enter via endocytosis and also via fusion with the target cell membrane. On entry to the cell cytoplasm, the liposome breaks down and is able to release the DNA contents into the cell cytoplasm before being transported into the nucleus for transcription. **Adenovirus** – On binding of the viral fibres to the coxsackie adenovirus receptor (CAR) on the target cell surface, the Adenovirus is transported into the cell membrane via clathrin mediated endocytosis, during which the fibres disassociate from the viral capsid. Following release of the endosome contents into the cell cytoplasm, the adenovirus loses its protein coat before introducing the DNA into the host nucleus via docking at the NPC. Here the capsid can be completely disassembled and viral genome can be transported into the nucleus where it can then be transcribed. **Adeno-Associated Virus (AAV)** – Following binding of the AAV to heparin or other primary receptors, as well as co-receptors such as integrin on the target cell membrane, the AAV is internalised into the cytoplasm via endocytosis.

Following endosomal trafficking the AAV viral capsid escapes from the late endosome and is uncoated before translocation to the NPC. On entry into the nucleus, the viral genome is converted from a single stranded (ssDNA) to a double stranded (ds) genome and integrates into the host genome. **Retrovirus & Lentivirus** – Upon binding to binding and fusion receptors on the cell surface, the viral envelope fuses with the cell membrane. From here the viral capsid can enter the cell cytoplasm where it is uncoated, allowing the single strands of viral RNA to be converted into double stranded DNA by the viruses own reverse transcriptase enzyme. Following intracellular trafficking, the viral DNA is transported into the nucleus where it can then permanently integrate into the host cell DNA using an integrase enzyme, permitting the transcription of the viral genome. For retroviral vectors, this process can only occur in cells undergoing mitosis. However, lentiviral vectors have no dependence on the target cell being in cell cycle and can thus also infect non-dividing calls.

1.2.2.3 Other Approaches

The apparent challenges and risks associated with the use of viral vectors for gene therapy and the low targeting efficiency of non-viral vectors such as liposomes warrants the development of alternative vectors and gene delivery systems. Around 10% of gene therapy clinical trials in 2016 were undertaken using alternative methods of gene therapy delivery (http://www.wiley.com/legacy/wileychi/genmed/clinical/). One of these alternative methods of gene delivery was the use of bacteria to deliver DNA to host cells, known as bactofection mediated gene therapy. Bactofection occurs, in the case of the most commonly used attenuated *S. typhimurium,* when the carrier bacteria invades the host cell, escapes the vacuole into the cytosol where it then lyses, releasing the carrier plasmid. The first indication that this would be possible was in 1980 with the delivery of cloned genes to mammalian cells in vitro [401] and has since been developed for use in DNA vaccines [402-404], gene therapy (in particular, for cancer treatment and targeting immune cells within cancerous tumours [405-407]) and for the production of therapeutic proteins (also known as alternative gene therapy) in which the host genome is not affected [408, 409].

However, despite the use of bacteria for gene delivery having a number of advantages; one being the ability of the bacteria to transfer a large cargo to the host cell and another being the ability to trigger and stop the transference/expression of the bacterial delivered DNA using the appropriate antibiotics, thus increasing the safety for the use of this vector in a clinical setting, the use of bacteria in gene therapy clinical trials still remains relatively low.

This may be due to the lower transfection efficiency of the bacteria in vivo alongside the lack of knowledge regarding the differences in animal immunoresponse and the toxicity of these vectors, with a number of differences in results from studies focussed on rodent vs human tissues and cells. To our knowledge, this approach has not yet been used for successful gene delivery in the testis.

Another way in which bacterial components have been applied to gene transference is the use of bacterial magnetic particles (BMP). BMPS are created by organelles called magnetosomes in magnetotactic bacteria believed to aid the bacteria in reaching regions of optimum oxygen concentration. When used in combination with other gene delivery techniques, they have also been shown to improve targeting efficiency of vectors upon application of a magnetic field. This technique has been utilised in the testis in which BMPs were combined with nanoparticle vectors and delivered to the testis in vivo with the goal of producing transgenic mice [410]. Results suggested an increased efficiency of the BMP delivered DNA for the production of transgenic mice in comparison to liposome only delivered DNA, indicating that a combination of the numerous vectors available may be beneficial for future application of DNA delivery techniques.

1.2.2.4 Applications of Gene Editing Technology in Male Reproductive Health – Future for Gene Therapy in the Testis

Alongside the development of a wide range of vectors for DNA delivery, there has also been a number of key developments in gene editing technology making the prospect of gene editing based therapies an increasingly likely concept. The three most common nucleases used for gene editing are zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) – associated nuclease Cas9 (CRISPR/Cas9), which all consist of engineered DNA binding domains and are capable of producing double stranded breaks (DSB) in the target/host DNA.

ZFN utilise zinc finger modules (of around 30 amino acids in size) as DNA binding domains which recognise a specific 3bp sequence in the target DNA and, following module binding to the target DNA, the FokI nuclease mediates DNA cleavage. TALENs also use the FokI nuclease, however, the customisable DNA binding domain contains 34-35 amino acids (derived from transcription activator like effectors) per module and can relate to individual bases in the target DNA sequence. Unfortunately, both techniques require the complicated engineering

of a protein which is able to recognise the specific DNA sequence that is being targeted making the process complicated and relatively expensive.

More recently, the development and exploitation of the CRISPR/Cas9 system has revived the gene editing prospects advancing the rate at which gene editing therapies could be developed for clinical use. The CRISPR/Cas9 system is a bacterial antiviral immune system modified for genome editing. Using small guide RNAs, which bind to a complementary sequence of the target DNA, a set of nucleases (Cas9) is guided to cleave the target DNA specifically at the target site. The system provides the rapid development of gene editing therapies due to the simplicity of the manufacturing process, and for this reason amongst others they are currently the most common gene editing tool in non-clinical development [411].

Among the first studies using CRISPR/Cas9 technology to correct a genetic disorder in vivo were Wu and colleagues, who, by injecting Cas9 mRNA and guide RNA into zygotes of a mouse model of cataracts, were able to rescue the mutation in the affected gene, a correction which was also transmitted to the next generation through the germline [412]. Further studies also demonstrated the ability of the CRISPR/Cas9 system to both correct genetic mutations and introduce humanised mutations in adult organs in vivo, as well as for a number of different disease models including blood disorders, muscular dystrophy and liver disease [413-418].

Gene editing nucleases have been used for gene editing experiments in spermatogonial stem cells, using ZFNs [419], TALENS [420] and CRISPR/Cas9 [420-422] nucleases, providing models for studying spermatogenesis, for generating targeted germ line mutations and potentially for the prevention of genetic disorders passed through the germ line. Due to the controversy of germ cell alteration in humans, whether, when combined with delivery vectors, these gene editing systems could be applicable to a clinical gene therapy needs to be further examined. To date, there has been no application of this technology for the manipulation of the somatic cells in the testis. The use of these technologies for the identification of factors impacting testis function and fertility is paramount for increasing understanding and thus affording the opportunity to apply this knowledge for the benefit of men impacted by male reproductive disorders including hypogonadism and infertility.

One of the risks of using any of these nucleases for gene editing is that, with a systemic gene therapy protocol, multiple organs could be targeted with the same gene editing technology. Using cell-specific delivery vectors, it is possible for gene editing nucleases and their components to be targeted to a specific cell type, or to be delivered separately to increase the safety of their use [423] (Figure 1.7). This technique has already been demonstrated with CRISPR/Cas9 in combination with AAV vectors. In one study, a modified Cas9 ortholog was delivered intramuscularly and shown to be capable of correcting disruptive mutations and partially recover functional deficiencies in a mouse model of Duchenne muscular dystrophy [424].

Depending on the requirement of the gene editing by the patient, the delivery vector should be chosen giving particular attention to potential off target effects of multiple organ delivery of both the nuclease and the vector itself. Therefore, future applications of both gene editing technologies such as CRISPR/Cas9 and vector systems should tailor the strategy used to the application required paying particular attention to the potential for off-target toxicity from both of these powerful biotechnological tools.

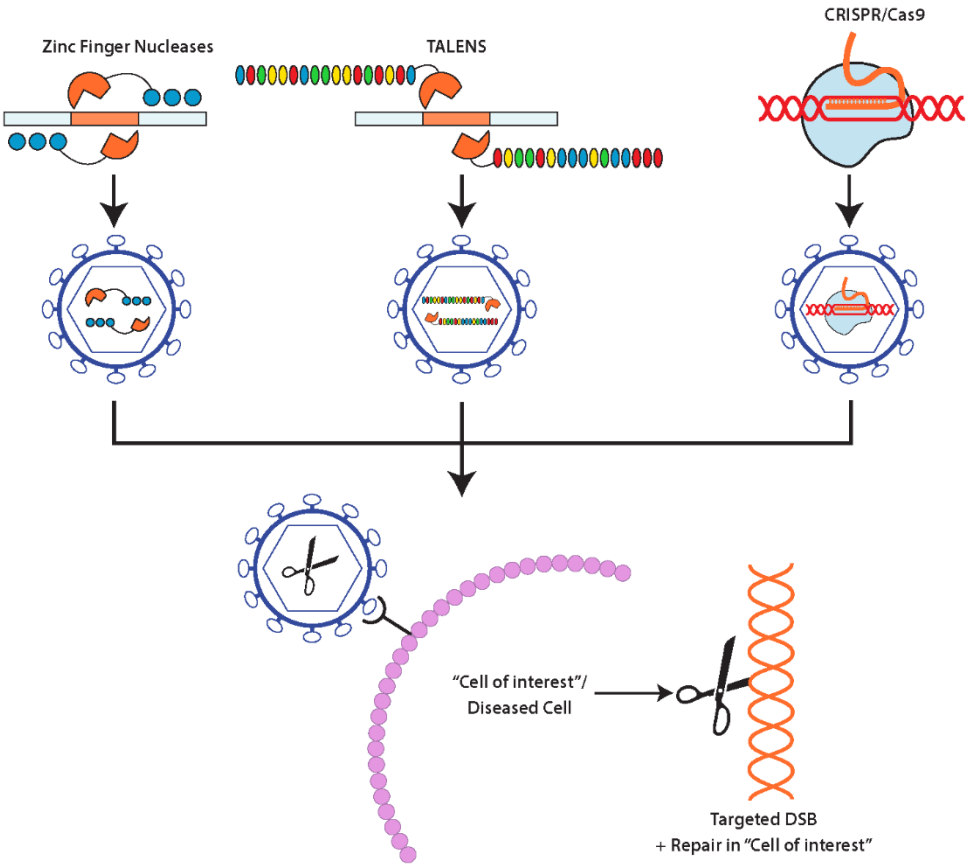


Figure 1.7 Schematic representation of the combinatory use of cell specific delivery vectors - including lentiviral vectors, and gene editing nucleases. This would allow the targeted DSB and/or repair in a cell of interest or for use as a therapy in a diseased cell.

1.3 Hypothesis, Aims & Approach

1.3.1 Hypothesis

The overarching hypothesis of the experiments described is that **the optimisation of viral vector delivery into the testis will permit targeting the adult testicular somatic cells *in vivo,* providing a tool from which (i) further insights into testicular function can be determined and (ii) the development of therapeutic techniques to treat male reproductive disorders could be established.**

1.3.2 Aims

1. To establish and validate viral vector delivery and targeting of the adult testicular somatic cells.

2. To generate a model of testicular dysfunction in which the potential of the optimised viral vector techniques could be determined and which could be also utilised to gain further insight into androgen signalling in the adult testis.

3. To determine the potential of viral vectors to rescue the phenotype described in an adult model of testicular dysfunction.

1.3.3 Approach

To optimise and validate viral vector targeting in the adult testis, vehicle (PBS or serum free media), dye, adenoviral and lentiviral vectors were injected into the interstitial and tubular compartments of the testis using different volumes, locations and injection pressures dependant on the cell type being targeted. Testis were then collected and analysed at different time points post injection to determine the most appropriate methodology for future attempts at gene delivery/editing.

Using Cre/loxP technology, novel models of double testicular somatic cell specific ARKOs were generated for repair with viral vector delivered transgenes. The adult testicular phenotype of the model was also characterised in the context of single testicular somatic cell

specific ARKOs to determine the synergistic and additive AR mediated relationships between the testicular somatic cells.

To confirm the practical uses of viral vector delivery to the testis, lentiviral vectors carrying mouse AR were delivered to adult SC-ARKO testis *in vivo*. Rescue of AR expression and testicular function was analysed to establish the potential of lentiviral vectors to rescue a phenotype, giving insight into their potential for use in therapeutics for male reproductive disorders

Chapter 2 Materials & Methods

2.1 Animals

2.1.1 Welfare

Mice were bred and maintained in the Biomedical Research Facility based at Little France, University of Edinburgh on a 12hr light/dark cycle with temperature and humidity maintained at between 20°C and 25°C and at 55% respectively. Mice were screened for pathogens and certified clean prior to their use in experiments. Soya-free chow and drinking water were continually available. All procedures were carried out in accordance with UK Home Office regulations under project licences 60/4200 and 70/8804 held by Professor Lee B. Smith and were compliant with the with the Animals (Scientific Procedures) Act, 1986. Daily animal husbandry, technical assistance and timed matings were performed by Mr. Michael Dodds (Senior Animal Technician for the Central Biomedical Research Facility at the University of Edinburgh).

2.1.2 Timed Matings

When animals at specific embryonic ages were required, timed matings were set up in which one male and one female were paired in the afternoon. At 8am on the following morning, females were examined for the presence of a post copulatory vaginal plug with the expectation that mice mate in the middle of their dark period. As a result of this, presence of a post-copulatory vaginal plug would allow the calculation of the gestation stage with the first morning being designated embryonic day (e) 0.5.

2.1.3 Transgenic Lines Used

Transgenic animals used in this study were generated using the Cre-loxP system. This system utilises the Cre recombinase enzyme, a 38-kDa protein originally derived from the P1 bacteriophage [273, 425]. The Cre protein is able to efficiently promote recombination at specific 34bp sites called loxP sites. On recognition of these loxP sites, the recombinase catalyses site specific recombination, leading to precise deletion of the DNA flanked by the loxP sites (said to be the ‘floxed’ region of DNA). As the mammalian genome is lacking in high affinity loxP sites, this has made Cre-loxP technology an invaluable source for gene editing, whether this be insertion, deletion or modification, in mammalian research.

To enable cell or tissue specific DNA modifications, the Cre recombinase enzyme must be expressed in the cell/tissue type of interest. To do so, the exogenous Cre recombinase gene must be placed downstream of a cell/tissue specific promoter, limiting gene expression and subsequent DNA modification to this cell type. Using the same methodology, the Cre recombinase enzyme can also be expressed at specific time points in a life span, depending on when the promoter is expressed; although it is important to note that upon a cells expression of the Cre recombinase gene, daughter cells deriving from the cell will also bear the consequential phenotype.

2.1.3.1 Tomato Red Cre Reporter Mice

Mice ubiquitously expressing a loxP flanked (floxed) stop cassette-controlled gene for red fluorescent protein (RFP) tdTomato (Gt(Rosa)26Sor$^{tm14(CAG-tdTomato)Hze}$) [426] were obtained from a colony maintained onsite by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh) gratefully received from Professor Neil Henderson (MRC Centre for Inflammation Research, University of Edinburgh). Animals were genotyped as later described in section 2.2.

2.1.3.2 Yellow Fluorescent Protein Reporter Mice

Mice expressing a loxP flanked (floxed) stop cassette-controlled gene for yellow fluorescent protein (YFP) under the ubiquitously expressed ROSA26 locus were used for tracing Cre expression in the testis. These mice were obtained from a colony maintained onsite by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh) [427]. Animals were genotyped as later described in section 2.2.

2.1.3.3 Complete Androgen Receptor Knock Out Mice

For ubiquitous ablation of Androgen Receptor (AR), female mice heterozygous for the X-linked hypoxanthine phosphoribosyltransferase-Cre transgene were mated to male mice bearing a floxed AR allele to produce females carrying one deleted allele and one wild-type (WT) allele of the X-linked AR gene. These females were subsequently mated to produce ARKO males and control littermates [428]. Archived tissues were used in these studies. Breeding of these animals and collections were carried out by Dr Michelle Welsh (Centre for Reproductive Health, University of Edinburgh)

2.1.3.4 Conditional Androgen Receptor Knock Out Mice

To selectively ablate expression of AR from single and/or multiple cell types in the testis, a number of different Cre expressing mouse lines were used. For each line, a Male mouse, expressing Cre recombinase in a specific cell type was mated with a female mouse homozygous for a floxed Androgen Receptor to ensure every male offspring produced from the cross will inherit an X chromosome bearing a floxed Androgen receptor allele (as androgen receptor is located on the X chromosome, male offspring only carry one copy of the androgen receptor allele).

For ablation of AR expression in Sertoli cells, a mouse line in which Cre recombinase was expressed under the control of the Anti-Müllerian Hormone (*Amh*) promoter was used. This mouse line (Tg(AMH-Cre)^{1Flor} was obtained from a colony maintained on site by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh) [48, 429].

To generate mice with AR ablation in Sertoli and/or peritubular Myoid cells, male mice heterozygous for smMHC-eGFP-Cre were mated with female mice heterozygous for Tg(AMH-Cre)^{1Flor} to generate offspring that expressed Cre recombinase under both promoters. Male mice positive for both Cre recombinases were then mated to a female mouse homozygous for a floxed Androgen Receptor. These matings gave rise to double positive (expressing both Cre recombinases), Amh-Cre positive (and MH-Cre negative), MH-Cre positive (and Amh-Cre negative) and wild type (expressing no Cre recombinases) offspring. The offspring derived from these matings are the animals used for the analysis performed in these studies. Breeding of these animals and collection of tissue was carried out by Mr. Martin O'Neill (Centre for Reproductive Health, University of Edinburgh).

For ablation of AR expression in Leydig cells, a mouse line in which Cre recombinase was expressed under the control of the Fatty acid binding protein 4 (Fabp4 or Ap2) promoter was used. This mouse line was obtained from a colony maintained on site by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh) [108, 430]. Breeding of these animals and collection of tissue was carried out by Dr Laura O'Hara (Centre for Reproductive Health, University of Edinburgh).

For ablation of AR expression in both Sertoli and Leydig cells, mice expressing Cre under the Cyp19/Aromatase promoter were used. This mouse line was obtained from Dr Sophie

Fouchécourt (INRA, UMR85 Physiologie de la Reproduction et des Comportements, F-37380, Nouzilly, France) [17].

All animals were genotyped as described in section 2.2.

2.2 Genotyping of Animals

To correctly identify animals inheriting genes for both the Cre and the floxed alleles, ear clips taken for identification at weaning (around d21) were retained for genomic DNA extraction and subsequent polymerase chain reaction (PCR) assays. All routine genotyping (unless stated otherwise) was carried out by the technical support available in the team of Professor Lee Smith.

2.2.1 Genomic DNA Extraction

For genomic DNA extraction, ear clips taken at weaning and/or tail tips taken at dissection were individually submerged and digested with 2 µl Proteinase K added to 25µl TE-Tween supplemented and incubated for one hour at 55°C on a TProfessional Thermocycler (Biometra GmbH, Germany). Samples were incubated for a further seven minutes at 97°C to denature any remaining Proteinase K in the solution and then cooled to 25°C. Samples were then vortexed and briefly centrifuged at low speed. The resulting supernatant was then diluted at 1:10 with sterile water for future downstream PCR assays.

2.2.2 PCR & Determination of Genotype Using QIAxcel System

PCR reactions were performed using either Biomix PCR reaction buffer (Bioline Reagents Ltd, UK) or Type-It Mutation Detect PCR kit (QIAGEN Ltd, UK) according to manufacturer's instructions. Forward and reverse primer sequences, annealing temperatures and buffers used for specific assays are listed in **Table 2.1**. PCR cycles were executed on a TProfessional Thermocycler (Biometra GmbH, Germany) and resulting PCR products were analysed using the QIAxcel capillary electrophoresis system (QIAGEN Ltd, UK). Positive, negative and no template controls were included in each PCR reaction to confirm validity of results and to ensure reagents were free from contaminating nucleic acids.

Table 2.1 Details of Primers used for Genotyping Assays.

Gene	Forward Primer	Reverse Primer	Annealing Temp (˚C)	Product Size
Amh-Cre	CACATCAGGCCCAG CTCTAT	GTGTACAGGATCG GCTCTGC	59	239bp
smMHC-GFP-Cre/GFP	GACGTAAACGGCC ACAAGTT	GGTCTTGTAGTTGC CGTCGT	59	264bp
Aromatase-iCre	CCTGGAAGATGCTC CTGTCTG	AGGGTGTTGTAGG CAATGCC	65	400bp
Interleukin 2	CTAGGCCACAGAAT TGAAAGATCT	GTAGGTGGAAATTC TAGCATCATCC	-	330bp

2.3 Surgery, Injections and Treatments

2.3.1 Lentiviral Vectors

2.3.1.1 Production of Lentiviral Vectors

To ensure safety of lentiviral vectors and their production, individual components required for the production of the lentiviral structure were split across multiple plasmids. In brief, shuttle vectors were packaged with a third-generation lentiviral vector plasmid pseudotyped for VSV-G, produced at a viral titre of >1x10^9. Information regarding lentiviral vectors used in these studies, including details of viral titre and specific viral components can be found in Table **2.2**. All Lentiviral vectors were produced by Dr Pamela Brown (Head of Biomolecular Core for the Shared University Research Facilities core (SuRF) at the University of Edinburgh).

Table 2.2 Details of Viral Vectors used.

Viral Vector	Nomenclature	Lentivirus Description	Source	Titre
LV-cppt-CMV-eGFP-opre	LV-CMV-emGFP	VSVG pseudotyped Lentiviral vector expressing enhanced GFP under the CMV promoter	Biomolecular Core, SuRF, University of Edinburgh	1×10^8
LV-cppt-EF1α-eGFP-f-puro	LV-EF1α-emGFP	VSVG pseudotyped Lentiviral vector expressing enhanced GFP under the EF1α promoter	Biomolecular Core, SuRF, University of Edinburgh	5.5×10^7
LV-cppt-CMV-mKate2-negmiRx2	LV-CMV-mKate2-negmiR	VSVG pseudotyped Lentiviral vector expressing mKate2 and a scrambled miR under the CMV promoter	Biomolecular Core, SuRF, University of Edinburgh	1.5×10^9
Ad-CMV-eGFP	Ad-eGFP	Human adenovirus (serotype5, E1/E3) expressing enhanced GFP under the CMV promoter	Vector Biolabs, USA	1×10^{10}
Ad-CMV-Cre-IRES-eGFP	Ad-Cre-eGFP	Human adenovirus (serotype5, E1/E3) expressing Cre recombinase and enhanced GFP (separated with an IRES component) under the CMV promoter	Vector Biolabs, USA	1×10^{10}
LV-cppt-CMV-P2A-moeGFP-opre	LV-moeGFP	VSVG pseudotyped Lentiviral vector expressing monomeric enhanced GFP downstream of the CMV promoter and a P2A cleavage site	Biomolecular Core, SuRF, University of Edinburgh	1.8×10^{10}
LV-cppt-CMV-mAR-P2A-moeGFP-opre	LV-mAR-moeGFP	VSVG pseudotyped Lentiviral vector expressing mouse androgen receptor and a monomeric enhanced GFP downstream of the CMV promoter and separated with a P2A cleavage site	Biomolecular Core, SuRF, University of Edinburgh	5.1×10^9

2.3.2 Surgical Procedure for Interstitial Injections

Lentiviral and Adenoviral vectors (Vector Biolabs, USA) and vehicle controls were injected into the interstitial compartment of adult testis. The procedure was performed under anaesthesia by inhalation of isoflurane and under aseptic conditions. Testis were exposed through an incision in the scrotum before between 10ul and 20ul of Lenti/Adeno-virus + 0.4% Trypan Blue (ThermoFisher Scientific, United States) (for visualisation of viral injection) was injected just under the tunica albuginea into the interstitial compartment using a 0.3mL 25g insulin needle (BD Bioscience U.K Ltd, United Kingdom). In some cases, animals underwent orchidectomy to remove one uninjected testis. Exposed testis were then replaced back into the scrotal compartment before the abdominal incision was closed with sterile sutures. Mice were injected subcutaneously with Vetergesic (active ingredient: Buprenorphine) 0.05mg/kg (Ceva Animal Health Ltd, United Kingdom), whilst anaesthetised and allowed to recover whilst being monitored. Mice were then closely monitored over the next 24 hours for any welfare problems, and twice daily from then onwards. Initial lentiviral vector injections were performed by Dr. Cornelia Roesl (Centre for Reproductive Health, University of Edinburgh).

2.3.3 Surgical Procedure for Rete Injections

Lentiviral vectors (or vehicle controls) were introduced into the rete compartment of the adult testis. As with the interstitial injections, the procedure was performed under anaesthesia by inhalation of isoflurane and under aseptic conditions. Testis were exposed through an incision in the abdominal cavity. Adipose tissue was carefully removed from the areas surrounding the rete testis, efferent duct and vasculature before between 10ul and 20ul of Lentivirus + 0.4% Trypan Blue (ThermoFisher Scientific, United States) (for visualisation of viral injection) was injected into the rete testis using a glass micropipette (outer diameter: 80µm (bevelled) (Biomedical Instruments, Germany) and a microinjector (Eppendorf FemtoJet; Eppendorf, Germany). Exposed testis were then replaced back into the scrotal compartment before the abdominal incision was closed with sterile sutures. Mice were injected subcutaneously with Vetergesic (active ingredient: Buprenorphine) 0.05mg/kg (Ceva Animal Health Ltd, United Kingdom), whilst anaesthetised and allowed to recover whilst being monitored. Mice were then closely monitored over the next 24 hours for any welfare problems, and twice daily from then onwards.

2.4 Tissue Collections & Imaging

2.4.1 Culling of Animals

Animals were sacrificed using as per conditions outlined in the Animals (Scientific Procedures) Act 1986; by exposure to carbon dioxide gas in a rising concentration. Foetal, larval and embryonic animals were decapitated following the sacrifice of the pregnant dam.

2.4.2 Measurement of Gross Anatomy

Following sacrifice and blood collection (detailed in 2.3.3), gross body weight of animals was measured using a digital balance. In some cases, anogenital distance was also recorded using digital callipers. Animals were observed for any gross abnormalities, in particular, presence of nipples and whether testis were located within the scrotum (for male animals).

2.4.3 Blood Collection & Dissection

Immediately following sacrifice, blood was collected via cardiac puncture using a 23G needle (BD Bioscience U.K Ltd, United Kingdom) and 1mL syringe (BD Bioscience U.K Ltd, United Kingdom) coated with heparin (LEO Laboratories Ltd., United Kingdom) prior to use. Blood samples were kept on ice before centrifugation at 17136g for 10 minutes at 4°C in order to harvest the plasma. Plasma was then stored at -80°C until required for analysis.

Both testis, epidimides and seminal vesicles were removed, cleared of excess adipose tissue and weight recorded. In some circumstances, extra gonadal tissues were collected including the pituitary, brain, adrenal, kidney, liver and spleen.

2.4.4 Imaging of Organs Post Dissection

Following removal from the cadaver, organs to be imaged were placed on ice in cold phosphate buffered saline (PBS) (detailed in 2.9.2). Organs were imaged in cold PBS using a Leica MZ-FLIII fluorescence stereomicroscope (Leica Microsystems, Germany) prior to either fixation or snap freezing (both of which are detailed in 2.4.5).

2.4.5 Tissue Processing

For histological analysis of tissues, organs were fixed in Modified Bouins Solution (Clin-Tech Ltd, United Kingdom). Testis (and other organs collected to be fixed) were submerged in Bouins solution for around six hours (applying a rule of around one hour of fixation time per millimetre of tissue). After the first four hours of fixation, testis were cut in half along the transverse plane and re-submerged in the Bouins solution to allow adequate fixative

penetration into the centre of the testis. Following fixation, samples were transferred to 70% ethanol (EtOH) (VWR International Ltd, United States) until required for further processing and embedding. Samples for embedding underwent an automated tissue processing procedure using the Leica ASP300 S Tissue Processor (Leica Biosystems, Germany) in which the samples were dehydrated in a series of EtOH solutions of increasing concentration, cleared with xylene and then infiltrated with histological wax (Shandon™ Paraffin; Thermo Scientific, United Kingdom). Once infiltrated with the wax, the samples were embedded into molten wax to form blocks and stored at room temperature until required for sectioning. All tissue processing and embedding was performed by Mr Garry Menzies (Technical Officer for Shared University Research Facilities core (SuRF) at the University of Edinburgh).

2.5 Analysis of Tissue Histology

2.5.1 Tissue Sectioning

Tissues embedded in paraffin blocks were cut into 5um sections using a Leica rotary microtome (Leica RM2125RT) and mounted onto positively charged microscope slides (Leica Biosystems, UK). Slides were then incubated in a drying oven, maintained at 55°C, overnight (for around 16 hours). Following dehydration, slides were stored at room temperature for use in downstream applications.

2.5.2 Dewaxing and Rehydration

In order to utilise tissues for downstream procedures detailed in sections 2.5.3 and 2.5.4, sections must be free of histological wax to allow penetration of aqueous solutions. To do so, slides were submerged in Xylene for the removal of the paraffin wax before being passed through a series of decreasing EtOH concentrations ending with tap water to both displace the Xylene solution and to rehydrate tissue sections.

2.5.3 Haematoxylin & Eosin Staining

To examine cellular components of tissue sections, haematoxylin and eosin stains were used to distinguish between nuclear and cytoplasmic structures. Following dewaxing and rehydration, slides were immersed in Harris' Haematoxylin solution (Leica Biosystems, UK) to stain nuclei (negatively charged cellular components, e.g. nucleic acids found in the

nucleus) for up to 5 minutes. After washing any residual haematoxylin stain, slides were briefly placed in 1% acid-alcohol (Leica Biosystems, UK) to further remove excess stain from tissues and to define nucleic staining. To increase contrast of haematoxylin staining by converting staining colour from purple to blue, slides were then immersed in Scotts tap water solution (Cell Path, UK). For staining of cytoplasm (positively charged cellular components, e.g. amino groups in proteins within a cells cytoplasm), slides were immersed in Eosin Y solution (Leica Biosystems, UK). Residual stain was washed from slides in tap water before being passed through a series of increasing EtOH concentrations to dehydrate tissue and cleared of residual alcohol with submersion in Xylene. Slides were then mounted with glass coverslips (Leica Biosystems, UK) using a non-aqueous mounting medium, Pertex (Cell Path, UK) and left to dry within a fume cupboard.

2.5.4 Immunohistochemistry

Immunohistochemistry (IHC) methods were applied to confirm and visualise both presence and localisation of specific proteins in fixed tissue samples. Depending on downstream experiments required from the tissue (for example, whether the stained sections were to be used for imaging or for stereology/cell counts), different methods of protein revelation were applied. For simple detection and for tissue/proteins requiring stereology, chromogenic IHC was used and for detecting more than one protein in the same section of tissue; fluorescent IHC was used. Details of antibodies, revelation, antigen retrieval and detection methods following optimisation can be found in Table 2.3. For all immunohistochemistry experiments, positive tissue and negative (in which primary antibody incubation was omitted) controls were included.

2.5.4.1 Antigen retrieval

During tissue fixing, antigenic sites on proteins are masked as a consequence of cross linking proteins. To reveal these antigenic sites and thus enable the detection of proteins, antigen retrieval is often a required step in IHC. For the majority of antibodies used in this thesis, heat induced epitope retrieval were used (unless stated otherwise in Table 2.3). This consisted of incubating slides (following dewaxing and dehydration) in either pH6 citrate buffer or pH9 Tris-EDTA buffer within a pressure cooker (InstantPot, Canada) for 5 minutes before cooling in water.

2.5.4.2 Labelling of Samples with Antibodies

A number of steps were included in the immunohistochemistry protocol to prevent non-specific immunostaining of tissue sections. Slides were subjected to an incubation of 0.03% hydrogen peroxidase (Fisher Scientific, UK) in TBS for 30 minutes at room temperature to quench any activity of endogenous peroxidases. Following this, slides were rinsed with water and sections were incubated with normal serum blocking solution for 30 minutes in a humidity chamber at room temperature (species of serum was the same species in which the secondary antibody was raised). This prevented the binding of the primary antibody to any non-specific epitopes.

Tissue sections were then incubated with the primary antibody (diluted in the normal serum blocking solution) in a humidity chamber at 4°C overnight. Following all subsequent incubations with antibodies and reagents slides were washed in TBS for three 5 minute incubations.

2.5.4.3 Chromogenic detection

After removal of the primary antibody with the TBS washes, either a biotin-conjugated secondary antibody (species dependant on primary antibody – see Table 2.3) diluted in normal serum blocking solution or with ImmPRESS™ peroxidase polymer detection reagent (one drop per section) (Vector Labs, USA) were then added to tissue sections for 30 minutes in a humidity chamber at room temperature. If the ImmPRESS™ peroxidase polymer detection reagent was not being used, tissue sections were then incubated with streptavidin-peroxidase conjugate (SA-5004, Vector Labs, USA) diluted in TBS for a further 30 minutes in a humidity chamber at room temperature. The chromogen used for visualisation was ImmPACT DAB peroxidase substrate (Vector Labs, USA); a DAB (3, 3 -diaminobenzidine) horse radish peroxidase substrate, according to manufacturer's instructions. Development of the dark brown colour reaction was monitored and timed on control slides and reaction time of experimental slides was based on these controls. Reaction and colour development was stopped by immersing slides in tap water. Slides were then rinsed again with tap water before counterstaining with Harris haematoxylin, dehydrated, cleared and mounted (as earlier in 2.5.3, omitting staining with Eosin Y solution).

2.5.4.4 Fluorescent detection

For fluorescent detection of proteins, primary antibody was removed as with chromogenic detection before the addition of the appropriate peroxidase conjugated secondary antibody (diluted in normal serum blocking solution) (Table 2.3) to tissue sections for 30 minutes in a humidity chamber at room temperature. Tor visualisation of proteins, tissue sections were then incubated with Tyramide Signal Amplification (TSA) working solution (PerkinElmer, UK) for five minutes in a humidity chamber at room temperature; as per manufacturer's instructions.

This system was also utilised to visualise multiple proteins in the same tissue section to aid investigation into co-localisation of proteins, for example. To do so, following detection of the first primary antibody, tissue sections were subjected to further antigen retrieval, blocking of endogenous peroxidases and non-specific epitopes to prevent background staining and overnight incubation with a different primary antibody as described in preceding sections (2.4.5.1 and 2.4.5.2). Detection of subsequent primary antibodies was performed using the TSA system, ensuring different fluorophores to those used for previous proteins were being used to distinguish detected proteins from one another.

Following detection of all proteins, tissue sections were incubated with either SYTOX™ nucleic acid stain (Life Technologies, UK) or DAPI nucleic acid stain (Sigma-Aldrich, USA) diluted in TBS for 10 minutes in a humidity chamber at room temperature. Slides were then mounted with glass coverslips (Leica Biosystems, UK) using an aqueous mounting medium; Lab Vision™ PermaFluor™ (ThermoFisher Scientific, UK) and stored in the dark at 4°C until required for imaging and analysis.

Table 2.3 Details of antibodies used for immunohistochemistry.

Target	Antigen Retrieval	Primary Antibody			Secondary Antibody				Detection Method
		Supplier	Catalogue Number	Dilution Used	Reagent	Supplier	Catalogue Number	Dilution Used	
3-Beta-Hydroxysteroid Dehydrogenase (HSD3β)	Citrate pH6	Santa Cruz Biotechnology Inc, USA	sc30820	1/2000	Chicken Anti- Goat IgG-HRP	Santa Cruz Biotechnology Inc, USA	sc-2961	1/200	Tyramide
Androgen Receptor (AR)	Tris-EDTA pH9	Spring Bioscience Corporation, USA	M4070	1/400	ImmPRESS™ HRP Anti-Rabbit IgG	Vector Labs, USA	MP-7401	n/a	DAB
Cleaved Caspase 3	Citrate pH6	Cell Signalling Technology (New England Biolabs),	9661S	1/500	Chicken Anti- Goat IgG-HRP	Santa Cruz Biotechnology Inc, USA	sc-2961	1/200	Tyramide
Cytochrome P450 17A1 (Cyp17a1)	Citrate pH6	Santa Cruz Biotechnology Inc, USA	sc-46081	1/1000	Chicken Anti- Goat IgG-HRP	Santa Cruz Biotechnology Inc, USA	sc-2962	1/200	Tyramide
Green Fluorescent Protein (enhanced) (eGFP)	Citrate pH6	Abcam, UK	ab6556	1/4000	Goat Anti Rabbit IgG-HRP	Vector Labs, USA	PI-1000	1/200	Tyramide
Green Fluorescent Protein (monomeric enhanced) (moeGFP)	Tris-EDTA pH9	Abcam, UK	ab6556	1/3500	Goat Anti Rabbit IgG-HRP	Vector Labs, USA	PI-1000	1/200	Tyramide
Insulin-like 3 (Insl3)	Citrate pH6	Santa Cruz Biotechnology Inc, USA	s-134587	1/500	Biotinylated Goat Anti-Rabbit IgG	Vector Labs, USA	BA-1000	1/500	DAB
Galectin-3/Mac2	Citrate pH6	Cedarlane Labs, Canada	CL8942AP	1/2000	ImmPRESS™ HRP Anti-Rabbit IgG	Vector Labs, USA	MP-7401	n/a	Tyramide
Phosphoglycerate Kinase 1 and 2 (PGK1/2)	Tris-EDTA pH9	Santa Cruz Biotechnology Inc, USA	sc-28784	1/300	ImmPRESS™ HRP Anti-Rabbit IgG	Vector Labs, USA	MP-7402	n/a	DAB
Red Fluorescent Protein (RFP)	None	Evrogen, Russia	AB233	1/1000	Biotinylated Goat Anti-Rabbit IgG	Vector Labs, USA	BA-1000	1/500	DAB
Red Fluorescent Protein (RFP)	None	Evrogen, Russia	AB233	1/4000	Goat Anti Rabbit IgG-HRP	Vector Labs, USA	PI-1000	1/200	Tyramide
SRY-Box 9 (Sox9)	Citrate pH6	Merck Millipore, USA	AB5535	1/500	Biotinylated Goat Anti-Rabbit IgG	Vector Labs, USA	BA-1000	1/500	DAB

2.5.5 Microscopy and Image Capture

Imaging of Haematoxylin & Eosin and chromogenic (DAB) stained tissue sections was performed on an Olympus Provis AX70 microscope (Olympus, Japan) and/or an Axio Scan Z.1 SlideScanner (Carl Zeiss Ltd, UK). Fluorescent stained sections were imaged using an LSM 710 confocal microscope (Carl Zeiss Ltd, UK). All microscope/imaging systems were used with Zen imaging software (Carl Zeiss Ltd, UK).

2.5.6 Stereological Analysis

Cell counts and histological measurements were determined using Image-Pro Plus 7.0 software (Media Cybernetics, UK) with a Zeiss Axio Imager A1 Microscope (Carl Zeiss, UK), Qimaging QICAM Fast 1394 digital camera (Qimaging, Canada) and a Prior ProScan automated stage (Prior Scientific Instruments Ltd, UK). For all measurements, a tiled image of the entire testis section was captured and from this an area of interest was manually highlighted. Within this area of interest, up to 200 random fields of view were generated for analysis at higher magnification.

For cell counts, this magnification was at 60x resulting in a counting grid of 18x24 points being overlaid the random field of view. Using the 'count' function, the points within the nuclei and the cytoplasm of either HSDβ3 (for Leydig cell counts) and SOX9 (for Sertoli cell counts) were counted. From this the percentage of the random view recorded as cell nuclei over the total number of points counted was calculated using the following equation (noted as % Cell Nuclei for downstream calculations):

$$\%\ \text{Cell Nuclei} = \left(\frac{\text{Total Number of Nuclei Counted}}{\text{Total Number of Points in Counting Grid (18x24)}}\right) \text{x } 100$$

These measurements, along with the testis weight noted at dissection, were then used to calculate the absolute volume of the cell within the testis using the following formula:

$$\text{Absolute Volume of Cell Nuclei (mg)} = \frac{(\%\ \text{Cell Nuclei}) \text{ x Testis Weight (mg)}}{100}$$

Within the same field, the mean nuclear volume of the cell type of interest was also measured using the 'nucleator' function included with the software. Here, the centre of the nucleus of the cell of interest is selected. This generates three random lines intersecting the centre of the nucleus. The points in which each of these lines cross the nuclear membrane were selected, generating measurements of the nuclei radius; this allowing the nuclear volume to be calculated.

The total number of cells (either Leydig cells or Sertoli cells) within a testis was then calculated using the absolute volume of the cell nuclei and the mean nuclear volume of the cell type with the following formula:

$$Total\ Number\ of\ Cells = \left(\frac{Absolute\ Volume\ of\ Cell\ Nuclei\ (mg)}{Mean\ Nuclear\ Volume\ (\mu m^3)}\right) x100$$

For seminiferous tubule and lumen diameters, analysis magnification was at 10x and the 'nucleator' function was utilised in the same manner as with mean nuclear volume measurements (generating measurements from the centre of the tubules as opposed to the centre of the nuclei). Only whole, round, tubules visible in the random field of view were measured.

For analysis of dye distribution following interstitial injection of Biomark Tissue Marker Dye (Biostain Ready Reagents Ltd, UK). Tissue Marker Dye injected testis sections were images using an Axio Scan Z.1 slide scanner (Carl Zeiss Ltd, UK) with Zen imaging software (Carl Zeiss Ltd, UK). Percentage of coverage by the Tissue Marker Dye was quantified using Image J software (National Institutes of Health, USA) using the instructions taken from Image J; "Quantifying Stained Liver Tissue" (https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html) and applied to the Tissue Marker Dye testis sections. In brief, images were split into red, green and blue channels and threshold was manually adjusted until Tissue Marker Dye was highlighted in red (threshold was adjusted according to the blue channel given that the blue channel exhibited the best separation of tissue from dye in testis sections). The whole section was then selected and termed the "area of interest" before measurements were set. The fraction of area with Tissue Marker Dye detected was then

calculated within the limit of the previously set threshold, producing a percentage of area occupied by Tissue Marker Dye. Uninjected testis were also included as negative controls.

For analysis of testis injected at different pressures, haematoxylin and eosin stained sections of testis injected at a range of pressures (20hPA-50hPA) were analysed subjectively for the presence of vacuoles, distended tubules and sloughing of germ cells. Each of these features were graded separately on a scale as follows; 0=feature not observed in testis section, 1=feature observe d on one occasion in testis section, 2= feature observed in a small number of tubules, 3= feature observed in an estimated quarter of tubules, 4= feature observed in estimated half of tubules, 5= all/majority of tubules in testis section have feature present. Grades were then added together to give a total score out of 15. Scores were averaged from each of the testis in each pressure group.

2.6 Hormone Analysis

Luteinising hormone (LH) quantification in mouse plasma was performed using the MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel (RPTMAG-86K; Merk Millipore, USA) according to the manufacturer's instructions. Assays were read on a Bio-Plex 200 suspension array system using Bio-Plex Manager software (Bio-Rad Laboratories Ltd, UK). Assays for LH and FSH were carried out by Dr Laura Milne at the University of Edinburgh.

Quantification of testosterone in mouse plasma was carried out using a competitive Enzyme Linked Immunosorbent assay (ELISA). A competitive ELISA differs from a direct or sandwich ELISA as they involve a step which mixes the sample with another reagent prior to its addition to the ELISA plate. First 96 well ELISA plates were coated with Donkey Anti Rabbit serum at 4°C overnight. 0.5% bovine serum albumin phosphate buffer (BSA PBS) was then added to prevent any non-specific binding of proteins to the ELISA plate. Samples (and standards) were then mixed with testosterone conjugated with Horse Radish Peroxidase (HRP) (at a dilution of 1 in 20,000) (Astra Biotech, Germany) in 0.1% BSA PBS and Assay buffer and added to the ELISA plate and mixed using a plate shaker. Anti-Rabbit Anti-Testosterone Antibody was then added at a dilution of 1 in 200,000 in 0.1% BSA PBS Assay Buffer. The reactions were then incubated for two hours at 28°C on a shaker. After removal of any remaining unbound antibody with washing steps, reactions were incubated with tetramethylbenzidine for 10 minutes. Any wells containing testosterone bound HRP will boast a colour change from clear to blue due to the enzymatic action of HRP on tetramethylbenzidine. This reaction was

stopped with the addition of 1N sulphuric acid; changing the reaction colour from blue to yellow in the process. The wells were then read with a Multiskan EX plate reader (Thermo Scientific, US) with a filter of 450nm. Samples containing high levels of testosterone will contain less testosterone conjugated with HRP bound to the Donkey Anti Rabbit Serum coating the plate compared to samples with low levels of testosterone. Consequently, a reduction in the levels of HRP enzyme will result in a reduced conversion of the clear tetramethylbenzidine to a blue substrate. Therefore, testosterone concentration in samples was inversely related to the colour change measured with a Multiskan EX plate reader (Thermo Scientific, US). Testosterone assays were carried out by Dr Forbes Howie (Head of Specialised Assay Service, Shared University Research Facilities, University of Edinburgh).

2.7 mRNA Expression Analysis

2.7.1 RNA Extraction and Quantification

Before extraction, up to 30mg of testis was collected and weighed from a whole snap frozen testis. To this, a proportionate amount of luciferase RNA (5ng/testis; Promega, UK) was added for use as an external reference gene before being homogenised with a ball bearing using a TissueLyser LT tissue disrupter (QIAGEN, UK). RNA was extracted and purified from the lysate using the RNeasy Mini Kit (QIAGEN Ltd, UK) following manufacturer's instructions. The optional on column DNase treatment step was included to minimise genomic DNA contamination. Extracted RNA was analysed for both quality and quantity using a NanodropOne spectrophotometer (ThermoFisher Scientific, UK) with a 260:280 absorbance ratio of around 2.0 being considered as pure RNA. Extracted RNA was stored in RNase free tubes at -80°C until required for further downstream applications.

2.7.2 cDNA Synthesis

cDNA was reverse transcribed from up to 2µg of the extracted RNA described in 2.5.6.1 using the SuperScript® VILO™ cDNA synthesis kit (ThermoFisher Scientific, UK) according to manufacturer's instructions. A reaction free of the reverse transcriptase enzyme was also included as a control (-RT control) to ensure reagents were free of nucleic acid contamination. All reactions were performed on a TProfessional Thermocycler (Biometra GmbH, Germany) and resulting cDNA was stored at -20°C.

2.7.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To analyse and quantify expression of specific mRNA within samples, qRT-PCR was performed using the Universal Probe Library (UPL; Roche Diagnostics Ltd, UK) on cDNA (2.5.6.2). The UPL is based on short hydrolysis probes labelled with fluorescein (FAM) at the 5' end and with a dark quencher dye at the 3' end. These probes are capable of detecting 8- and 9-mer motifs that prevalent in the relevant organism's transcriptomes (for example, there are 90 probes configured for the Mouse transcriptome). Assays were designed using the ProbeFinder Assay Design Software (Roche Diagnostics Ltd, UK) in which the design software suggests a number of suitable PCR primers and UPL probes specific to the gene of interest. Where possible, primers spanning introns were chosen to avoid amplification of contaminating genomic DNA (as cDNA is synthesised from mRNA, it does not contain sequences derived from introns, unlike genomic DNA which consists of sequences from both introns and exons).

During the qRT-PCR reaction, the short UPL probe sequence is complementary to the one of the strands of the amplicon. During each annealing stage of the PCR cycles, the probe binds to the amplicon and as the polymerase extends from primer to the amplicon, it displaces the fluorescein (at the 5' end) from the quencher (at the 3'end), resulting in an increase in fluorescence emitted.

Theoretically, PCR doubles the number of molecules or strands with each amplification cycle; amplifying DNA exponentially. The exponential phase of the PCR cycle, when reagents are in excess, the DNA polymerase is still highly efficient and when final DNA product is still relatively low so as not to compete with the primers annealing capability, is when the threshold cycle (C_t) is set. At this point, the fluorescent signal being emitted from the PCR reaction is distinguishable from background signal. As the C_t value is the point at which the fluorescent signal crosses the threshold from background to relevant and significant signal, the C_t value is inversely related to the amount of amplicon present in a reaction; the greater the expression of mRNA in the cDNA sample, the lower the C_t value.

qRT-PCR reactions were set up in triplicate (technical replicates of each sample) using the Applied Biosytems™ Taqman™ Universal PCR Master Mix (Applied Biosystems, Life Technologies, UK) according to manufacturer's instructions and were run on the ABI 7900HT

Real-Time PCR system with SDS 2.4 software (Applied Biosystems, Life Technologies, UK). No template and reverse transcriptase negative controls were included to ensure reactions were free of contamination. The external Luciferase control gene was detected using a probe labelled with NED at the 5' end (as opposed to FAM with the UPL probe).

Details of assays used for this thesis, including primer sequences, UPL probe number and details of the Luciferase external control assay can be found in **Table 2.4**.

Table 2.4 Details of primers and probes used in qRT-PCR assays.

Gene	Forward Primer	Reverse Primer	UPL Probe
Aquaporin-8 (Aqp8)	CCATTCTCCATTGGCTTCTC	CAGGTCCAAAGGCACGAG	50
Desert Hedgehog (Dhh)	CACGTATCGGTCAAAGCTGAT	GTAGTTCCCTCAGCCCCTTC	75
Desmin	GCCACCTACCGGAAGCTACT	GCAGAGAAGGTCTGGATAGGAA	15
Endothelin-1 (ET-1)	CTGCTGTTCGTGACTTTCCA	AGCTCCGGTGCTGAGTTC	50
Smooth Muscle Actin (SMA)	ACTCTCTTCCAGCCATCTTTCA	ATAGGTGGTTTCGTGGATGC	58
HMG-CoA reductase (HMGCR1)	TGCGTAAGCGCAGTTCCT	TTGTAGCCTCACAGTCCTTGG	19
Steroidogenic Acute Regulatory Protein (StAR)	TTGGGCATACTCAACAACCA	ACTTCGTCCCCGTTCTCC	11
Cytochrome P450 11a1 (Cyp11a1)	AAGTATGGCCCCATTTACAGG	TGGGGTCCACGATGTAAACT	104
3 beta-hydroxysteroid dehydrogenase 1 (HSD3B1)	GAACTGCAGGAGGTCAGAGC	GCACTGGGCATCCAGAAT	12
Cytochrome P450 17a1 (Cyp17a1)	CATCCCACACAAGGCTAACA	CAGTGCCCAGAGATTGATGA	67
17 beta-hydroxysteroid dehydrogenase 3 (HSD17B3)	AATATGTCACGATCGGAGCTG	GAAGGGATCCGGTTCAGAAT	5
Insulin-like 3 (Insl3)	AAGAAGCCCCATCATGACCT	TTTATTTAGACTTTTTGGGACACAGG	10
3 beta-hydroxysteroid dehydrogenase 6 (HSD3B6)	ACCATCCTTCCACAGTTCTAGC	ACAGTGACCCTGGAGATGGT	95
Luciferase	GCACATATCGAGGTGAACATCAC	GCCAACCGAACGGACATTT	NED Probe

2.7.3.1 qRT-PCR Assay Optimisation and Validation

With the assumption that a 100% efficient PCR reaction is doubling the amplicon with each cycle, assays were tested for efficiency to ensure C_t values were suitable for quantitative analysis.

For these studies, assays with an efficiency between 90 and 110% were considered acceptable. Assays were validated for efficiency using a standard curve in which a serial dilution of cDNA (from tissue known to express the gene of interest) was added to reactions and resulting C_t values were plotted against the log dilution factor. The efficiency of the reaction was then calculated using the equation of the linear regression line (produced from the plotted C_t values) using the following formulas:

$$\text{Exponential Amplification(E)} = 10^{\frac{-1}{(\text{Coefficient of X})}}$$

$$\text{Efficency of Reaction}(\%) = (\text{E} - 1)\ \text{x}\ 100$$

For each gene of interest, a number of assays were designed and tested for efficiency before the most efficient was utilised for experimental tissues.

2.7.3.2 Analysis of qRT-PCR Assays

Real time PCR data was analysed using the comparative C_t method to give a quantification of mRNA expression relative to expression of a control mRNA. This method is also known as the $2^{-\Delta\Delta Ct}$ method [431].

Using this method, the C_t values of the experimental samples (for example androgen receptor knock out testis) are compared to control samples (for example, wild type testis tissue). These C_t values are first normalised to the external control gene by subtracting the C_t value of the gene of interest from that of the external control gene (ΔC_t) and then corrected to control groups to give the $\Delta\Delta C_t$ value. The fold change of the of mRNA expression relative to control mRNA expression was calculated with the following formula:

$$\text{mRNA Expression Fold Change} = 2^{-\Delta\Delta Ct}$$

2.8 Statistical Analysis

Statistical analysis of data was carried out using GraphPad Prism 7.02 (GraphPad Software Inc., USA). Data sets were tested for normality (assuming the data approximates a Gaussian distribution) using the D'Agnostino & Pearson Normality Test. A p value of ≥ 0.05 indicates derivation from the Gaussian distribution is no more that would be seen with chance alone (i.e. the data is normally distributed) and a p value of ≤ 0.05 indicates that the data set was not sampled from a Gaussian distribution.

If data was considered 'normally distributed'; it was determined to be acceptable to use parametric tests to compare data sets. For comparison of two data sets alone, an unpaired, two tailed t-test was performed. For analysis of differences of means between three or more groups, a one-way ANOVA was performed and when groups were subjected to two experimental factors, a two-way ANOVA was performed. Multiple comparisons of means were performed using post-hoc analyses; Dunnet's or Turkeys depending on whether means were compared to every other mean or to a control mean respectively.

If data was not 'normally distributed, data was either log transformed to enforce Gaussian distribution before performing parametric tests or, in cases where this was not possible, non-parametric tests were used for comparison. For comparison of two data sets alone, a Mann-Whitney U Test was used. For comparison of three or more groups, a Kruskal-Wallis test was applied with means of groups being compared using a Dunn's post hoc analysis.

For these studies, a p value of ≤ 0.05 was considered statistically significant for both parametric and non-parametric analyses.

2.9 Commonly Used Buffers and Solutions

2.9.1 Tris Buffered Saline

For one litre of a 20x stock of Tris buffered saline (TBS) (0.4M Tris, 3M NaCl, pH7.6) 48g Tris HCl, 11.2g Tris base and 176g NaCl were dissolved in 900mL of deionised water. Once at room temperature, the pH was then adjusted to pH7.6 (pH was then adjusted to 6.0 with Hydrochloric Acid (to decrease pH) or sodium hydroxide (to increase pH) and final volume adjusted to one litre using distilled water. 20x stock was kept at 4°C until required. For a working stock of TBS solution (20mM Tris, 150mM NaCl, pH7.6), 500mL of 20x TBS was added 9.5L of deionised water and then stored at room temperature.

2.9.2 Phosphate Buffered Saline

For one litre of 1x Phosphate buffered saline (PBS)(10mM Phosphate Buffer, 137mM NaCl, 2.7mM KCl, pH7.4), 5 PBS tablets (Sigma-Aldrich, UK) were dissolved in one litre of deionised water. The solution was stored at room temperature.

2.9.3 Genomic DNA Extraction

2.9.3.1 Tris Stock Solution

For one litre of Tris (1M Tris, pH8.0), 121.14g Tris base (Sigma Aldrich, UK) was dissolved in 800mL deionised water. pH was adjusted to pH8.0 with Hydrochloric Acid (to decrease pH) or sodium hydroxide (to increase pH) and final volume adjusted to one litre using distilled water. The solution was stored at room temperature.

2.9.3.2 Ethylenediaminetetraacetic Acid (EDTA)

For one litre of EDTA stock (0.5M EDTA, pH8.0), 185g EDTA (Sigma-Aldrich, UK) was dissolved in 800mL deionised water. pH was then adjusted to pH8.0 and volume adjusted to one litre with deionised water. The solution was stored at room temperature.

2.9.3.3 Tris-EDTA-Tween

For 50mL of Tris-EDTA-Tween, 100µl 0.5M EDTA (pH8.0) and 250µl TWEEN® 20 were added to 2.5mL of 1M Tris (pH8.0) before adjusting the volume to 50mL with deionised water. The solution was the filter sterilised through a 0.2µm tube top filter disc (Merk Millipore, USA) into a fresh sterile falcon tube. The solution was stored at room temperature.

2.9.4 Immunohistochemistry

2.9.4.1 Citrate Buffer for Heat Induced Citrate Retrieval

For one litre of 10x stock of 0.1M Citrate Buffer, 21.01g Citric Acid monohydrate was added to 875mL deionised water. Concentrated Sodium Hydroxide was then added until the pH reached pH5.5. Final volume was then adjusted to one litre with deionised water and pH adjusted to 6.0 with Hydrochloric Acid (to decrease pH) or sodium hydroxide (to increase pH). The solution was then stored at room temperature

2.9.4.2 Tris-EDTA for Heat Induced Citrate Retrieval

To make a 20x stock of Tris-EDTA buffer (0.2M Tris Base, 20mM EDTA solution, pH9), 1.21g of Tris and 0.37g of EDTA was added to 50mL of deionised water and mixed to dissolve. For

a working 1x stock of Tris-EDTA buffer (10mM Tris Base, 1mM EDTA solution, 0.05% TWEEN® 20, pH9), 50mL of the 20x stock and 0.5mL of TWEEN® 20 (Sigma-Aldrich, UK) was added to one litre of deionised water before mixing well. The solution was then stored at room temperature for up to three months or at 4°C for longer periods of time.

2.9.4.3 Normal Sera & Blocking Solutions

2.9.4.3.1 Normal Sera

Normal sera (Biosera, UK) was obtained through the SuRF histology facility. For the immunohistochemistry experiments used in these studies, Normal Goat (NGS), Chicken (NChS), Horse (NHS) or Donkey (NDS) were used.

2.9.4.3.2 Blocking Solutions

For 200mL of blocking solution, 2g bovine serum albumin (BSA; Sigma-Aldrich, UK) was dissolved in 140mL of TBS (2.5.8.1), 20mL of normal serum (2.5.8.4.3.1) was added before the final volume was made up to 200mL with TBS. The solution was then filtered through a 0.22µm syringe filter unit (Merk Millipore, USA) before being dispensed into aliquots of up to 15mL and stored at -20°C.

Chapter 3 Optimisation and Validation of Targeting the Testicular Somatic Cells Using Viral Vectors

3.1 Introduction

The somatic cells of the testis are essential for maintaining both the endocrine and the reproductive functions of the adult testis. In particular, the Sertoli cells have been noted to be master regulators of both of these testicular functions with studies demonstrating their requirement for spermatogenesis and to maintain the Leydig cells numbers and steroidogenic function [7-9].

It is believed that a number of male infertility cases are as a result of genetic mutations and that these genetic mutations can result in Sertoli and Leydig cell dysfunction [238, 239, 432]. However, understanding of the genetic mechanisms in both testis function and dysfunction is currently incomplete. This is, in part due to routine genetic testing of men presenting with reproductive disorders being limited to karyotyping, specific microdeletions and gene mutations (Azoospermia factor (AZF) microdeletions and Cystic fibrosis transmembrane conductance regulator (CFTR) mutations) which have already been identified as issues [433]. As a result of this, the causes for the majority of cases of male reproductive disorders remain idiopathic.

Currently, treatments available to men presenting with infertility or hypogonadism are limited to treating the symptoms as opposed to the underlying causes of disorders; using in vitro fertilisation and/or ICSI and hormone replacement therapy respectively. A number of studies have been published disputing the potential adverse effects of using androgen replacement therapy (ART) to treat the symptoms of hypogonadism as a result of the potential adverse effects on the cardiovascular system [264, 266, 434, 435]. Alongside this, there is some debate regarding the increased likelihood of producing offspring bearing a congenital defect such as anencephaly and limb abnormalities in pregnancies resulting from assisted reproductive technology in comparison to ‘natural pregnancies’ [436-438]. This may be due to the assisted reproductive technology techniques bypassing the normal checkpoints reached by fertilisation that prevent abnormal genetic replication, essentially allowing genetic abnormalities to ‘slip through the net’ [8]. However, the introduction of pre-

implantation genetic diagnosis has resulted in a steady decline in the incidence of these congenital abnormalities [439].

The diagnosis and treatment of both male infertility and hypogonadism could be improved by targeting the causative mutations underlying the condition. There are currently over 2500 gene therapy clinical trials worldwide (http://www.abedia.com/wiley/countries.php) highlighting the accelerating advancement in gene targeting technologies. Recent technological advances in both gene modification and their delivery systems are making the idea of both increasing the understanding of and subsequently the treatment of male reproductive conditions a reality.

Targeting the testicular somatic cells *in vivo* with gene modification has been approached in a number of ways; using both non-viral and viral vectors. Non-viral methods such as electroporation with naked DNA and using liposomes carrying naked DNA have proven to result in a low efficiency of targeting and, due to a lack of integration of the DNA into the host/target cell DNA, relatively short lived targeting [317, 318, 440]. The majority of studies using these methods were carried out with the purpose of targeting of the germ cell lineage as opposed to targeting the somatic cells of the testis, though expression of the plasmid in the offspring was in circular form. Further studies have demonstrated that the use of electroporation (a technique in which an electrical pulse is applied in order to increase the permeability of membranes, commonly used with naked and liposome DNA delivery) on the testis can negatively impact spermatogenesis, making it incompatible for the application of gene therapy and/or repair [302].

The use of viral vectors have been used more extensively in the testis to target both somatic cells and the germ cell population. Studies suggesting successful targeting of germ cells with viral vectors are controversial with a number of publications reporting low targeting efficiencies and episomal location of the inherited transgene [346, 363, 396]. Greater success has been found with using viral vectors to target somatic cells, with studies demonstrating viral vector targeting of both Leydig [175, 330, 334, 364] and Sertoli cells [45, 330, 336, 347, 397, 399, 441-443]. Using this method, viral vectors have been used to generate cell ablation through the knockout of essential genes (through Cre expressing adenoviral injections [175]), to generate an acute adult knockout of Androgen receptor (using lentiviral vectors expressing

Cre recombinase) [45]) and to repair mouse models of infertility through reintroduction of genes with viral delivery [396, 397]. However, some studies have reported some negative side effects as a result of injecting viral vectors into the testis indicating that the use of viral vectors for gene delivery may not be a 'one fits all system' and further refinement is required depending on the purpose of viral vector delivery [336].

3.2 Hypothesis & Aims

3.2.1 Hypothesis

It is hypothesised that delivery of viral vectors (Lentiviral and Adenoviral) to the testis will target the somatic cells. Specifically the Leydig or Sertoli cells of the testis will be targeted and upon refinement of the delivery technique, that this will be suitable for the repair of dysfunctional testis in that there will be little or no side effects as a result of the introduction of viral vectors.

3.2.2 Aims

The overarching aim of the experiments described in this chapter was to determine the best methods/protocols for targeting these two somatic cell types utilising methods and protocols published previously and modifying these protocols for the purpose of gene therapy/repair of testis (inducing as little impact to testicular histology and function as possible). Specifically, the following aims were established:

Aim 1: To establish a protocol for injection of vectors into the testis that would result in a wide coverage of the interstitial compartment with the goal of targeting the Leydig cells.

Aim 2: To confirm whether injection of a viral vector into the interstitial compartment of the testis would result in the production of transgenic offspring through viral targeting of the germ cells.

Aim 3: To determine the efficiency of lentiviral targeting of Leydig cells using interstitial injections.

Aim 4: To determine the efficiency of adenoviral targeting of Leydig cells using interstitial injections.

Aim 5: To establish a protocol for injection of vectors into the rete compartment of the testis, with the overarching aim of targeting the Sertoli cells with a reduction in the impact on testicular histology.

3.2.3 Approach

To achieve these aims, vehicle (PBS or serum free media), dye, adenoviral and lentiviral vectors were injected into the interstitial and tubular compartments of the testis using different volumes, locations and injection pressures dependant on the cell type being targeted. Testis were then collected and analysed at different time points post injection to determine the most appropriate methodology for future attempts at gene delivery/editing.

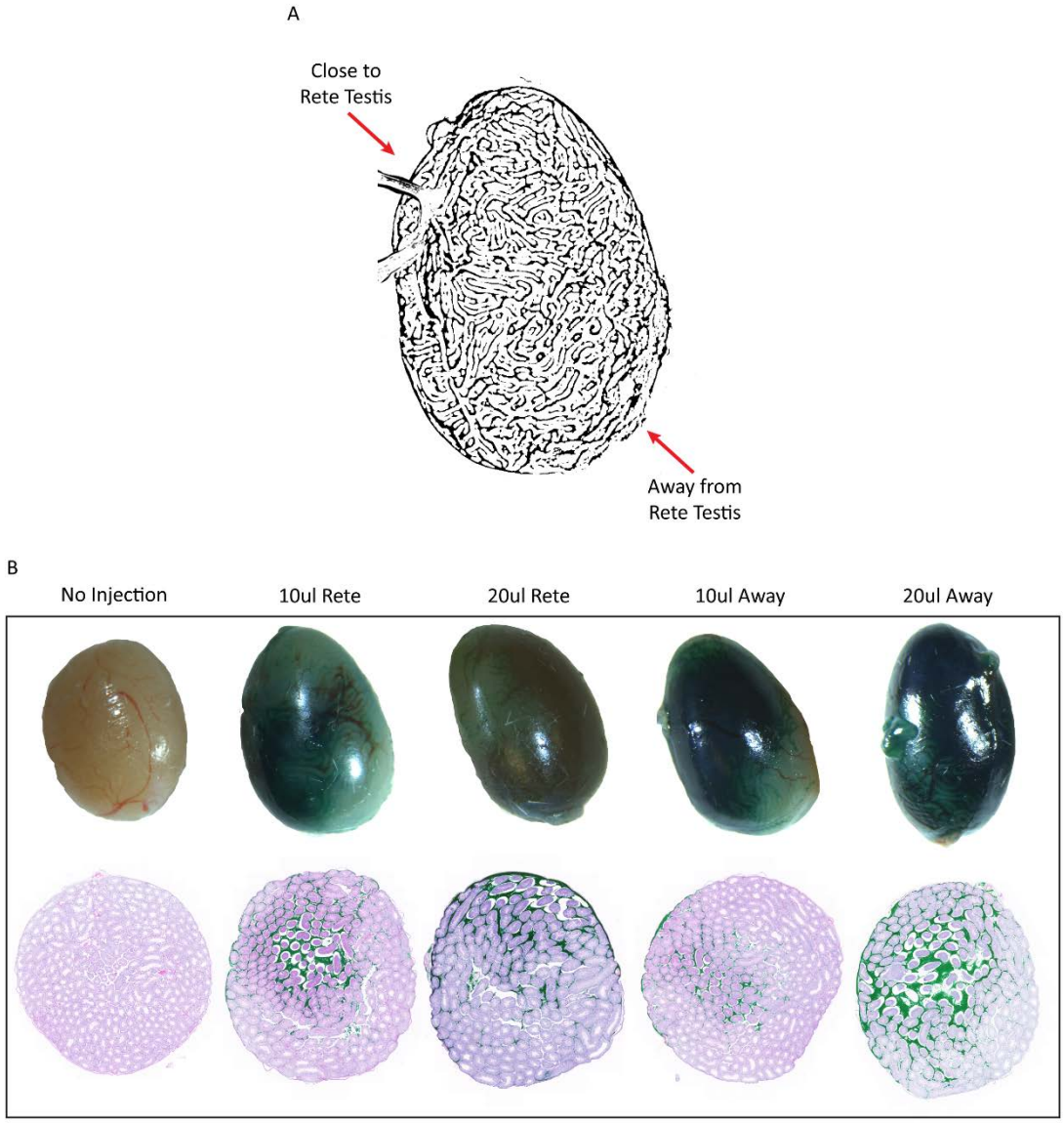
3.3 Results

3.3.1 Using Interstitial Injections to target Leydig cells in the Adult Testis

3.3.1.1 Optimising Injection Volume and Site

With the aim of targeting cells in the interstitium, first the optimal injection volume and site for interstitial injections had to be determined. Injection sites chosen for investigation were around the area close to the rete testis and the area furthest away from the rete testis; allowing consistent positioning of injections (due to the ease of location of the rete testis) (Figure 3.1A). To determine the optimal volume (defined as being the volume resulting in the widest distribution of injected solution through the testis), testis were injected with either 10µl or 20 µl of Tissue Marker Dye (a dye capable of withstanding tissue processing and histological procedures) at either of two injection sites (close to or away from the rete testis) and collected 30 minutes post injection. Injection procedures were performed as described in section 2.3.2. At the time of collection, distribution of the Tissue Marker Dye was clearly visible particularly in regions close to the injection site (Figure 3.1B). Cross sections through the testis gave further insight into the distribution of the dye through the testis with those injected with 20µl of dye appearing to have a wider distribution of dye compared to those injected with 10 µl of dye (Figure 3.1B). The percentage of cross section surface area with dye present was then calculated for comparisons (as described in 2.5.4.6). Uninjected testis were included in order to set a negative baseline for the analysis. Analysis confirmed that

injections of 20µl of dye localised at the rete testis resulted in a significantly greater proportion of testis section positive for the dye in comparison to testis injected with 10µl of dye (Figure 3.1C). For testis injected in the area furthest away from the rete, there was no difference between injection volumes (Figure 3.1C). Given the wider distribution of the dye at higher volumes and the lower variation of values, 20µl volume injections localised close to the rete were regarded as the most suitable for future interstitial injections. Closer inspection of H&E stained, vehicle injected testis sections also indicated that there were no significant impacts on testicular histology following injection with either volume (Figure 3.1D), further confirming the suitability of using a higher volume for injections, echoing methods used and published by Kojima et al (2003) and Penny et al (2017) [175, 330].



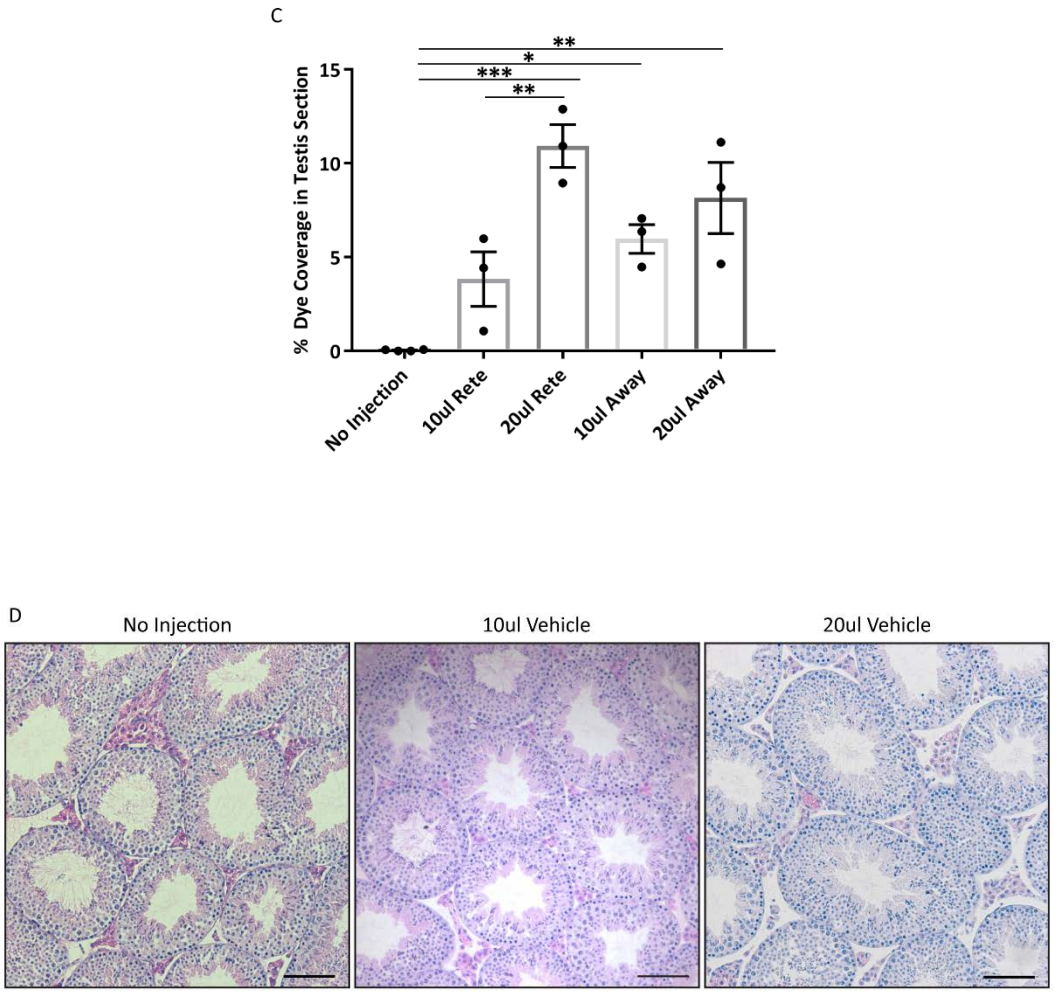


Figure 3.1 Optimisation of Interstitial Injection Volume and Site. (A) Schematic of mouse testis demonstrating locations of interstitial injections being investigated; Close to the rete testis and Away from the rete testis. (B) Representative images of freshly dissected testis injected with different volumes of Tissue Marker Dye at the two different locations (Not to Scale) and corresponding whole cross sections of H&E stained testis directly below. (Not to Scale) (C) Analysis of Tissue Marker Dye coverage within interstitial injected testis sections (One way ANOVA; * significant at $p<0.05$, ** significant at <0.005, *** significant at $p<0.001$). Bars represent average ± SEM. n number = 3 for each experimental group and n=4 for non-injected controls. (D) Representative images H&E stained testis injected with either 10µl or 20µl of vehicle, illustrating that there were no significant impacts on testicular histology following interstitial injection with either volume. Scale bars = 100µm.

3.3.1.2 Using Interstitial Injections to target Leydig cells in the Adult Testis

To date, targeting of Leydig cells in the interstitial compartment of the testis has been successfully demonstrated using adenoviral [175, 330], gamma-retroviral vectors [364] and, in brief, lentiviral vectors [400]in the mouse and sheep respectively. Having confirmed the optimal technique for injection into the interstitial compartment, preliminary studies using testis injected interstitially with lentivirus, (including archived tissue sections injected by Dr Cornelia Roesl) into the interstitial compartment were performed.

3.3.1.2.1 Ensuring Germ cells are not being targeted by Lentiviral vectors

Two published studies have reported targeting of the germ cell population following viral vector injection into the interstitial compartment of the testis [394, 395, 444]. Given that human germline modification is currently banned in 29 countries [445], and the overarching aim of this study to investigate a technique which could ultimately be applicable to human male reproductive disorders, we then sought to confirm whether lentiviral vectors were indeed capable of transducing the germ cell population. Sehgal *et al* (2011) successfully utilised a lentiviral vector containing human elongation factor-1 (EF-1) promoter driven eGFP to generate transgenic offspring, following previous reports suggesting lentiviral vectors expressing cytomegalovirus (CMV) promoter driven GFP were unsuccessful [394, 444]. A follow up study by Qin *et al* (2015) confirmed successful generation of transgenic offspring using both the EF-1 and the CMV promoter but found that the delivered transgene was silenced in offspring derived from males injected with the lentiviral vector containing the EF-1 promoter [395]. This was attributed to the delivered transgene being suppressed by DNA methylation. With a number of studies attempting to target the germ cell population and with many of these studies contradicting each other, we then recapitulated the protocol as published by Seghal [394] (Figure 3.2A) and analysed offspring produced for GFP expression following interstitial injection with lentiviral vectors expressing GFP under either the CMV or EF1α promoters . GFP expression in offspring would indicate targeting of the spermatogonial stem cell population in the testis suggesting that this technique may not be suitable for the treatment of men with reproductive disorders.

Collected embryos were imaged under BF and GFP fluorescent filters adjacent to negative control tissue (Figure 3.2B) before being frozen for analysis of genomic DNA (Figure 3.2C).

Both imaging and genotyping of a total of 263 embryos (Figure 3.2D&E) derived from ten lentivirus injected males (n=5 for each promoter) confirmed that there was no transmission of GFP to offspring indicating that the lentiviral vectors were not targeting the spermatogonial stem cell population in the injected testis.

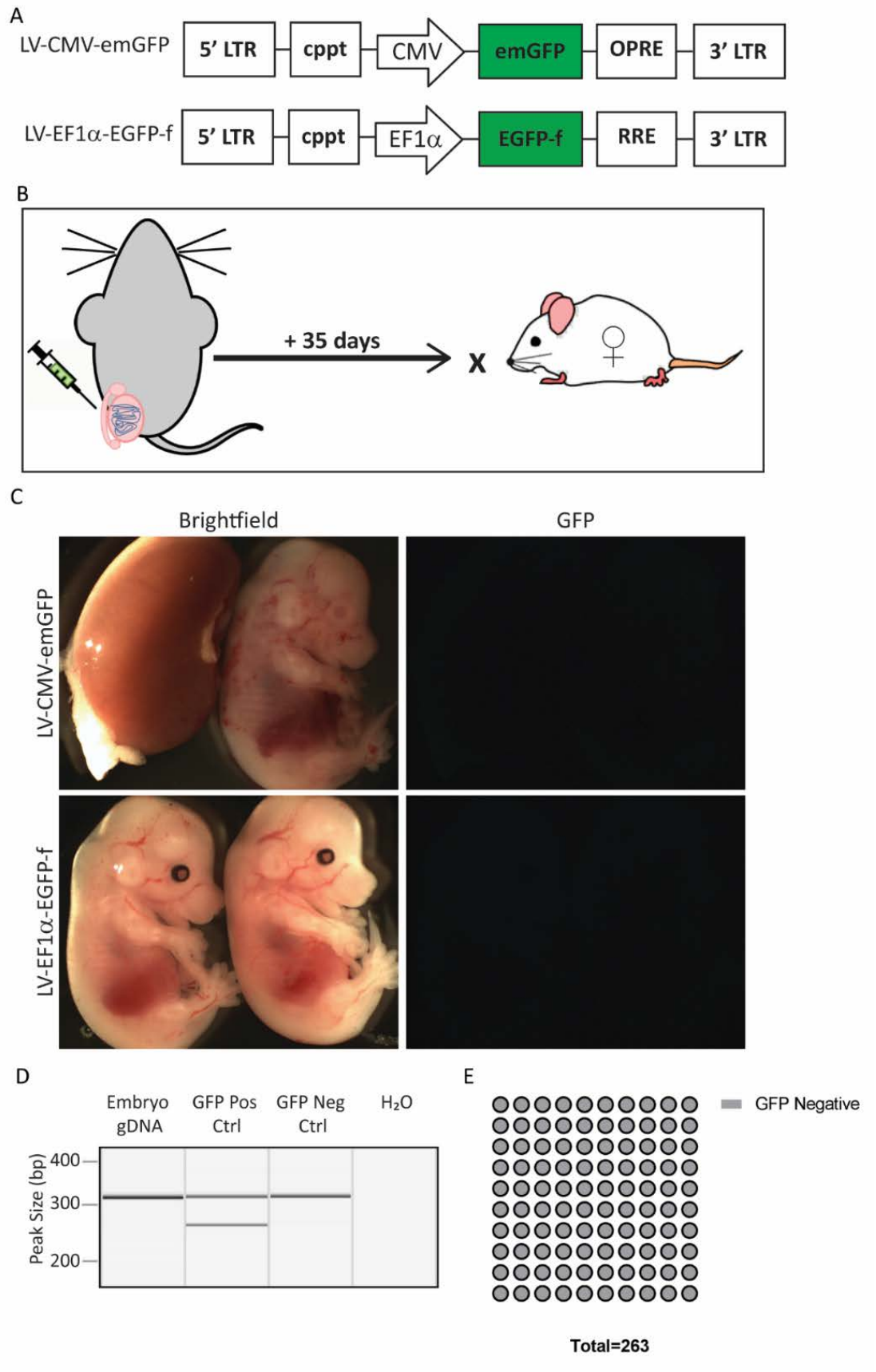

A
LV-CMV-emGFP
5' LTR
cppt
CMV
emGFP
OPRE
3' LTR
LV-EF1α-EGFP-f
5' LTR
cppt
EF1α
EGFP-f
RRE
3' LTR
B
+ 35 days
X
C
Brightfield
GFP
LV-CMV-emGFP
LV-EF1α-EGFP-f
D
Embryo gDNA
GFP Pos Ctrl
GFP Neg Ctrl
H2O
Peak Size (bp)
400
300
200
E
GFP Negative
Total=263

Figure 3.2 Ensuring Germ cells are not being targeted by Lentiviral vectors. (A) Schematics of the Lentiviral vectors expressing GFP under either the CMV or EF1α promoter. (B) Schematic of the experimental procedure; wild type male mice (at 28 days postpartum) were injected with either of the lentiviral vectors into the interstitial compartment of the testis. The contralateral testis was removed. 35 days post procedure, the males were mated with wild type females and any resultant embryos were collected for analysis. (C) Images of freshly dissected embryos (and negative control tissue – located on the left of the image) derived from males injected with lentiviral vectors. Images were taken under brightfield and GFP fluorescent lamps. (Brightfield images taken at 0.5 seconds of exposure, GFP fluorescent images taken at 5.5 seconds of exposure) (D) PCR analysis of genomic DNA isolated from embryos derived from males injected with lentiviral vectors. GFP positive, negative and water controls were included. Primers for a positive control gene; Interleukin 2 were also included to ensure successful amplification of DNA. (Interleukin2; 330bp, GFP; 264bp). (E) Occurrence of GFP positive or GFP negative results in 263 embryos derived from males injected with either LV-CMV-emGFP or LV-EF1α-EGFP.

3.3.1.2.2 Injection of Lentiviral Vectors into the Interstitial Compartment Results in Targeting of 9.3% of the Adult Leydig Cell Population

To determine the cell type targeted by lentiviral particles in the interstitial compartment. Lentiviral vectors carrying a GFP reporter were injected into the interstitial compartment of the testis and collected seven days post injection for analysis. Immunostaining for GFP and Leydig cell expressed protein HSD3β confirmed targeting of Leydig cells by the injected lentiviral vectors with co-localisation of GFP and 3βHSD observed in the interstitial compartment (Figure 3.3A). Estimates of targeting efficiency (from counts of GFP+HSD3β positive cells) suggested that up to 9.3% of Leydig cells were being transduced by the lentiviral particles (with a maximum volume delivered) (Figure 3.3B) 7 days post injection (dpi) (allowing time for both integration of the viral particles and sufficient expression of the delivered transgene) with numbers of cells being targeted increasing with increasing injection volume.

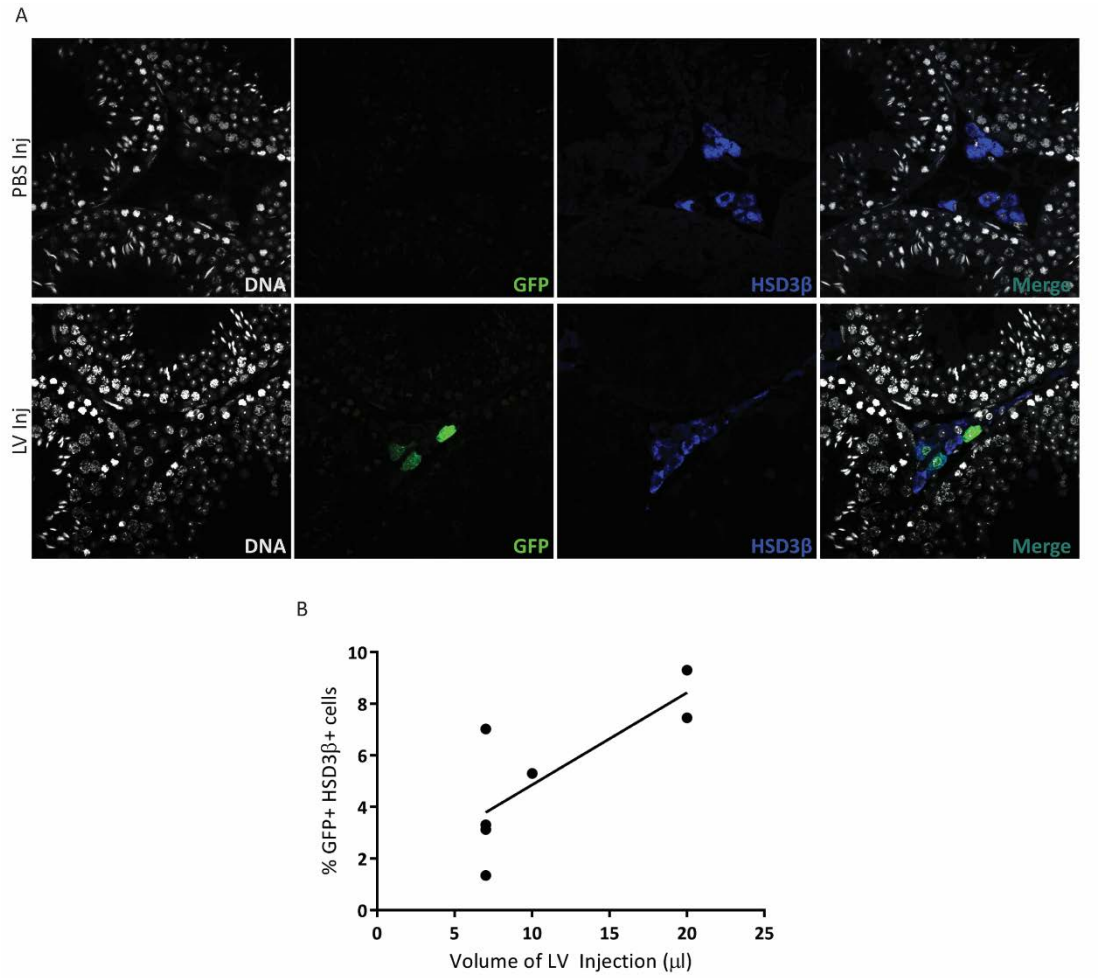


Figure 3.3 Investigating targeting in testis injected interstitially with Lentiviral vectors carrying GFP. (A) GFP and 3bHSD expression in testis 7 days post interstitial injection with LV-CMV-emGFP demonstrating targeting of Leydig cells with the lentiviral vector (B) Percentage of GFP positive HSD3β positive cells in the testis injected with 7.5µl (n=4), 10µl (n=1) or 20µl (n=2) of lentiviral vectors carrying GFP.

3.3.1.2.3 Longevity of delivered gene expression

It is well documented that lentiviral vectors have the ability to integrate into the host cell DNA, allowing potentially permanent expression of a delivered transgene [366]. To assess the longevity of gene expression in adult Leydig cells transduced with lentivirus *in vivo,* testis were collected 3.5, 7 and 10 days post injection (dpi) of GFP expressing lentiviral particles and analysed for GFP expression using immunohistochemistry. At 3dpi and 7dpi , GFP expression is visible in the interstitial compartment of the injected testis, however at 10dpi there were no GFP positive cells present in the injected testis suggesting that targeted cells

had either stopped expressing/silenced the transgene or the that the cells expressing the delivered transgene had been cleared from the testis (Figure 3.4). Immunohistochemistry for cleaved caspase 3 (a marker of apoptosis) revealed that GFP positive cells were co-expressing this marker (Figure 3.4) and, therefore, cells targeted by the lentiviral particles were undergoing apoptosis by 7dpi resulting in clearance and absence of the targeted cells by 10dpi.

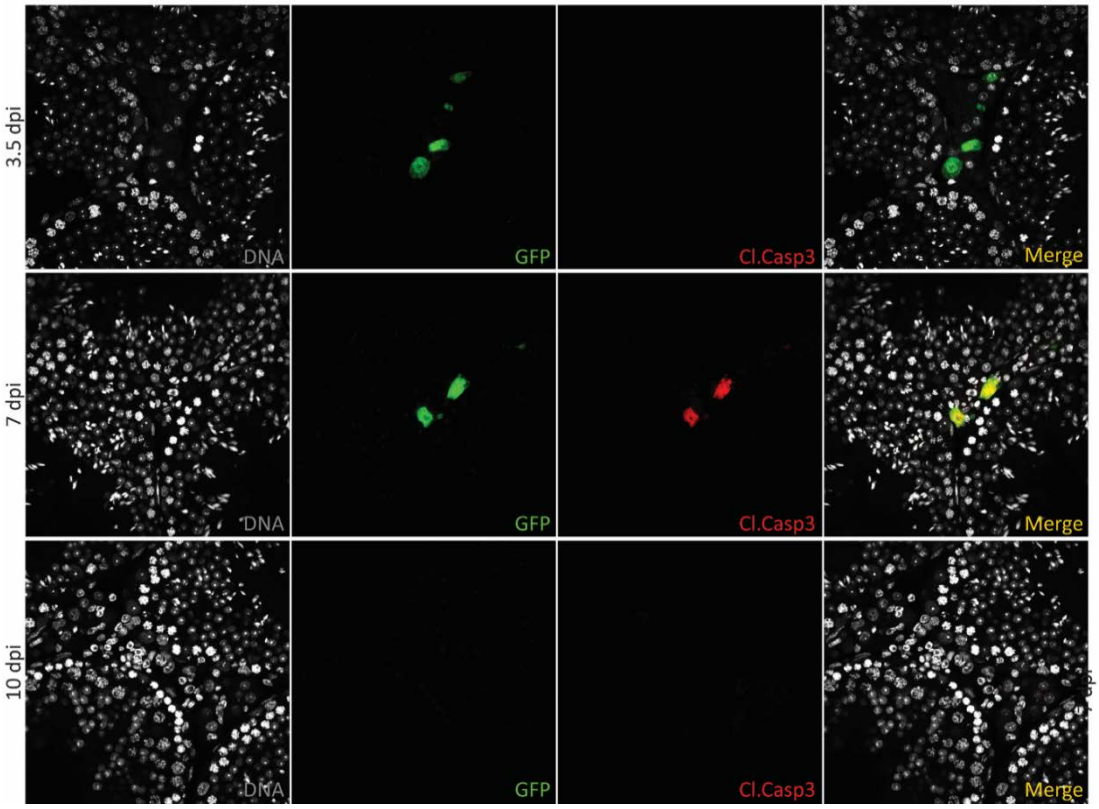


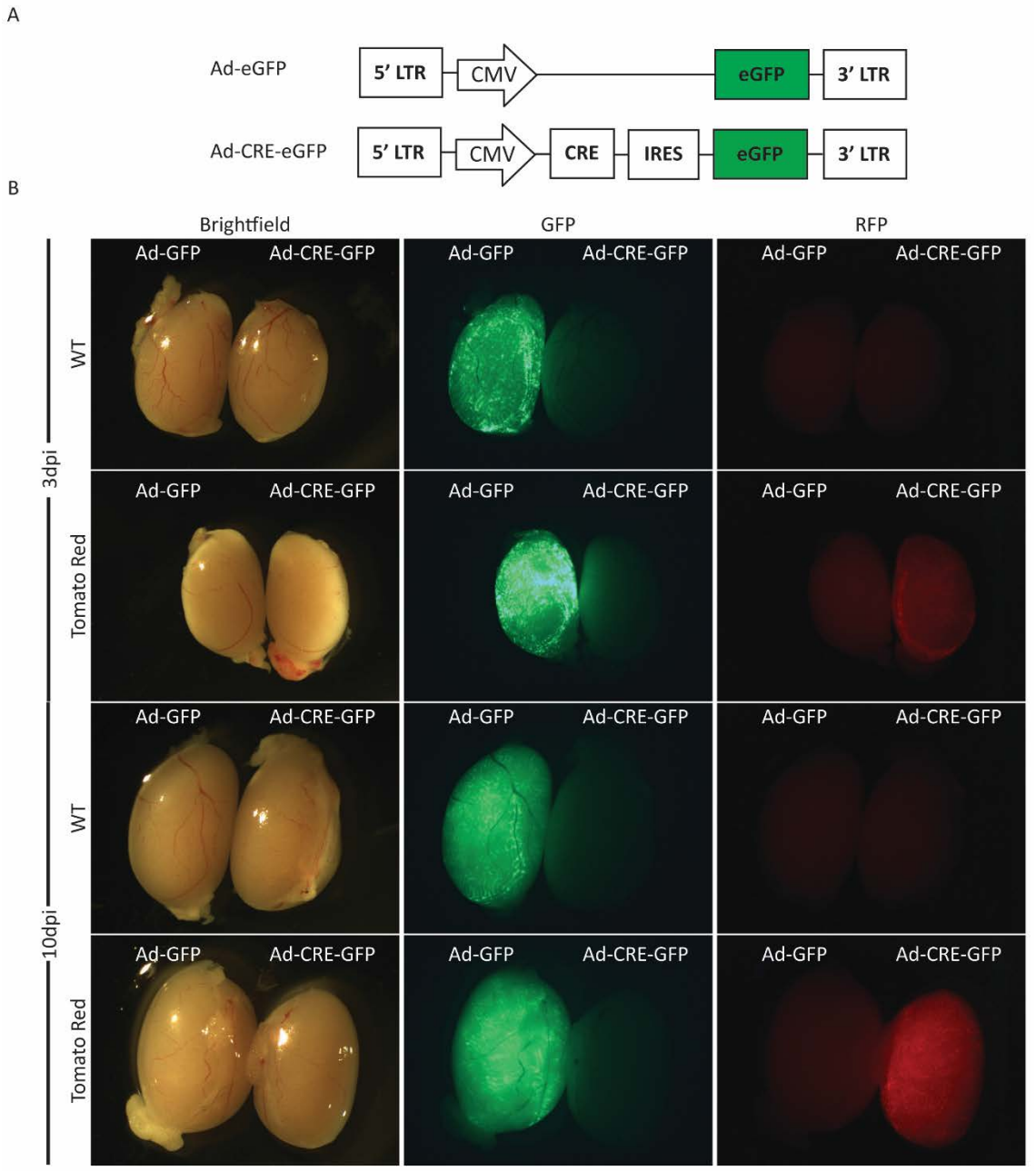
Figure 3.4 Investigating the longevity of lentiviral delivered gene expression in Leydig cells. GFP and Cleaved caspase 3 expression in testis collected 3.5 days (n= 4), 7 days (n= 3) and 10 days (n=5) post interstitial injection with LV-CMV-emGFP demonstrating targeting of Leydig cells with the lentiviral vector and resultant cleaved caspase expression exclusively in cells targeted by the lentiviral vector. Consequently, targeted cells were cleared from the interstitial compartment by 10 days post injection (dpi).

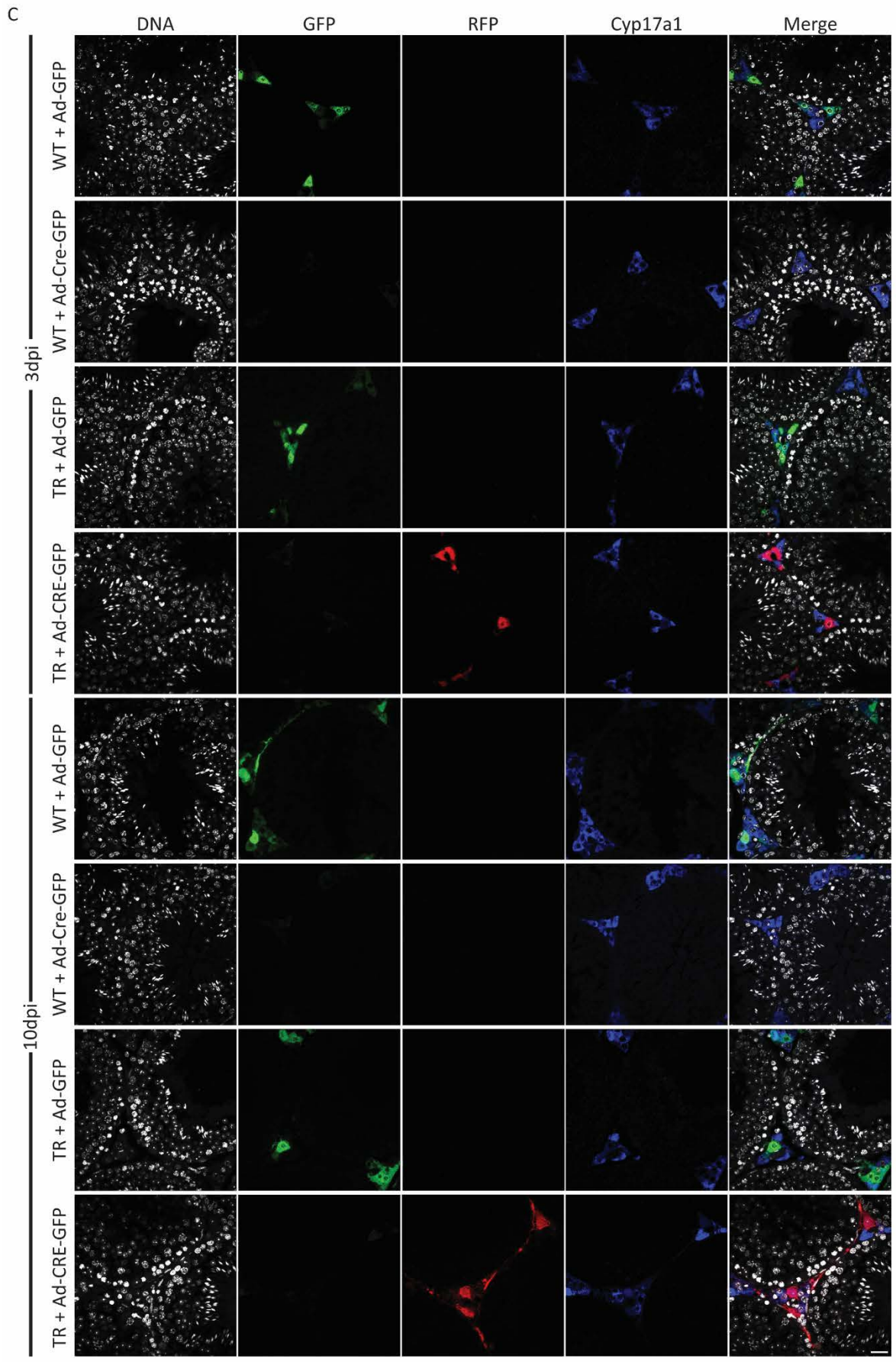
3.3.1.3 Injecting Adenoviral vectors into the interstitial compartment

3.3.1.3.1 Cell Type being targeted and Longevity of delivered gene & Efficiency of targeting

With lentiviral vector targeted Leydig cells undergoing apoptosis, an alternative vector to target these cells would be required. Following completion of experiments determining the targeting efficiency of lentiviral vectors in the interstitium, a study was published demonstrating successful targeting of Leydig cells with an Adenoviral vector, with the purpose of probing Gata4 function [175]. To confirm targeting of Leydig cells with adenoviral vectors and to determine whether expression of transgene would persist for longer than those delivered by lentiviral vectors, wildtype and RFP reporter testis were injected with either a GFP expressing adenoviral vector (Ad-eGFP) or a Cre-GFP expressing adenoviral vectors (Ad-Cre-eGFP) into the interstitial compartment and collected at 3dpi and 10dpi (Figure 3.5A).

Post collection, testis were imaged under brightfield, GFP and RFP fluorescent filters (Figure 3.5B). From these images it was clear, at both 3 and 10dpi that targeted cells were still expressing the delivered transgene and that targeted cells appeared to be located in the interstitial compartment, as expected. It was also evident that, following injection of Ad-Cre-eGFP into RFP (Tomato Red) reporter mice; that the GFP transgene was not as highly expressed as the CRE transgene; a typical artefact of using a vector expression multiple transgenes separated with an IRES component [446]. On closer inspection, and using the Leydig cell marker Cyp17a1, we confirmed that the cell type being targeted in the interstitium was the Leydig cells (Figure 3.5C). When quantified, the number of Leydig cells being targeted was 15.39% at 3dpi and 19.31% at 10dpi (mean ±SEM). There was also no differences in the mean number of Leydig cells targeted after 10dpi when compared with testis collected 3dpi, namely due to the large variance in targeted Leydig cells counted between each injected testis (Figure 3.5D). The expression of the delivered transgene was unexpected at 10dpi, given the results shown by Penny et al (2017) who report a loss of reporter gene expression at 12dpi.


A
Ad-eGFP
5' LTR
CMV
eGFP
3' LTR
Ad-CRE-eGFP
5' LTR
CMV
CRE
IRES
eGFP
3' LTR
B
Brightfield
GFP
RFP
Ad-GFP
Ad-CRE-GFP
3dpi
WT
Tomato Red
10dpi
WT
Tomato Red


C
DNA
GFP
RFP
Cyp17a1
Merge
3dpi
10dpi
WT + Ad-GFP
WT + Ad-Cre-GFP
TR + Ad-GFP
TR + Ad-CRE-GFP
WT + Ad-GFP
WT + Ad-Cre-GFP
TR + Ad-GFP
TR + Ad-CRE-GFP

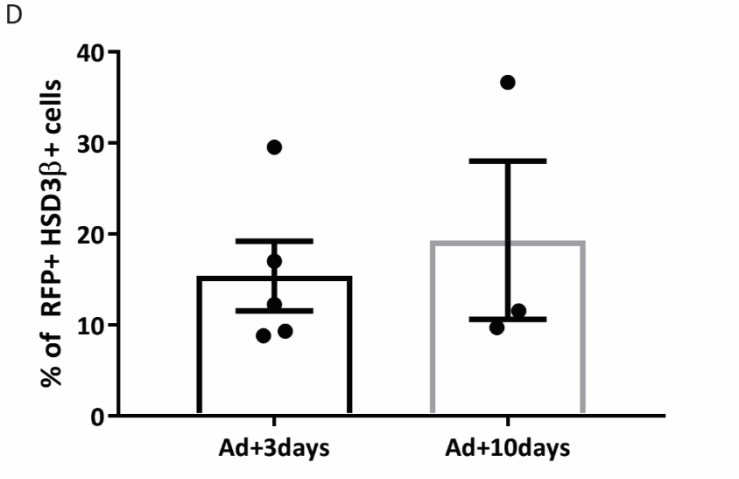
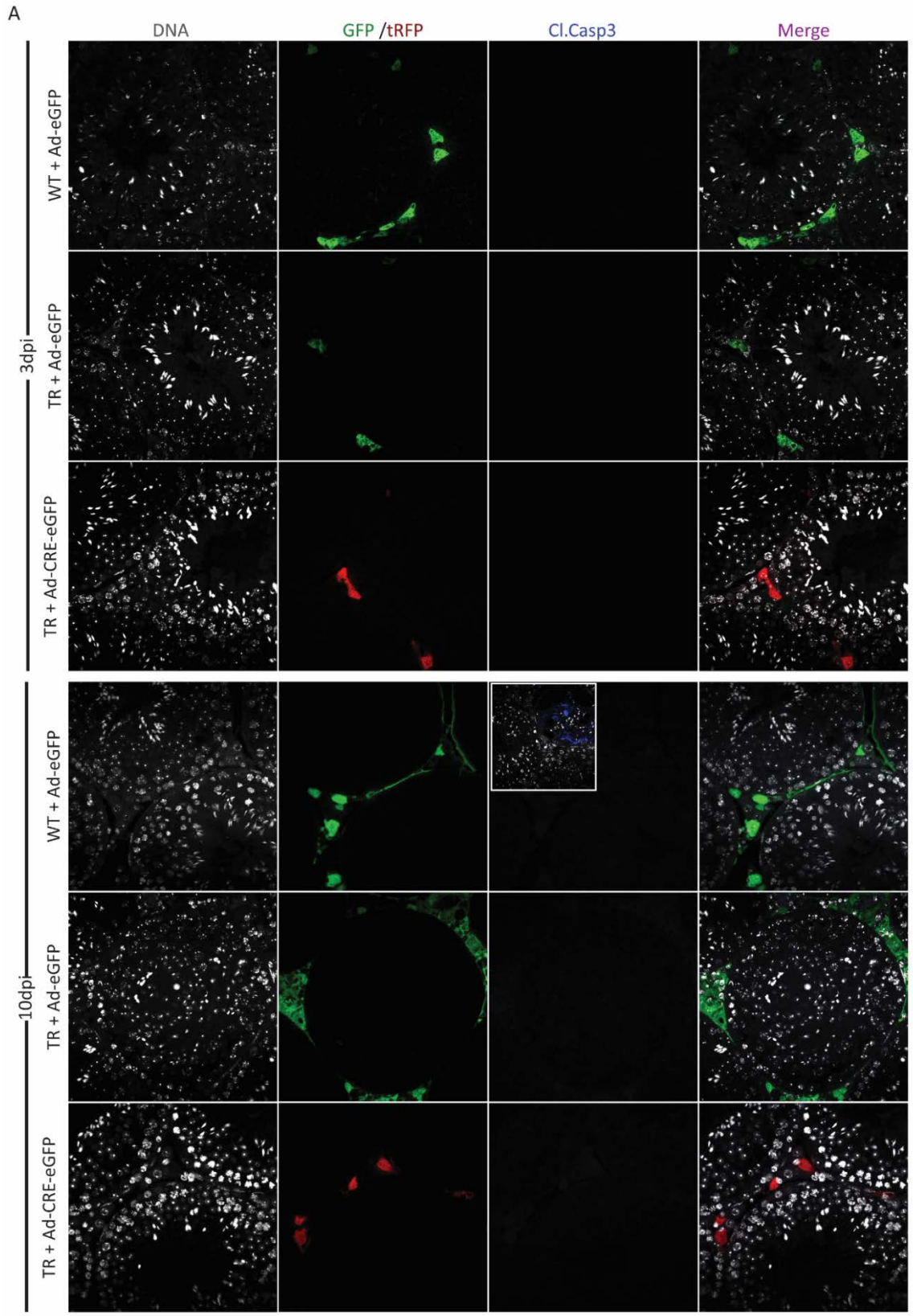


Figure *3.5* Injecting Adenoviral vectors into the interstitial compartment of Adult Mouse Testis. (A**)** Schematic diagrams of adenoviral constructs used. (B) Representative images of freshly dissected wild types and tomato red reporter testis under brightfield and GFP fluorescent lamps at 3 and 10 days post injection with either GFP or Cre-GFP expressing adenoviral vectors. (Brightfield images taken at 0.5 seconds of exposure, GFP fluorescent images taken after 5.5 seconds of exposure). 5 mice were included in each of the injected groups. **(C)** GFP, tRFP and Cyp17a1 expression in wildtype (WT) and tomato red reporter (TR) testis 3 and 10 days post injection (dpi) with GFP or Cre-GFP expressing adenoviral particles. Representative images clearly demonstrate uptake of adenoviral particles by Cyp17a1 expressing cells (Leydig cells) in the interstitial compartment of the testis. Scale bar = 20µm **(**D**)** Counts of cells co-expressing red fluorescent protein (RFP) and Leydig cell marker; HSD3β in tomato red reporter testis injected with Cre-GFP expressing adenovirus give an estimation as to the number of Leydig cells being targeted at both 3dpi (n= 5) and 10dpi (n=3). (Unpaired t-test; p=0.6479). Bars represent average ±SEM.

3.3.1.3.2 Immune Response to introduction of Adenoviral vectors into the interstitial compartment of the testis

Given the apoptosis observed previously in lentiviral targeted Leydig cells, immunohistochemistry was used to stain testis sections with cleaved caspase 3 (a marker of apoptosis) to determine whether adenoviral targeted Leydig cells also underwent cell death. At both 3dpi and 10dpi, in both WT and RFP reporter testis, there were no cleaved caspase 3 positive cells in the interstitial compartment, confirming that adenoviral infection did not induce Leydig cell death (Figure 3.6A).

As adenoviral vectors have an increased immunogenicity compared to other viral vectors, the immune response of testis post adenoviral injection was investigated. To give an idea of the impact of the injection alone, non-injected and vehicle-injected testis sections were analysed for the presence of macrophages using a Mac2 antibody; a general macrophage marker recognising mouse peritoneal exudate macrophages as well as tissue resident macrophages (Figure 3.6B). This confirmed presence of macrophages within the interstitial compartment regardless of treatment and time post procedure, suggesting that the procedure and introduction of solution into the interstitial compartment alone does not induce a dramatic response from testicular macrophages. Wild type testis injected with Ad-eGFP and RFP reporter testis injected with either Ad-eGFP or Ad-Cre-eGFP and collected 3dpi and 10dpi were immuno-stained for macrophage marker Mac2 (Figure 3.6C). There appeared to be no differences in Mac2 staining between the testis of different genotypes and testis injected with different viruses, though further quantification of Mac2 positive cells would confirm this. In testis collected 10dpi (regardless of viral construct injected), some targeted cells in the interstitium appear to be being encompassed and surrounded by Mac2 positive cells (Figure 3.6C, inset), suggest a response of the testicular immune cells to the introduction of adenoviral particles. This is similar to the immune delayed immune response observed in Penny et al (2017), who noted a delayed influx of T lymphocytes at 12dpi coinciding with a loss of reporter transgene expression. Whether this is a response to the delivered transgene or the virus itself it yet to be confirmed.


A
DNA
GFP /tRFP
Cl.Casp3
Merge
3dpi
WT + Ad-eGFP
TR + Ad-eGFP
TR + Ad-CRE-eGFP
10dpi
WT + Ad-eGFP
TR + Ad-eGFP
TR + Ad-CRE-eGFP

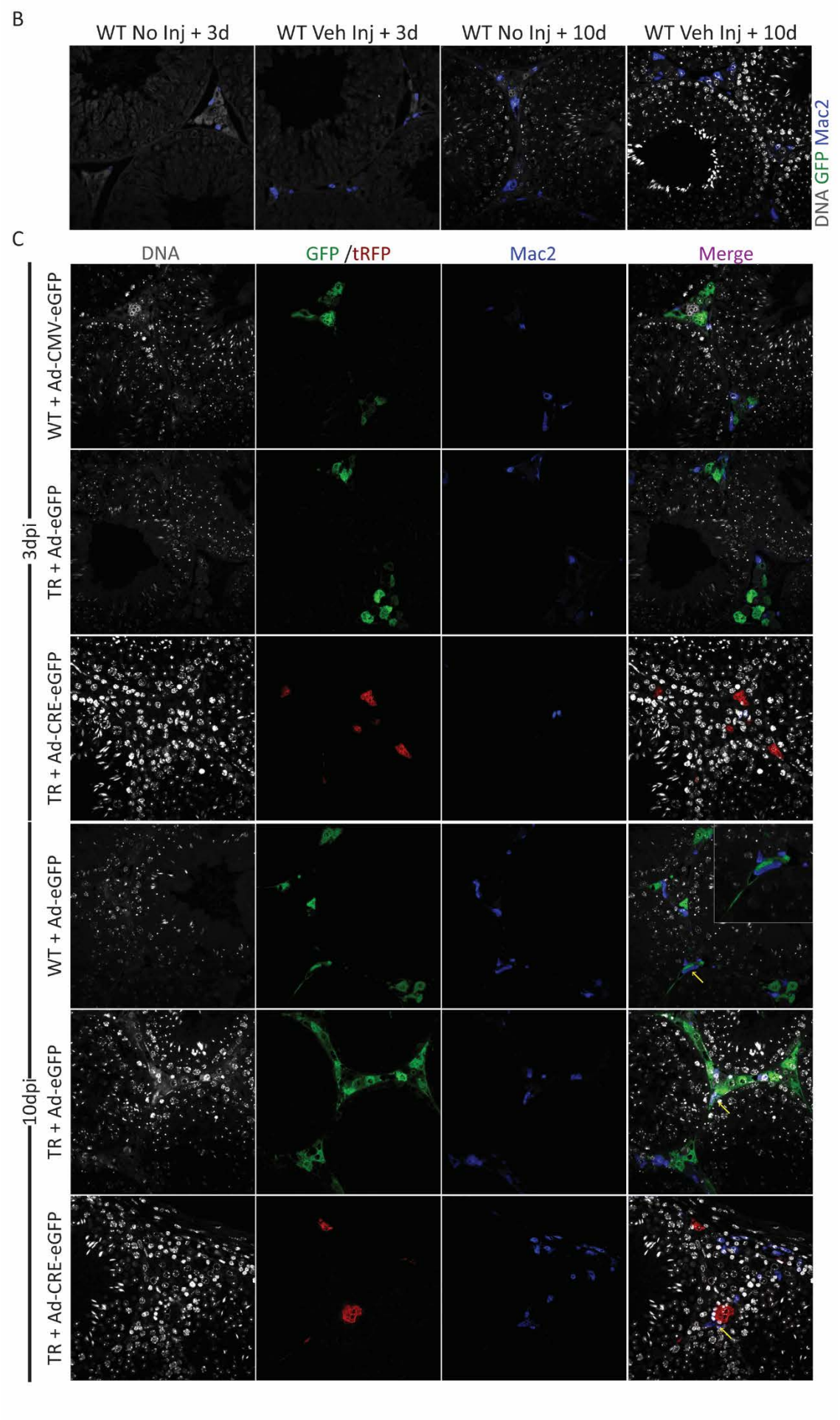

B
WT No Inj + 3d
WT Veh Inj + 3d
WT No Inj + 10d
WT Veh Inj + 10d
DNA GFP Mac2
C
DNA
GFP /tRFP
Mac2
Merge
3dpi
10dpi
WT + Ad-CMV-eGFP
TR + Ad-eGFP
TR + Ad-CRE-eGFP
WT + Ad-eGFP
TR + Ad-eGFP
TR + Ad-CRE-eGFP

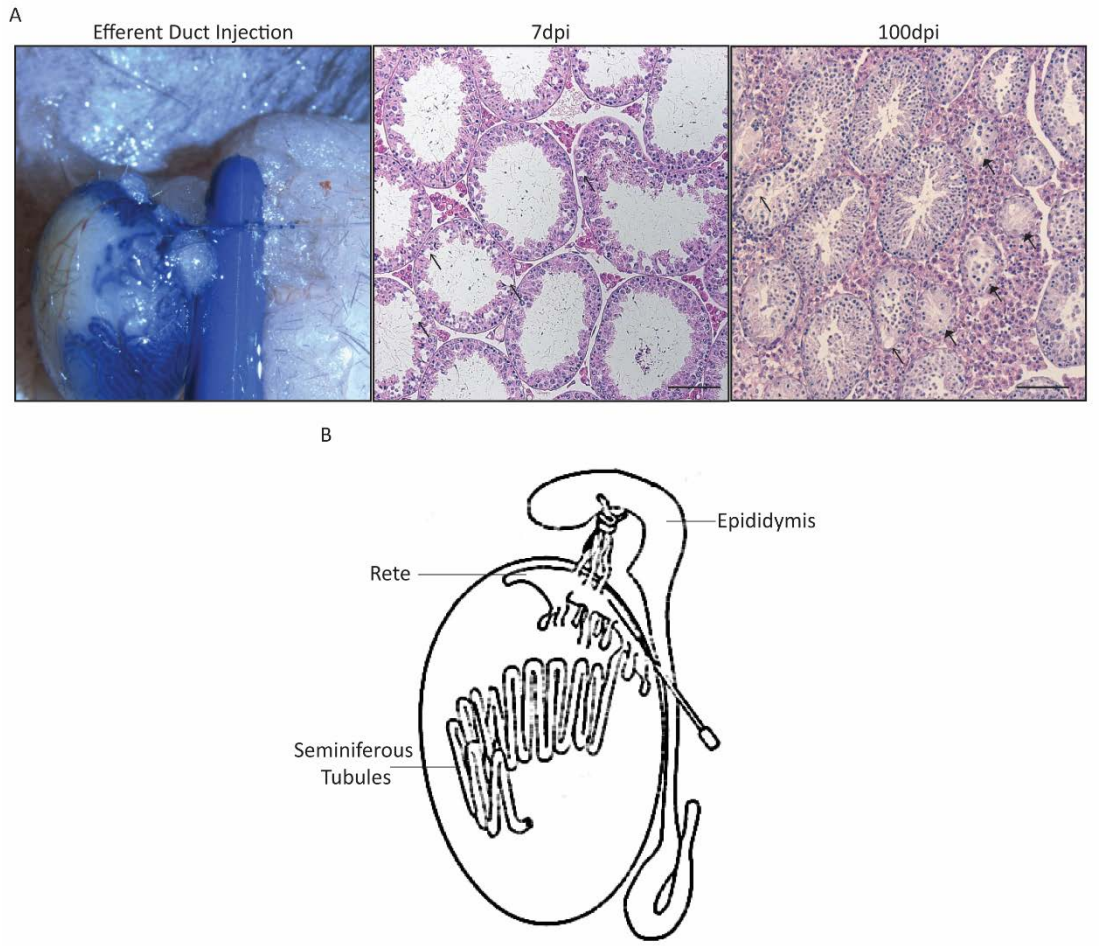
Figure 3.6 Immune Response to introduction of Adenoviral vectors into the interstitial compartment of the testis. (A) GFP/tRFP and Cleaved Caspase 3 expression in wildtype (WT) and tomato red reporter (TR) testis 3 and 10 days post injection (dpi) with eGFP or Cre-eGFP expressing adenoviral particles. Representative images indicate that there is no apoptosis occurring as a result of adenoviral targeted. Inset = positive cleaved caspase 3 staining as a result of blood testis barrier disruption serving as an internal positive control. (B) Mac2 expression in WT testis 3 and 10 days post procedure consisting of either no injection or injection with vehicle. Images reveal the presence of Mac2 positive cells in control testis. (C) GFP/tRFP and Mac2 expression in wildtype (WT) and tomato red reporter (TR) testis 3 and 10 days post injection (dpi) with GFP or Cre-GFP expressing adenoviral particles. Representative images reveal some interaction between targeted cells and Mac2 positive macrophages at 10dpi (highlighted with yellow arrows and inset zoomed in image). 5 animals were used in each of the injection groups.

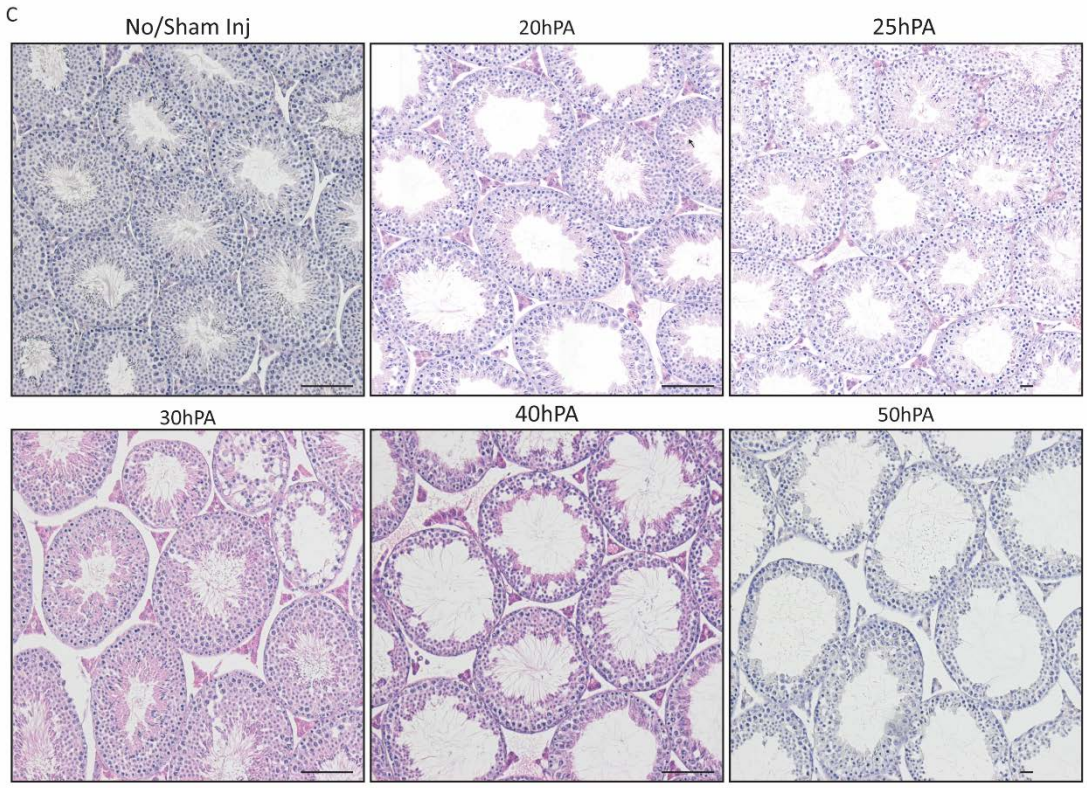
3.3.2 Seminiferous Tubule Injections to target Sertoli cells in the Testis

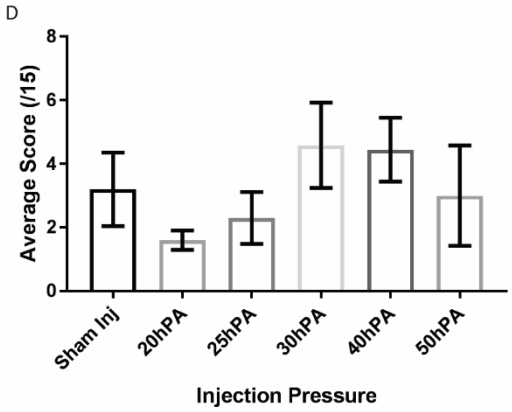
3.3.2.1 Determining the best pressure to use for injection into the rete compartment

Previously, lentiviral vectors have been delivered via the efferent ducts of the testis to target the Sertoli cells. Though this method can occasionally be effective (as described in Willems et al 2015 [45]), this delivery technique often resulted in distended seminiferous tubules, presumably as a result of blockage or damage to the fragile efferent duct (Figure 3.7A). To circumvent this, we first moved the injection site to the rete compartment of the testis, bypassing the need for over handling and dissection of the efferent duct during the injection procedure yet still providing good access to the seminiferous tubules [293] (Figure 3.7B). Blanchard and Boekelheide [441] observed that mechanical disruption of the rete resulted in seepage of the injected solution into the interstitial compartment, giving suggestion as to the delicate structure of the rete compartment. Therefore, injection pressure was then optimised using a microinjector (Eppendorf Femtojet; Eppendorf, Germany) to introduce vehicle into the seminiferous tubules at pressures ranging from 20hPA to 50hPA, the latter being the pressure previously used with efferent duct injections. H&E staining of testis sections indicated that a lower injection pressure resulted in a reduced likelihood of negative features such as vacuolisation and tubule distension being present (Figure 3.7C).

Quantification (using the methodology described in 2.5.4.6) confirmed that lower injection pressures resulted in a reduction in the likelihood of artefacts being present in the testis (Figure 3.7D), with a low score indicating fewer artefacts observed and a high scores indicated an increase in the negative impact in a testis section. However, it was noted during injection procedures that the time taken to complete the injection would increase as injection pressure was lowered. Because of this, an injection pressure of 25hPA was chosen for all future injections in order to both reduce the likelihood of negative impacts on the testis and to complete the injection in a timely manner (for animal welfare considerations). Figure 3.7E demonstrates the specific targeting of lentiviral vectors with presence of Sertoli cells expressing the lentiviral delivered tRFP transgene 50dpi and that testicular histology is maintained following injection at the lower pressure of 25hPa as opposed to that seen in the testis injected at 50hPA. Injection procedures were performed as described in section 2.3.3.




C
No/Sham Inj
20hPA
25hPA
30hPA
40hPA
50hPA


D
Average Score (/15)
0
2
4
6
8
Sham Inj
20hPA
25hPA
30hPA
40hPA
50hPA
Injection Pressure

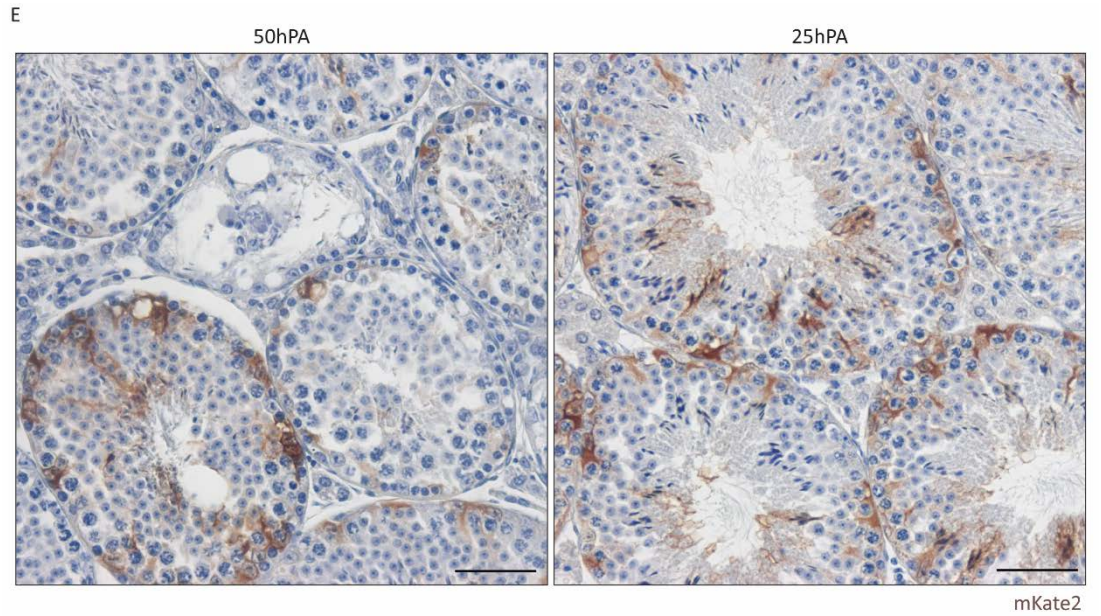


Figure 3.7 Determining the best pressure to use for injection into the rete compartment. (A) Injections via the efferent duct performed previously in the group have resulted in damage to testicular histology with presence of vacuoles at 7dpi and 100dpi (open arrows) and shrunken tubules 100dpi as evident from the representative images (closed arrowheads).[Injection performed by Dr C Roesl, Images taken by Mr N Jeffery] (B) Schematic illustrating injection into the rete compartment of the testis. (C) Representative images of H&E stained testis sections following either a sham injection or injection of vehicle at the following pressures; 20hPA, 25hPA, 30hPA, 40hPA and 50hPA. (D) Average scores of testis following either sham injection of injection of vehicle at the varying pressures. Scores are out of 15 and were determined following observation of vacuoles, germ cell sloughing and distended tubules, scoring on both presence and incidence of the histological factor. A higher score would indicate presence and higher incidence of negative testicular factors indicating an increase in the potential for testicular damage. Bars represent average score ±SEM (E) mKate2 (RFP) expression in testis injected with a lentiviral vector carrying mKate2 (LV-CMV-mKate2-negmiR) at 50hPA and 25hPA demonstrating the impacts of reducing rete injection pressure. (Scale Bars = 50µm). Between 5 and 7 animals were included in each of the groups examined.

3.4 Discussion

As yet, only a handful of published studies have focussed on targeting the somatic cells of the testis, with some success using viral vectors as a delivery mechanism [45, 175, 330, 334, 336, 347, 397, 399, 441-443]. Few of these studies have attempted targeting of Leydig cells using viral vectors; with studies utilising adenoviral, lentiviral and adeno-associated viral vectors to target these cell types [175, 330, 334, 347, 400]. With little previous insight into the most appropriate methodology for delivery of substances to the interstitial compartment of the testis, an injection protocol was first optimised in order to cover a wide range of this compartment whilst inducing as little disruption to the testis architecture as possible. Results indicated that injections of a higher volume, specifically at the rete compartment of the testis resulted in an increased proportion of testis section surface area being infiltrated with the dye and at this location, injections were more consistent. H&E stained sections of testis injected with either 10µl or 20µl of vehicle also confirmed that there appeared to be no severe phenotypes arising as a result of the introduction of substances into the interstitial compartment of the testis. In the literature, any studies describing injection of substances into the interstitial compartment of the testis have set their volume at 20µl, further confirming the observations described here [394, 395, 444] but have not described optimal location of testicular injections. As a result, all future injections were performed according to these standards to both increase targeting efficiencies and reduce negative impacts of injections.

Studies utilising lentiviral vector injections into the interstitial compartment have so far done so with the purpose of generate transgenic offspring *in vivo*, with conflicting results [394, 395, 444]. To establish whether lentiviral vectors were indeed capable of transducing the spermatogonial stem cell population (as described in the literature), we mated wildtype males 50dpi with lentiviral vectors carrying plasmids expressing GFP under either the EF1α or CMV promoters with wild type females. Both fluorescent imaging and genotyping of all offspring produced revealed that no offspring were positive for the GFP transgene, regardless of the promoter driving transgene expression. Sehgal *et al* (2011) [394, 444], described successful generation of transgenic offspring using the EF1α promoter but not the CMV promoter with Qin *et al* (2015) describing the contrary attributing lack of transgene expression in lentivirus carrying EF1α promoter driven GFP derived offspring to gene

silencing through DNA methylation. This may also be the case in the present study and could be confirmed by determining the presence or absence of proviral DNA in the DNA of the offspring. Retroviral delivered transgene silencing in stem cells has been well described in the literature [447, 448], however whether lentiviral delivered transgenes are also silenced is still subject to debate [449-451]. It has been suggested that transgene silencing is particularly prevalent in stem cell populations as a result of intrinsic defence mechanisms against exogenous DNA [451, 452], further evidence as to why the experiments performed in this study (and that of Sehgal *et al* (2011) and Li *et al* (2015)) fail to consistently produce transgenic offspring through *in vivo* targeting of the spermatogonial stem cell population. One way this may be circumvented is by incorporating a ubiquitous chromatin opening element into the lentiviral vector backbone, helping to establish stable transgene expression in the transfected cells [453]; potentially permitting the generation of transgenic offspring. However, in terms of somatic cell modification using lentiviral vectors with the purpose of therapeutic development, the difficulties presented with targeting the adult stem cell populations is advantageous, given that germline modification in humans is currently banned in 29 countries [445]

Having confirmed that interstitial injection of lentiviral vectors does not result in the production of transgenic offspring, testis injected interstitially with lentiviral vectors were analysed to determine the cell type(s) being targeted. Testes were analysed at three, seven and ten days post lentiviral injection into the interstitial compartment. Presence of the lentiviral reporter gene (GFP) was found in up to 9.3% of Leydig cells at 3dpi and 7dpi, but was absent from injected testis after 10 days as a result of targeted cells undergoing apoptosis. Increased apoptosis of cells exposed by lentivirus has also recently been reported in human hematopoietic stem and progenitor cells as a result of lentiviral vectors triggering signalling through Tumour Protein 53 (p53), a protein encoded by a tumour suppression gene, known to have a role in stem cell maintenance and in the initiation of apoptosis [454], though whether this is the mechanism through which lentiviral targeted Leydig cells and undergoing apoptosis is yet to be confirmed.

One type of viral vector that has shown some promise for the targeting of Leydig cells is adenovirus. In a recent study by Penny *et al* (2017) GFP and/or Cre expression adenoviral vectors were injected into the testicular interstitial compartment of mice carrying floxed

Gata2 and Gata4 alleles [175]. As a result, testes injected with GFP expressing adenoviral vectors demonstrated GFP positive Leydig cells and those injected with adenoviral vectors expressing Cre resulted in ablation of the Leydig cells as a result of the excision of the Gata2 and Gata4 alleles in the targeted cells. The study also noted that transgene expression persisted for up to 12 days before reporter expression was lost from the targeted cells and, to coincide with this, a delayed influx of immune cells into the testis at the same time point. To characterise adenoviral targeting of Leydig cells further, testes injected with these same adenoviral vectors were analysed at three and ten days post interstitial injection and we found that around 20% of Leydig cells. Though targeting efficiency of adenoviral vectors appeared to be greater than when using lentiviral vectors, variability was high with some injected testis exhibiting as low as 8.8% and as high as 36.6% of cells targeted, a 4 fold difference in targeting efficiency. This may be due to some technical issues related to the injection procedure as opposed to ability of the viral vector to target the Leydig cells. Another avenue which could be investigated would be to inject juvenile reporter testis with Cre expressing adenoviral vector in an attempt to target the proliferating cell populations of the Leydig cells found in juvenile testis. This would result in delivered transgene (Cre) expression in daughter cells deriving from the targeted juvenile cells, affording potential to increasing the final number of cells targeted. Transgene expression was still detectable at ten days post injection, though at this time point, targeted cells were interacting with Mac2+ macrophages, reflecting the delayed immune response reported by Penny et al (2017) and indicating a possible clearance of adenoviral targeted cells from the testis soon after ten days post injection. Collections at later time points post injection would also provide further information as to the longevity of the adenoviral vector delivered transgene expression with previous studies suggesting a loss of delivered transgene expression between ten and thirty days post injection in Leydig cells, though this may be dependent on host species with Blanchard *et al* investigating the use of adenoviral vectors in rat testis [175, 441].

The Sertoli cells are key regulators of both the endocrine and the spermatogenic functions of the testis [7-9]. As with both the germ cell and the Leydig cell populations, attempts to target the Sertoli cell population using viral vectors have been made [45, 330, 336, 347, 396, 397, 441, 443], the first being Blanchard and Boekelheide [441] who targeted rat Sertoli cells *in vivo* by introducing adenovirus into the tubular compartment via the rete testis with similar experiments in the mouse being performed. There has been some success using adenoviral

vectors for gene delivery; with evidence of partial restoration of spermatogenesis following adenovirus mediated gene delivery in Steel/Steel(dickie) mutant mouse testis (a mouse model that is otherwise infertile) [443]. However, a number of studies have reported an immune response as a result of adenovirus injection with some demonstrating cells undergoing apoptosis within the targeted seminiferous tubules. These observations were validated in a later study confirming that adenoviral vectors are unsuitable as vehicles for gene delivery to Sertoli cells [330, 336, 397, 441].

Using lentiviral vectors to target Sertoli cells has proven more suitable with no reports of testicular disturbances following introduction of lentiviral particles into the seminiferous tubules [45, 396, 397, 399]. The majority of published viral targeting of Sertoli cell studies have delivered viral particles to the seminiferous tubules via the efferent duct, the exception being Blanchard and Boekelheide [441] in the rat testis. In the study conducted by Li *et al* (2013), out of 24 males injected with lentivirus into the efferent duct, only 21 were fertile. This is not discussed at length nor attributed to a particular cause throughout the paper and no histological images of 'infertile' testes are provided following lentiviral injection. However, previous unpublished experiments from our group have suggested that efferent duct injections may result in negative testicular phenotypes such are distended tubules and vacuolisation presumably as a result of damage or blockage to the delicate efferent duct structures. By injecting directly into the rete compartment at low pressures, impacts on testicular histology have been circumvented and this has also been confirmed with the observation of both an uninterrupted testicular histology alongside lentiviral delivered transgene expression following delivery using low pressure injection. This improvement in delivery to the seminiferous tubules will assist in future experiments, ensuring that any testicular phenotypes present are a direct result of the delivered transgene expression as opposed to the injection itself.

The results obtained from the experiments described in this chapter have established a protocol for the injection of vectors into the interstitial compartment of the testis allowing the targeting of Leydig cells using either Lenti- or Adeno- viral vectors. Following this, results have demonstrated the lack of germline transmission following injection of Lentiviral vectors into the interstitial compartment, disputing previous studies suggesting that by using this technique, spermatogonial stem cells could be transduced. Injection of lentiviral vectors into

the interstitial compartment resulted in targeting of the adult Leydig cell population, however, the cells targeted by the lentiviral vector underwent apoptosis soon after injection. This was not the case for Leydig cells targeted with Adenoviral vectors, however, due to the increased immunogenicity and the transient expression of transgenes with these vector types, further investigation into the longevity of Adenoviral vector delivered transgenes would be beneficial.

Delivery of Lentiviral particles to Sertoli cells has previously been reported in the literature to be more appropriate than using Adenoviral vectors. Here, an improved methodology for the delivery of Lentiviral vectors has been described allowing delivery of lentiviral vectors with minimal side effects.

To determine the practical uses of using this methodology coupled with the delivery of lentiviral vectors, the subsequent chapters will describe the generation of a model for use as a 'testbed' for repair/rescue of a knock out gene.

Chapter 4 Identifying a model for Testicular Repair and Determining the Effects of Single and Double Somatic Cell Androgen Receptor Knock-Out Models

4.1 Introduction

Having established a technique to deliver viral vectors to the somatic cells of the testis, the key interest was to determine whether and how existing knowledge could be applied to exploit this technology to identify its potential for use for gene therapy or repair. To address this, a model of male infertility or hypogonadism would be required. An ideal model for proof of principle of gene repair or replacement would consist of a gene whose function within the testis has been well defined in the literature and whose impacts on testis function can be easily analysed; for example, presence or absence of ongoing spermatogenesis.

The effects of androgens through androgen receptor (AR) signalling are known to be essential for both the steroidogenic and spermatogenic functions of the testis as described [6, 45, 48-50, 52, 101, 102, 108, 109, 124, 455-460]. AR, a steroid nuclear hormone receptor, is expressed by a number of cell types in a wide range of tissues including in a number of the somatic cells located within the testis in the male. Genetically modified mouse models in which Androgen receptor has been completely knocked out (ARKO) have previously been generated. This resulted in overall feminisation, with reductions in body weight and impacts on testicular descent, development and function; with a decrease in spermatogenesis and testosterone production being observed in ARKO models [60, 101, 456].

Despite androgen signalling being crucial for spermatogenesis, germ cells themselves do not express AR; indicating that the regulation of spermatogenesis through androgens must be mediated through other testicular cell types within a more complex signalling network [39, 40, 461]. To dissect this androgen signalling network, testicular cell specific knock outs of androgen receptor have been well characterised, each with differing impacts on both the endocrine and reproductive functions of the testis.

Sertoli cell (SC) AR knock out (SC-ARKO) models have suggested that a loss of Sertoli cell AR may impact the development and proper function of Leydig cells (LC) as well as reduce the

capacity of Sertoli cells to support germ cells past the meiotic stages of spermatogenesis [6, 48, 49, 101, 457, 458]. Sertoli cell function was also found to be compromised in a peritubular myoid cell specific ARKO (PTM-ARKO) with a decrease in the expression of Sertoli cell specific androgen dependent genes and disruption in the orientation of Sertoli cells resulting in PTM-ARKO males being infertile [52]. Alongside this, some Leydig cells within the PTM-ARKO testis were also found to be unable to fully differentiate into adult Leydig cells further illustrating the importance of paracrine signalling in somatic cell function [124]. A Leydig cell AR knock out (LC-ARKO) revealed that AR was dispensable for the attainment of normal Leydig cell numbers but is vital for the functional maturation of the adult Leydig cell population. Unlike other testicular cell specific ARKO models, the LC-ARKO males were still fertile in early adulthood (post-natal day 80), however, when left to age further, wide spread loss of germ cells became increasingly apparent, suggesting that Leydig cell AR activity may (whether it be directly or indirectly) have a protective role throughout the cycle of spermatogenesis within the seminiferous tubules [108].

These testicular cell specific androgen receptor knock models were all generated using Cre/*Lox P* technology. In each of the models, the promoter driving Cre expression was active early in testicular development, allowing the investigation of the roles of AR throughout development. However, this made investigating the role of AR in the adulthood difficult as it was impossible to determine whether impacts are a result of perturbed testicular development or due to disrupted AR signalling specifically in adulthood. To circumvent this, one study introduced Cre expressing lentiviral vectors into the seminiferous tubules of AR floxed mice to generate an adult SC-ARKO model. In doing so, it became possible to dissect the roles of AR specifically in adult Sertoli cells and, due to the evident germ cell arrest during meiosis following injection of Cre expressing lentiviral particle, concluded that Sertoli cell AR is essential for complete spermatogenesis in the adult testis [45].

The number of studies investigating testicular cell specific androgen receptor roles clearly indicate that the testicular somatic cells work in concert to maintain steroidogenesis and spermatogenesis. However, the interactions between these cell types and the role that AR plays in these relationships appears to be complicated and is yet to be fully described as to whether these cell types complement or overlap each other's specific functions and whether a loss of AR from more than one cell type would generate an additive phenotypic effect.

4.2 Hypothesis & Aims

4.2.1 Hypothesis

Given the previously published findings on the roles of somatic cell AR expression in testicular function, it is hypothesised that loss of AR from both the Sertoli and Leydig cells will have a significant impact on adult testis function. Furthermore, the generation of this model will allow a comparison to both single and double somatic cell ARKOs to give further insight into the roles of AR on these specific cell types. Finally, analysis of this model, in context with the other cell specific ARKO models available will help to gain a wider perspective of AR signalling in the testis and allow the most suitable ARKO model for gene rescue to be identified.

4.2.2 Aims

From this hypothesis, a number of aims were established:

Aim 1: To establish the expression pattern of a recently published Aromatase-iCre using a fluorescent reporter mouse.

Aim 2: To generate and validate a Sertoli and Leydig cell AR knock out using the Aromatase-iCre for both the study of AR signalling in the testis and in context with previously generated single and double somatic cell AR knock outs.

Aim 3: To identify the most suitable cell specific ARKO model to determine the potential for lentiviral transgene delivery in the adult testis.

4.2.3 Approach

To achieve the aims described, single and double testicular somatic cell specific AR knock outs were generated as described in section 2.1.3.3. Tissue was collected at adulthood between pnd80-100 and compared to the wild type littermate controls of each respective cell-specific ARKO model.

4.3 Results

4.3.1 Androgen Receptor Expression in the Adult Testis

Immunostaining for AR shown in Figure 4.1 illustrates the expression of AR by Sertoli cells, Peritubular Myoid cells and Leydig cells (and lack thereof in the germ cell populations) in the adult testis.

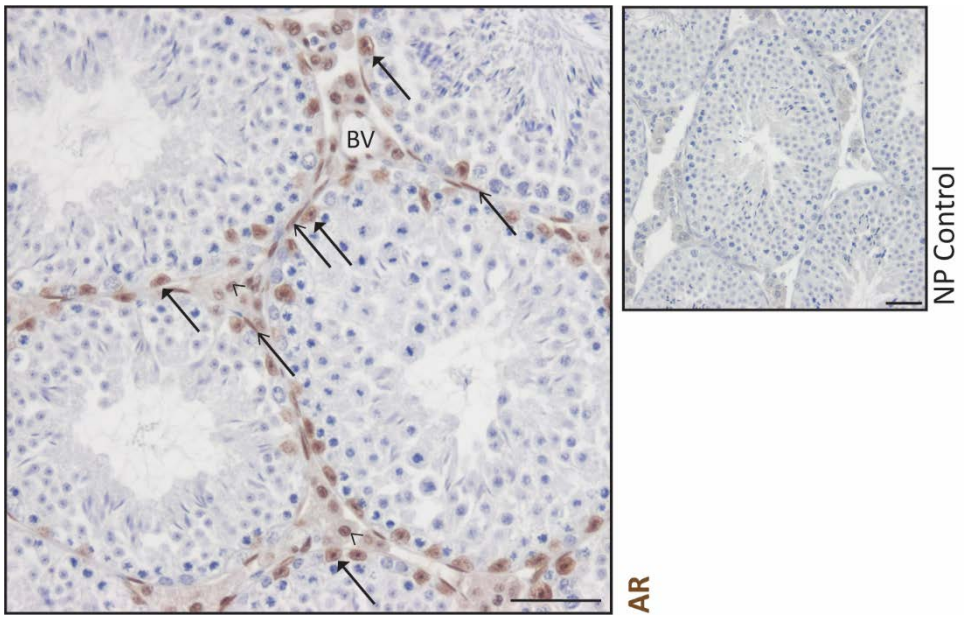


Figure 4.1 Expression of AR in a wild type Testis. Immunostaining for Androgen Receptor (AR) in a wild type testis reveals expression of AR in Sertoli cells (closed arrows), peritubular Myoid Cells (open arrows) and Leydig cells (open arrow heads). A Blood vessel (BV) in the interstitial compartment can also be seen to express AR. Scale Bar = 50µm.

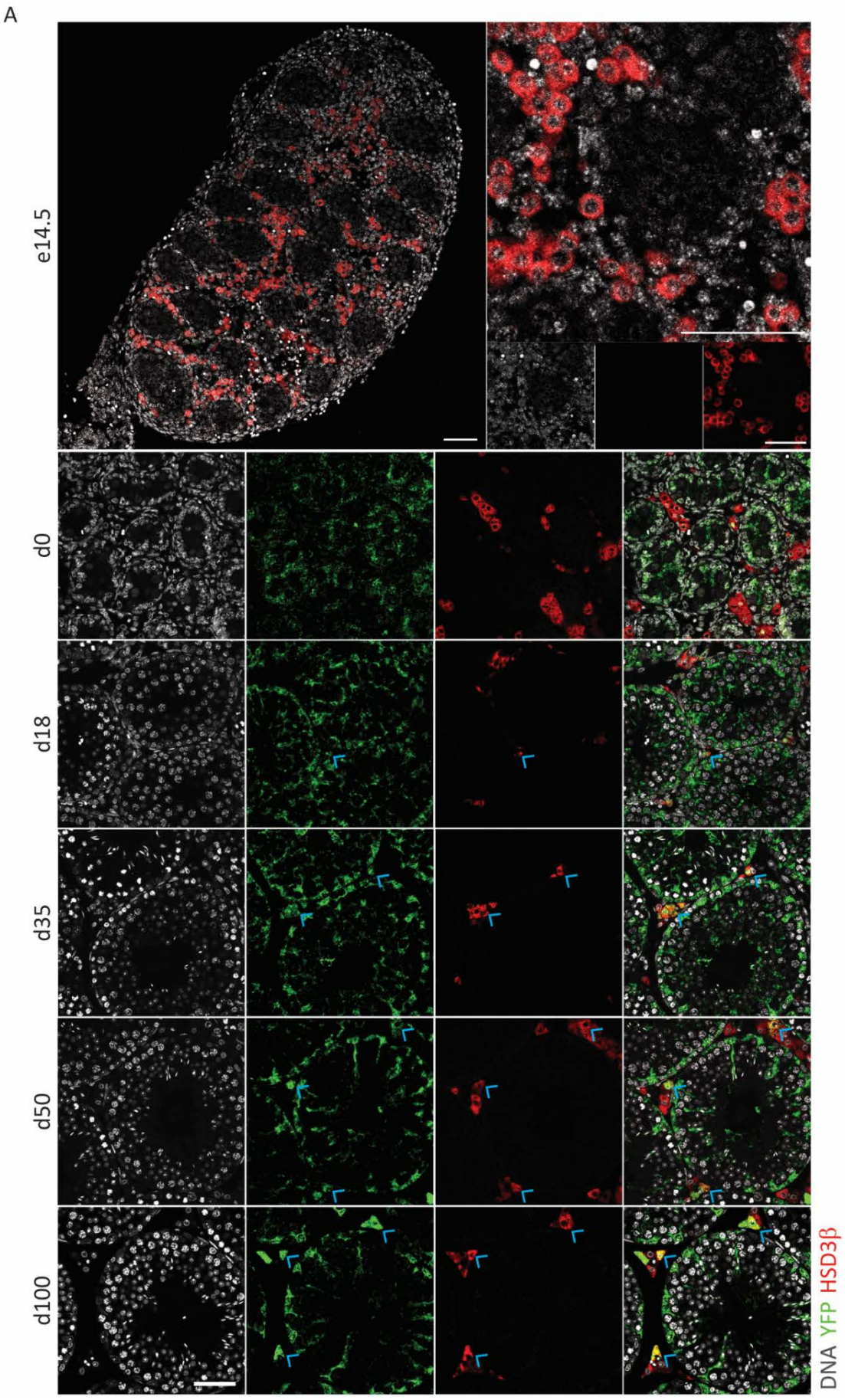
4.3.2 Generation of Conditional YFP Reporter Line using the Aromatase Cre

The suitability of the Aromatase-Cre for targeting Sertoli and Leydig cells was first established.

4.3.2.1 Characterisation of Aromatase Cre Expression using the Reporter

To characterise the expression of Cre recombinase driven by the human CYP19/Aromatase promoter, mice positive for the Aromatase-iCre were mated to yellow fluorescent protein (YFP) reporter mice (described in 2.1.3.2) and offspring was collected at differing age points

through development and into adulthood. The constructs used to generate the Aromatase-Cre mice used in these studies [17] contained two response elements, a steroidogenic factor 1- (SF1)- site and a CRE-like sequence binding CREB protein, critical for cyclic AMP induction of the human CYP19/Aromatase promoter known to be active in the testis and ovary; specifically within the somatic cells [462]. Double fluorescent immunostaining for YFP and HSD3β reveal Cre-mediated YFP expression in the Sertoli cells from postnatal day 0 and within the Leydig cells from postnatal day 18 (Figure 4.2A). YFP expression appeared to be in the majority of Sertoli cells at pnd100, though this was not confirmed with stereological methods. Quantification of YFP and HSD3β double positive cells in the testis at these different age points reveal that YFP expression continued to increase throughout testis development in 24.78% of the total Leydig cell population at postnatal d100 (Figure 4.2B). This suggests that the CYP19/Aromatase promoter activity may be activated early in Leydig cell development in progenitor Leydig cells (from postnatal day 10) and expression of YFP is subsequently being inherited following Leydig cell proliferation during the pubertal period, resulting in 24.78% of the final adult Leydig cell population expressing YFP [463].


A
e14.5
d0
d18
d35
d50
d100
DNA YFP HSD3β

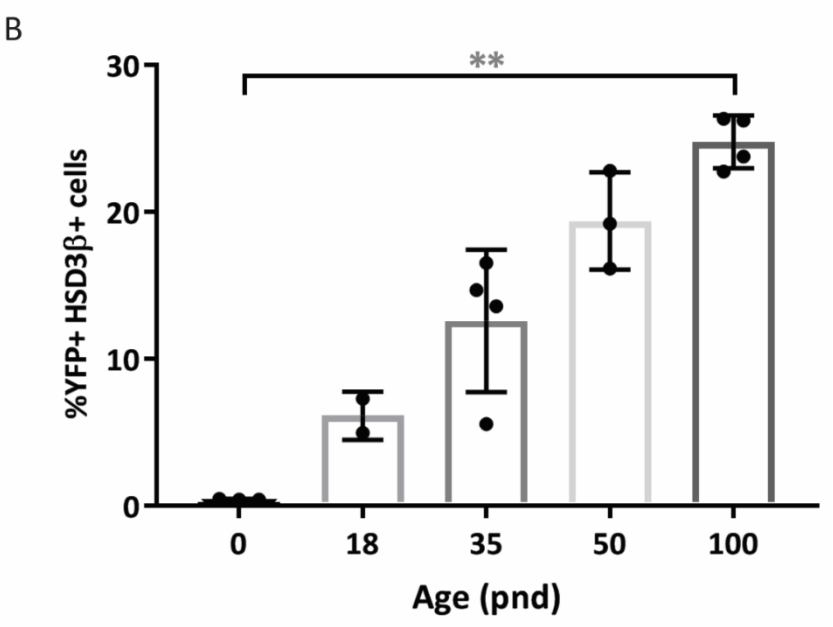


Figure 4.2 Analysis of Aromatase-iCre expression using a YFP reporter. (A) Immunostaining for yellow fluorescent protein (YFP; green) and hydroxysteroid dehydrogenase 3-beta (HSD3β; red) illustrate expression of Aromatase-iCre at different stages of testicular development. Blue open arrowheads identify areas of co-localisation, signifying expression of Aromatase-iCre in Leydig cells. YFP expression was also detected in the majority of Sertoli cells within the tubules. Scale Bars: 50µm. (B) Quantification of YFP+ HSD3β+ cells revealed an increase in cells positive for YFP as animals aged to pnd100. Kruskal-Wallis one-way ANOVAs were performed followed by a Dunn's multiple comparison testis to determine differences between groups (** denotes p=<0.01). Bars represent mean ±SEM. (pnd0: n=3, pnd18: n=2, pnd35: n=4, pnd50: n=3, pnd100: n=4).

4.3.3 Androgen Receptor Knock out in the Somatic cells of the Testis

Single and double testicular somatic cell specific AR knock out models were generated to explore the roles of AR expressed by the different testicular somatic cell types in adulthood and for the identification of suitable cell specific ARKO model for repair/rescue. To do so, adult males collected at around 3 months of age (pnd 80 - 100) were used from each of the ARKO models for analysis.

4.3.3.1 Generation of double and single cell AR knock out models using smMHC-eGFP/Amh-Cre and Aromatase-Cre

Single and double testicular somatic cell specific AR knock outs were generated as described in section 2.1.3.3. Using the Aromatase-iCre (of which the expression in the testis was characterised in 4.3.2.1), a double somatic cell knock out in both Sertoli (from pnd 0, though AR is not present in mouse Sertoli cells until around pnd 4 [464]) and Leydig cells (from as early as pnd 18) was generated (Figure 4.4A). PCR amplification of genomic DNA isolated from ear clips of these animals demonstrate the inheritance of the iCre (390bp band) alongside the internal positive control gene interleukin 2 (324bp band) (Figure 4.4B). This ARKO model will be referred to as the SC-LC-ARKO model from this point onwards. Littermates negative for iCre will also be referred to as either WT or WT littermates depending on the models being included in the analysis.

Animals deriving from the breedings using a smMHC-eGFP-Amh-Cre (illustrated in Figure 4.4C) generated offspring with AR knocked out in Sertoli cells (SC-ARKO), peritubular myoid cells (PTM-ARKO) or both Sertoli and peritubular myoid cells (SC-PTM-ARKO), thus generating both single and double testicular somatic cell AR knock outs as well as Cre negative (WT) littermates within one generation of offspring. The smMHC-eGFP/Amh-Cre is a combination of the smMHC-eGFP-Cre and the Amh-Cre, both of which have been characterised and utilised previously and published in the literature [48, 52, 60, 124, 429, 465]. Genotyping of these offspring confirmed Cre inheritance with PCR amplification of a 239bp product in SC-ARKOs, a 264bp product in PTM-ARKOs and both a 239bp and a 264bp product in SC-PTM-ARKO animals (Figure 4.4D). All lanes (except that of the water/no template control) also revealed the 324bp band representative of the internal positive control gene; interleukin 2 (Figure 4.4D). Genotyping was performed by technical staff in Professor Lee Smith's group, Centre for Reproductive Health, University of Edinburgh.

For a wider perspective on the impacts of single and double somatic cell ARKO, complete ARKO and LC-ARKO animals [108] were represented alongside SC-ARKO, SC-PTM-ARKO and the SC-LC-ARKO models. Where appropriate, WT littermate controls have been combined in analyses to aid comparisons between cell specific ARKO models. Time points at which Cre recombinase activity can be detected in each of the lines, as well as the time point of AR expression in each of the somatic cell types is represented in Figure 4.3.

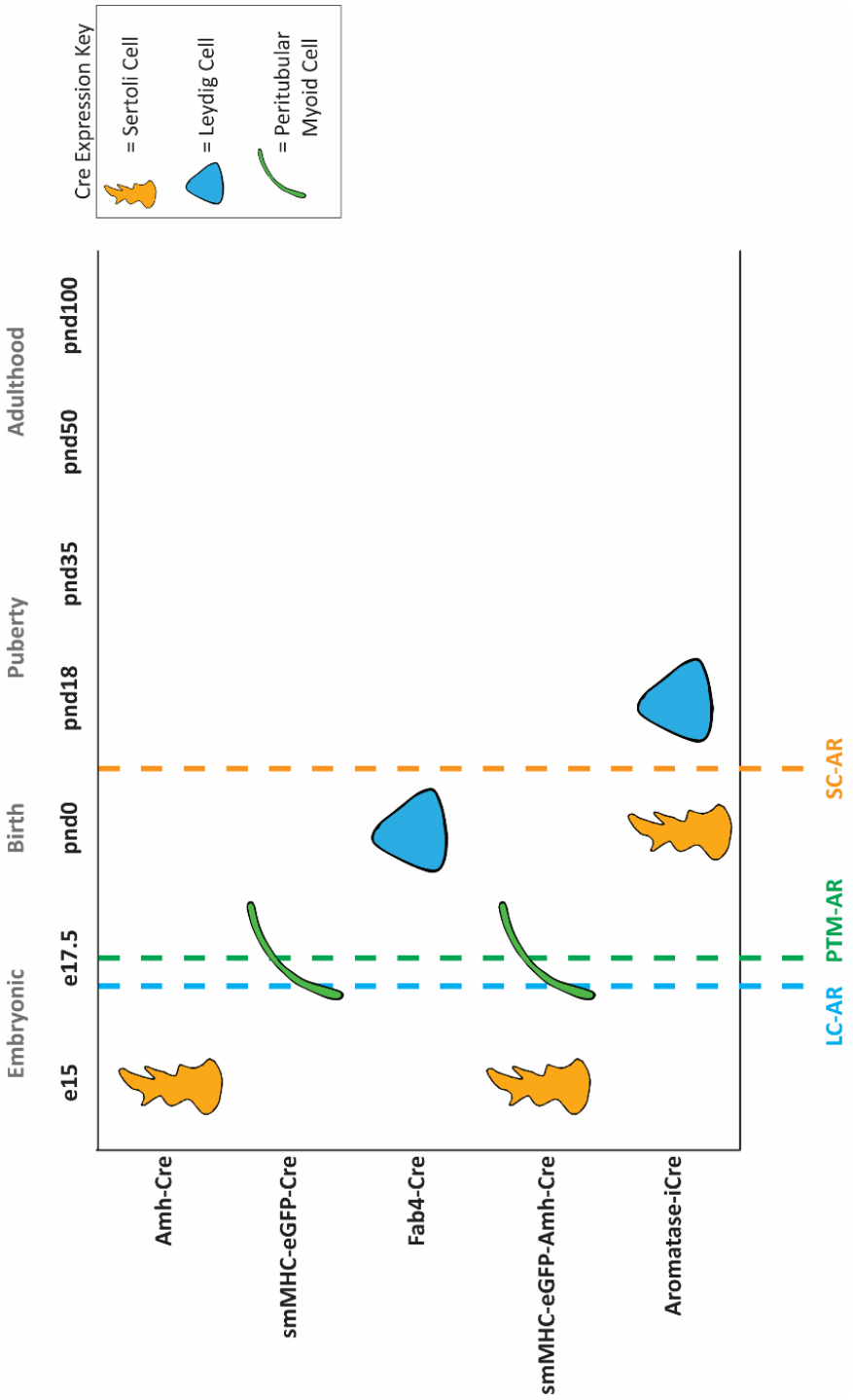


Figure 4.3 Cre Activity in lines used to generate cell specific ARKO models. Schematic representing the age points at which Cre is active in each of the lines used to generate the single and double somatic cell ARKO models. Cre activity in a cell type is indicated by schematic drawing of that cell type. Also represented is the point at which AR is expressed in a WT mouse testis in Leydig cells (LC-AR), peritubular myoid cells (PTM-AR) and Sertoli cells (SC-AR).

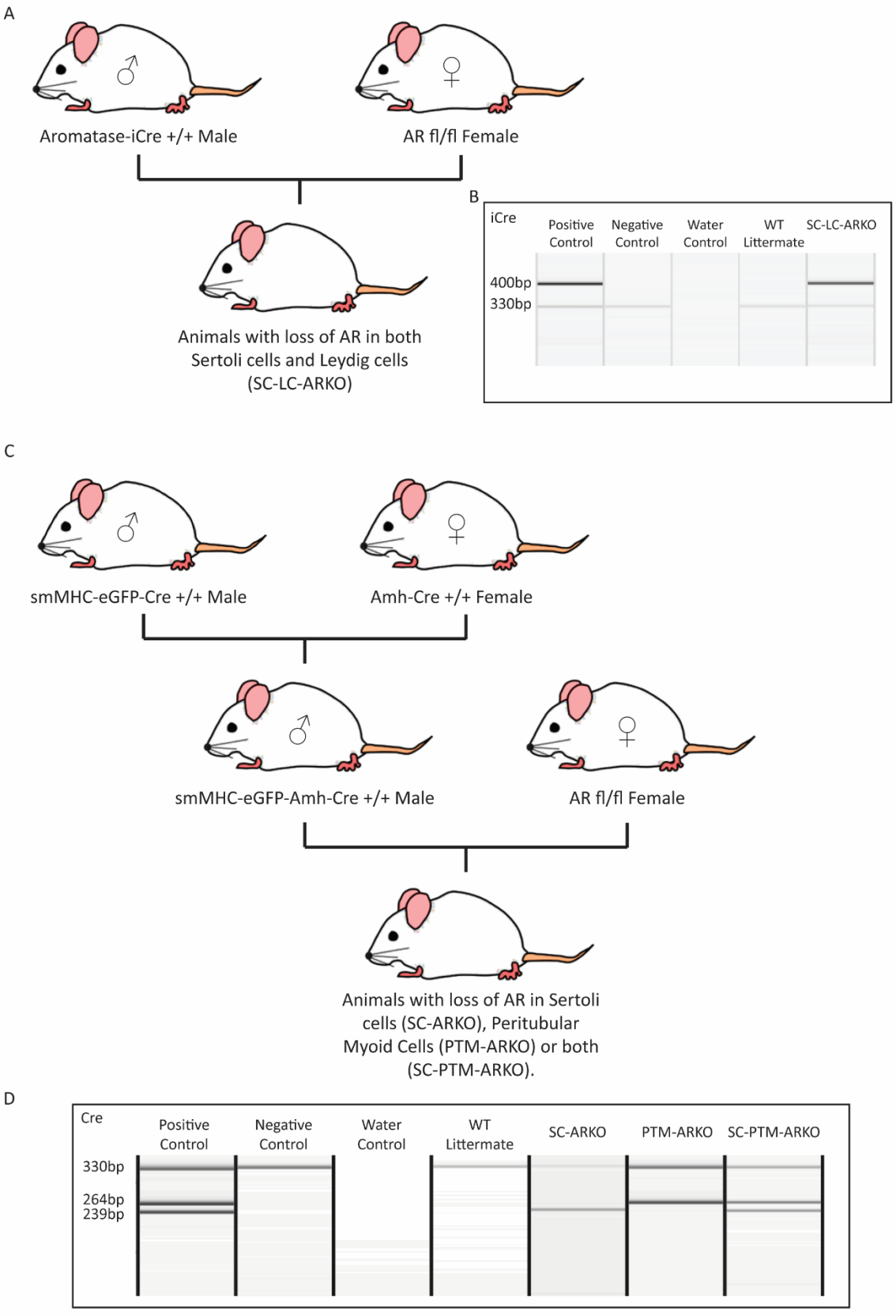

A
Aromatase-iCre +/+ Male
AR fl/fl Female
Animals with loss of AR in both Sertoli cells and Leydig cells (SC-LC-ARKO)
B
iCre
Positive Control
Negative Control
Water Control
WT Littermate
SC-LC-ARKO
400bp
330bp
C
smMHC-eGFP-Cre +/+ Male
Amh-Cre +/+ Female
smMHC-eGFP-Amh-Cre +/+ Male
AR fl/fl Female
Animals with loss of AR in Sertoli cells (SC-ARKO), Peritubular Myoid Cells (PTM-ARKO) or both (SC-PTM-ARKO).
D
Cre
Positive Control
Negative Control
Water Control
WT Littermate
SC-ARKO
PTM-ARKO
SC-PTM-ARKO
330bp
264bp
239bp

Figure 4.4 Generation of Single and Double Somatic cell Androgen Receptor Knock Out using Aromatase-iCre and smMHC-eGFP-Amh-Cre. (A) Generation of animals with a loss of AR in both Sertoli and Leydig cells (SC-LC-ARKO). (B) PCR analysis of genomic DNA isolated from WT littermate controls and SC-LC-ARKO ear clips. Bands at 330bp are representative of the Interleukin-2 internal positive control. Bands at 400bp indicate inheritance of Aromatase-iCre. No inheritance of Aromatase-iCre was detected in WT littermate and Negative controls. No amplification of DNA was detected in Water controls. (C) Schematic illustrating the breeding strategy to generate animals with a loss of AR in Sertoli cells (SC-ARKO), peritubular myoid cells (PTM-ARKO) or both (SC-PTM-ARKO). (D) PCR analysis of genomic DNA isolated from WT littermate controls, SC-ARKO, PTM-ARKO and SC-PTM-ARKO ear clips. Bands at 324bp are representative of the Interleukin-2 internal positive control. Bands at 239bp and/or 264bp indicates inheritance of Amh-Cre and/or smMHC-eGFP-Cre respectively. No inheritance of Amh-Cre or smMHC-eGFP-Cre was detected in WT littermate and Negative controls. No amplification of DNA was detected in Water controls

Table 4.1 Table describing ARKO models, Cre lines used to generate and the nomenclature used to reference each model.

ARKO Model	Cre Lines Used	Nomenclature
Sertoli cell Androgen Receptor knock out	Amh-Cre	SC-ARKO
Peritubular myoid cell Androgen Receptor knock out	smMHC-eGFP-Cre	PTM-ARKO
Leydig cell Androgen Receptor knock out	Fabp4-Cre	LC-ARKO
Sertoli and Peritubular myoid cell Androgen Receptor knock out	smMHC-eGFP/Amh-Cre	SC-PTM-ARKO
Sertoli and Leydig cell Androgen Receptor knock out	Aromatase-iCre	SC-LC-ARKO

4.3.3.2 Gross Morphology of Sertoli and Leydig cell double AR knock out alongside other ARKO models

Lack of Androgen signalling, whether through a knockout of androgen receptor or as a result of a mutation in androgen receptor, results in a feminised phenotype; including a reduction in overall body weight and feminised external genitalia. In contrast to the complete ARKO models, all single and double somatic cell ARKOs exhibited no differences in body weight in adulthood relative to WT controls (Figure 4.5A). However, anogenital distance (an indicator of androgen exposure during development) in PTM-ARKO and SC-PTM-ARKO was

significantly reduced (Figure 4.5B), suggesting a disruption in androgen signalling in the male reproductive tract during foetal development in these models.

Previously published studies using SC-ARKO, PTM-ARKO and LC-ARKO mice reported significant reductions in testis weight. As expected, this was also clearly evident upon dissection and following testis weight recording for both SC-PTM-ARKO testis (75.75% reduction) and SC-LC-ARKO (68.17% reduction), compared to their respective controls (Figure 4.5C&D). This would suggest that the cellular composition of testis in both double somatic cell ARKOs has been compromised as a result of the disrupted androgen signalling.

Cross sections of each of the somatic cell ARKO models illustrates the modifications in testicular histology as a result of AR loss. In particular the absence of elongated spermatids and loss of lumens in seminiferous tubules in both SC-PTM-ARKO and SC-LC-ARKO models mirroring that seen in both SC-ARKO and PTM-ARKO testis. Immuno-localisation of AR confirms a loss of AR expression in Sertoli and peritubular myoid cells in the SC-PTM-ARKO testis as well as some AR loss in Leydig cells in these testis. In SC-LC-ARKO, AR is seen to be lost in Sertoli cells and a proportion of Leydig cells, as expected.

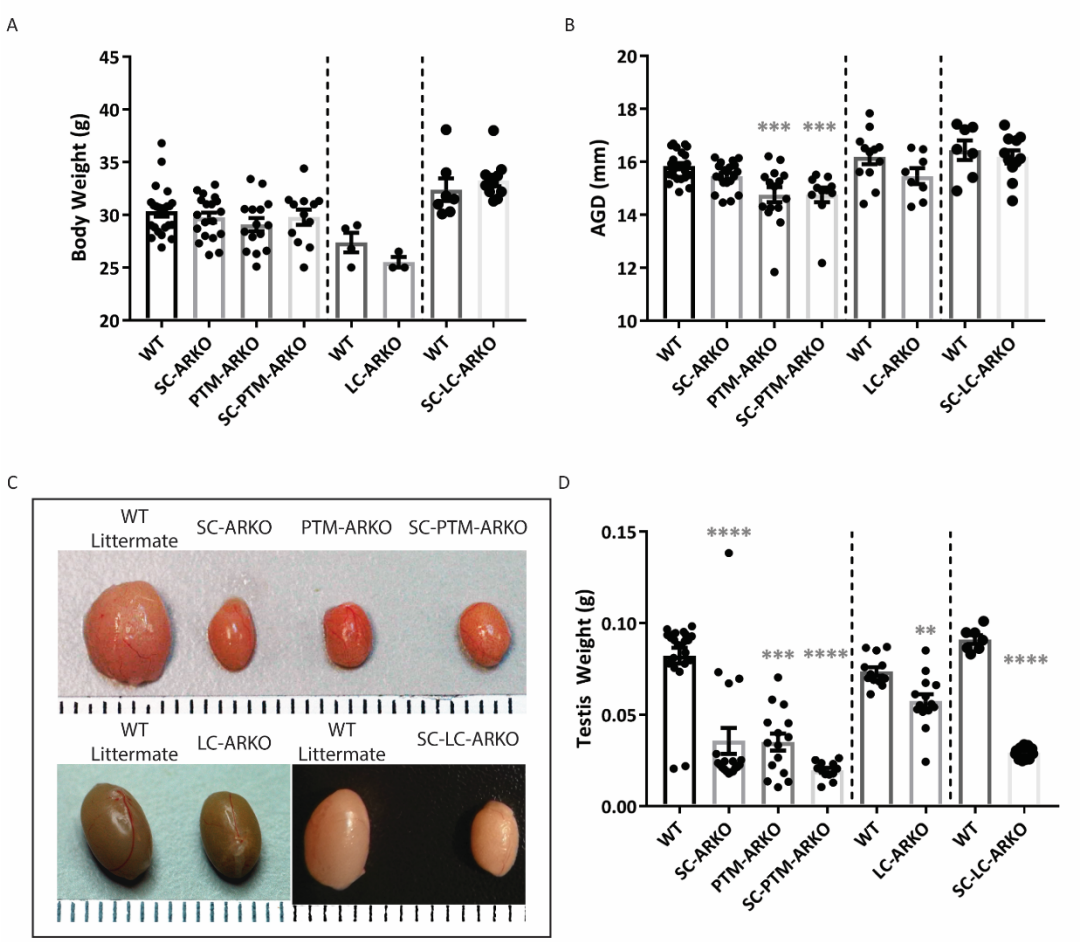


Figure 4.5 Gross Morphology of Adult Single and Double Somatic cell ARKOs. (A) Body weights of SC-ARKO (n= 19), PTM-ARKO (n= 15), LC-ARKO (n= 3) SC-PTM-ARKO (n= 12) and SC-LC-ARKO (n= 11) in comparison to the WT controls (n= 4-23) of each respective ARKO model. (B) Measurements of Anogenital Distance (AGD) of SC-ARKO (n= 19), PTM-ARKO (n= 15), LC-ARKO (n= 8), SC-PTM-ARKO (n= 11) and SC-LC-ARKO (n= 11) in comparison to the WT controls (n= 7-23) of each respective ARKO model. (C) Photographs of SC-ARKO, PTM-ARKO, SC-PTM-ARKO and SC-LC-ARKO and respective WT controls at dissection. (D) Testis weights of SC-ARKO (n= 19), PTM-ARKO (n= 15), SC-PTM-ARKO (n= 12) and SC-LC-ARKO (n= 11) in comparison to the WT controls (n= 7-23) of each respective ARKO model. Bars represent mean ±SEM. For measurements of SC-ARKO, PTM-ARKO and SC-PTM-ARKO, Kruskal-Wallis one-way ANOVAs were performed followed by a Dunn's multiple comparison tests to determine differences between groups. T-tests were used for WT vs LC-ARKO and WT vs SC-LC-ARKO measurements. (** denotes p=<0.01, *** denotes p=<0.001 and **** denotes p=<0.0001). Collection of tissue, measurements and staining's were performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and Annalucia Darbey (SC-LC-ARKO).

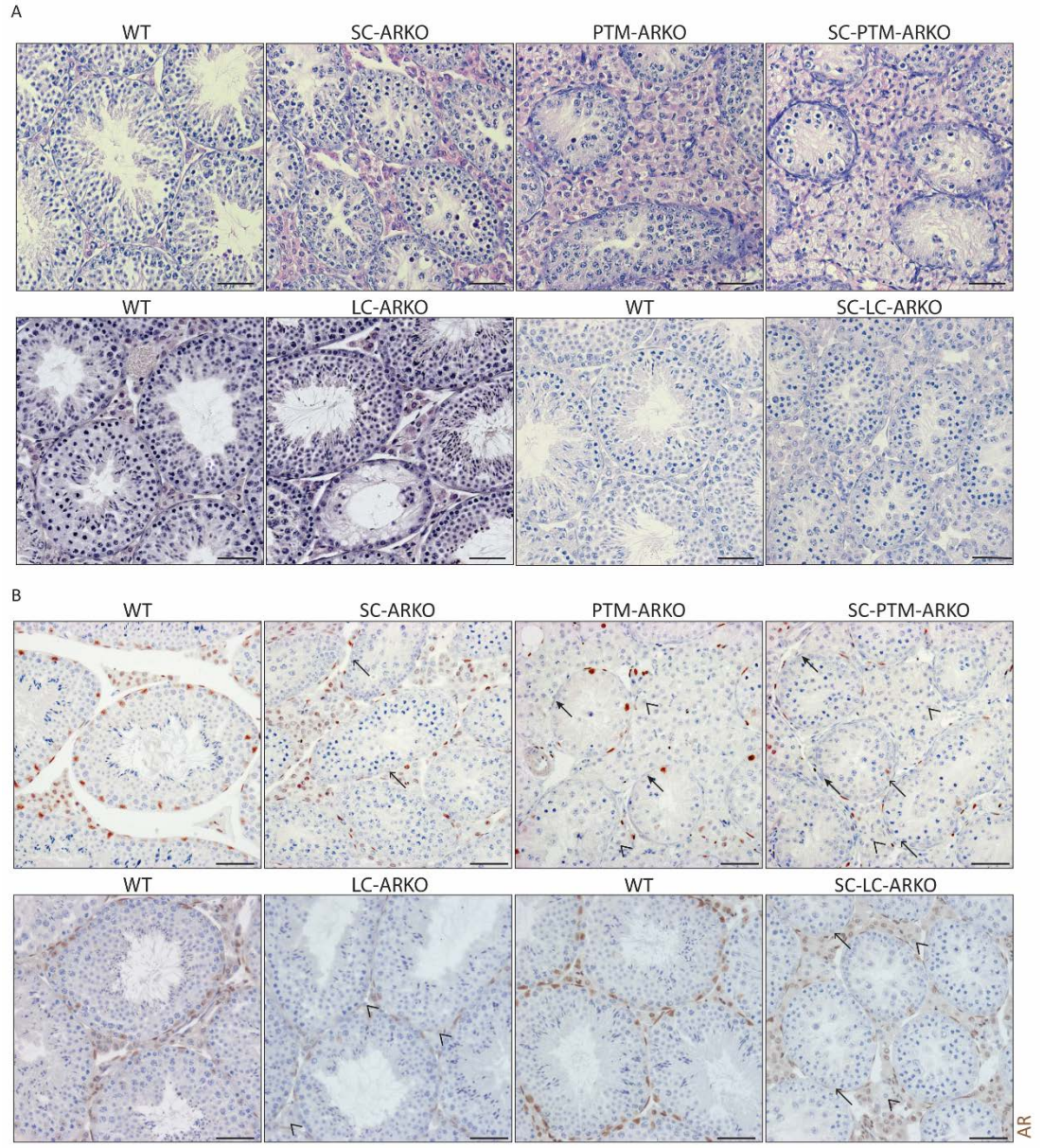


Figure 4.6 Testicular Histology of Single and Double Somatic cell ARKOs in Adulthood. (A) Representative H&E stained and (B) AR immuno-staining of testis sections from SC-ARKO, PTM-ARKO, LC-ARKO, SC-PTM-ARKO and SC-LC-ARKO adjacent to their respective WT littermate controls. (Open arrow = Sertoli cell with AR loss, closed arrow = Peritubular myoid cell with AR loss, open arrow heads = Leydig cell with AR loss – Note loss of AR from Leydig cells in PTM-ARKO and SC-PTM-ARKO testis) Scale Bars = 20µm.

4.3.3.3 Impacts of double somatic cell AR knockout on Sertoli Cells in Adult Testis

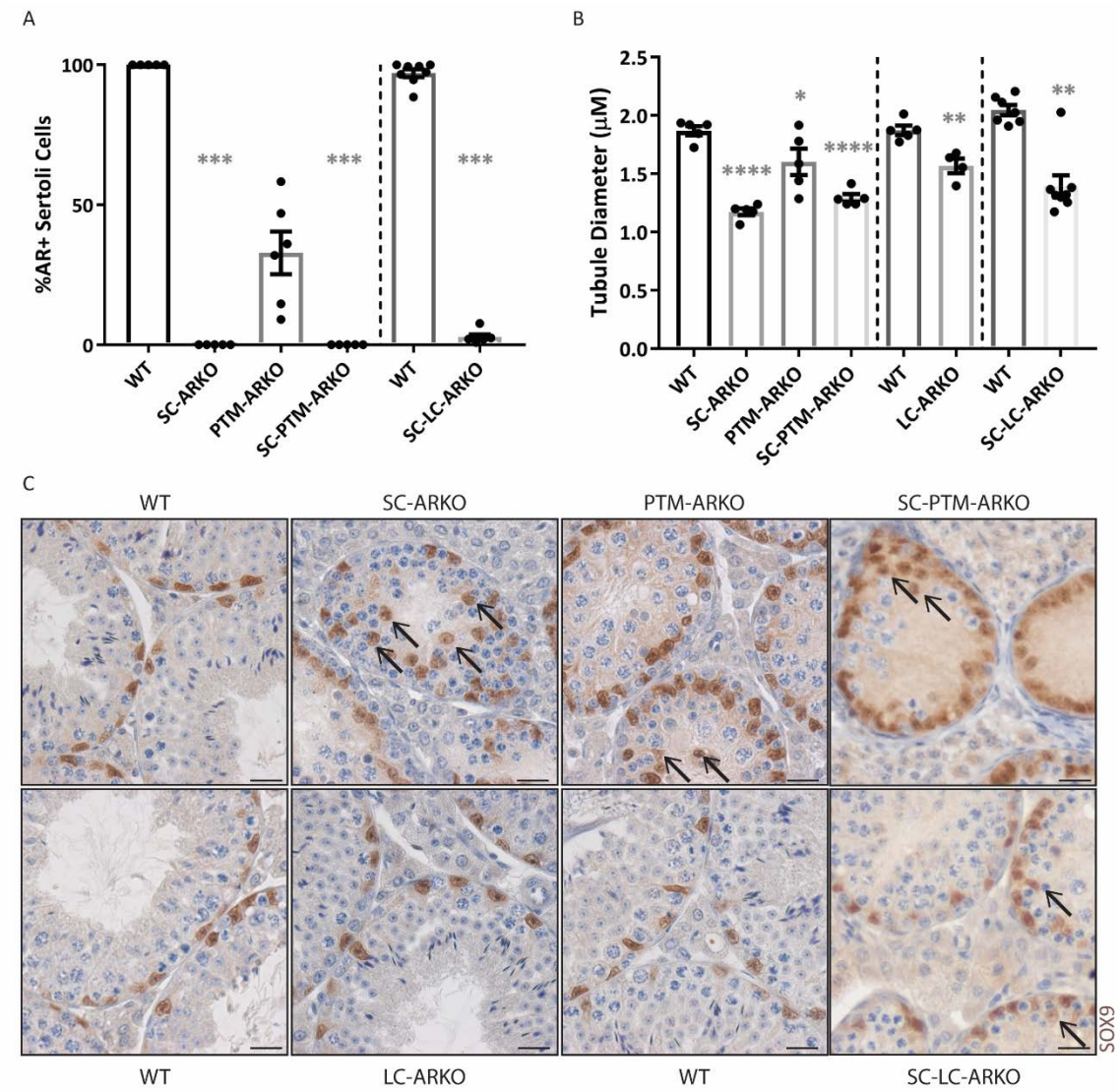
Quantification of AR in Sertoli cells confirmed a significant reduction of Sertoli cell AR expression in both SC-PTM-ARKO and SC-LC-ARKO (Figure 4.7A), resulting in a reduction in tubule diameter in both SC-PTM-ARKO and SC-LC-ARKO testis compared to their respective controls, suggesting a disruption in Sertoli cell functions such as support of germ cell development and secretion of fluid essential for support of germ cells and lumen formation (Figure 4.7B).

Signalling of androgens through Sertoli cell AR has been shown to be essential for progression of spermatogenesis past meiosis, potentially as a result of improper formation of the blood testis barrier (BTB) and reduced Sertoli cell fluid secretion into the lumen of the seminiferous tubules; reducing delivery of crucial factors necessary to support spermatogenesis [25, 48]. Alongside this, peritubular myoid cell AR has also been shown to impact Sertoli cell function with expression of Sertoli cell specific gene products being reduced in PTM-ARKO testis [52]. As seen in SC-ARKO and PTM-ARKO publications, the organisation of the seminiferous tubules appears to be disrupted in SC-PTM-ARKO and SC-LC-ARKO testis with immunolocalization of Sry box-9 (Sox9) demonstrating loss of polarity of the Sertoli cell at the basement membrane (Figure 4.7C) . The lack of polarity has been shown to influence the presence of germ cells in the seminiferous tubules due to impact on Sertoli cell functioning, reportedly via AR mediated peritubular myoid cell signalling. The disorganisation present in SC-PTM-ARKO and SC-LC-ARKO testis, would indicate that it may be a collaborative effort between these two major cell types that are influencing Sertoli cell positioning in the tubules (Figure 4.7C).

Expression of Sertoli cell specific genes *Desert hedgehog (Dhh)* (known to be implicated in proper germ cell development) and *Aquaporin-8 (Aqp8)* (a transmembrane water channel protein believed to be essential for regulation of water homeostasis in a cell [466] and recently confirmed to be Sertoli cell specific [467]) were analysed to give an indication as to the impacts of double somatic cell ARKO on Sertoli cell function. Analysis of *Dhh* mRNA expression revealed a significant reduction in expression in SC-PTM-ARKO but not SC-ARKO, PTM-ARKO and SC-LC-ARKO testis (Figure 4.7D). This would suggest that AR signalling through either directly through Sertoli cell AR or indirectly through peritubular myoid cell AR

is sufficient to maintain *Dhh* expression in Sertoli cells. *Aqp8* expression was also significantly reduced in all cell specific ARKOs excluding LC-ARKO (Figure 4.7E), suggesting that Leydig cell AR expression, has no impact on the fluid production functions of Sertoli cells, but that both Sertoli and peritubular myoid cell signalling are crucial for this role.

The Sertoli cell has a wide range of roles in both the spermatogenic and steroidogenic functions of the adult testis [5, 7, 9] and together these results indicate that AR expression, in particular in both Sertoli and peritubular myoid cells, may be involved orchestrating some of these functions; namely in supporting spermatogenesis and in the development of a lumen within the seminiferous tubules.



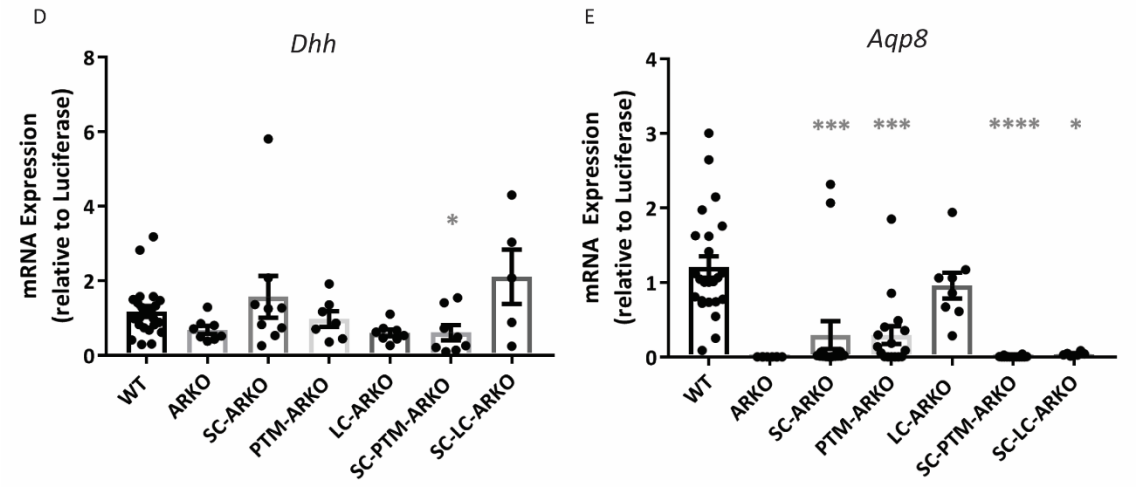


Figure 4.7 Impacts of double somatic cell ARKO on Sertoli cell function in the adult testis. (A) SC-AR expression in SC-ARKO (n= 5), PTM-ARKO (n= 6), SC-PTM-ARKO (n= 5) and SC-LC-ARKO (n= 7) and WT controls (n= 5-8). For measurements of SC-ARKO, PTM-ARKO and SC-PTM-ARKO, Kruskal-Wallis one-way ANOVAs were performed followed by a Dunn's multiple comparison testis to determine differences between groups. For WT vs SC-LC-ARKO comparisons, a Mann Whitney *U*-test was used. (** denotes p=<0.01, *** denotes p=<0.001 and **** denotes p=<0.0001). (B) Tubule Diameters (µm) in SC-ARKO (n= 5), PTM-ARKO (n= 5), SC-PTM-ARKO (n= 5), LC-ARKO (n= 4) and SC-LC-ARKO (n= 8) testis compared to respective WT controls (n= 5-7) (SC-ARKO, PTM-ARKO and SC-LC-ARKO testis were analysed for statistical differences compared to WT littermates using a one-way ANOVA followed by a Dunnet's multiple comparisons test, LC-ARKO testis; using an unpaired t-test and SC-LC-ARKO testis; using a Mann Whitney *U*-test). Bars represent mean ±SEM. (** denotes p<0.01 and **** denotes p=<0.0001). (C)Representative immunostaining of Sry box-9 (Sox9) WT, SC-ARKO, PTM-ARKO, LC-ARKO, SC-PTM-ARKO and SC-LC-ARKO testis sections. Arrows indicate Sox9 positive cells located away from the basement membrane of the seminiferous tubule. Scale Bars = 50µm mRNA expression of SC specific genes; (D) *Desert hedgehog (Dhh)* (one-way ANOVA followed by a Tukey's multiple comparisons test) and (E) *Aquaporin 8* (*Aqp8*) (Kruskal-Wallis followed by a Dunn's multiple comparison test) in WT (n= 23), SC-ARKO (n= 9), PTM-ARKO (n= 7), LC-ARKO (n= 8), SC-PTM-ARKO (n= 8) and SC-LC-ARKO (n= 5) adult testis. Bars represent mean ±SEM. (* denotes p<0.05 and **** denotes p=<0.0001). Measurements, staining's and qRT-PCR was performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and technical staff from Professor Lee Smiths group (SC-LC-ARKO). Analysis was performed by Annalucia Darbey.

4.3.3.4 Impacts of double cell AR knockout on Peritubular Myoid cells in Adult Testis

Peritubular myoid cells surround each of the seminiferous tubules and are known to be involved in the movement of tubular fluid aiding in the transport of spermatozoa within the tubules. Peritubular myoid AR signalling has been shown to have a significant impact on Sertoli cell function with a reduction in seminiferous tubule fluid, spermatogenesis and androgen dependant genes being reported in a PTM-ARKO testis [52]. However, it is unclear as to whether other testicular somatic cell AR have an impact on peritubular myoid cell function. Analysis of peritubular myoid cell specific genes, *Desmin* and *Endothelin-1* (*END1*) revealed a significant reduction in peritubular myoid cell function as a result of ARKO in PTM cells alone and in both Sertoli and peritubular myoid cells but not in other single somatic cell ARKOs (Figure 4.8 A&B). This would indicate that these genes and their downstream effects are being regulated through autocrine actions of AR on the peritubular myoid cells. Conversely, *Smooth Muscle Actin* (*SMA*) expression was also analysed and was found to be significantly reduced in SC-PTM-ARKO but not in PTM-ARKO testis alone (Figure 4.8C). This could suggest some influence of Sertoli cell on *SMA* expression and the main contractile apparatus of peritubular myoid cells; a key role of peritubular myoid cells in the adult testis.

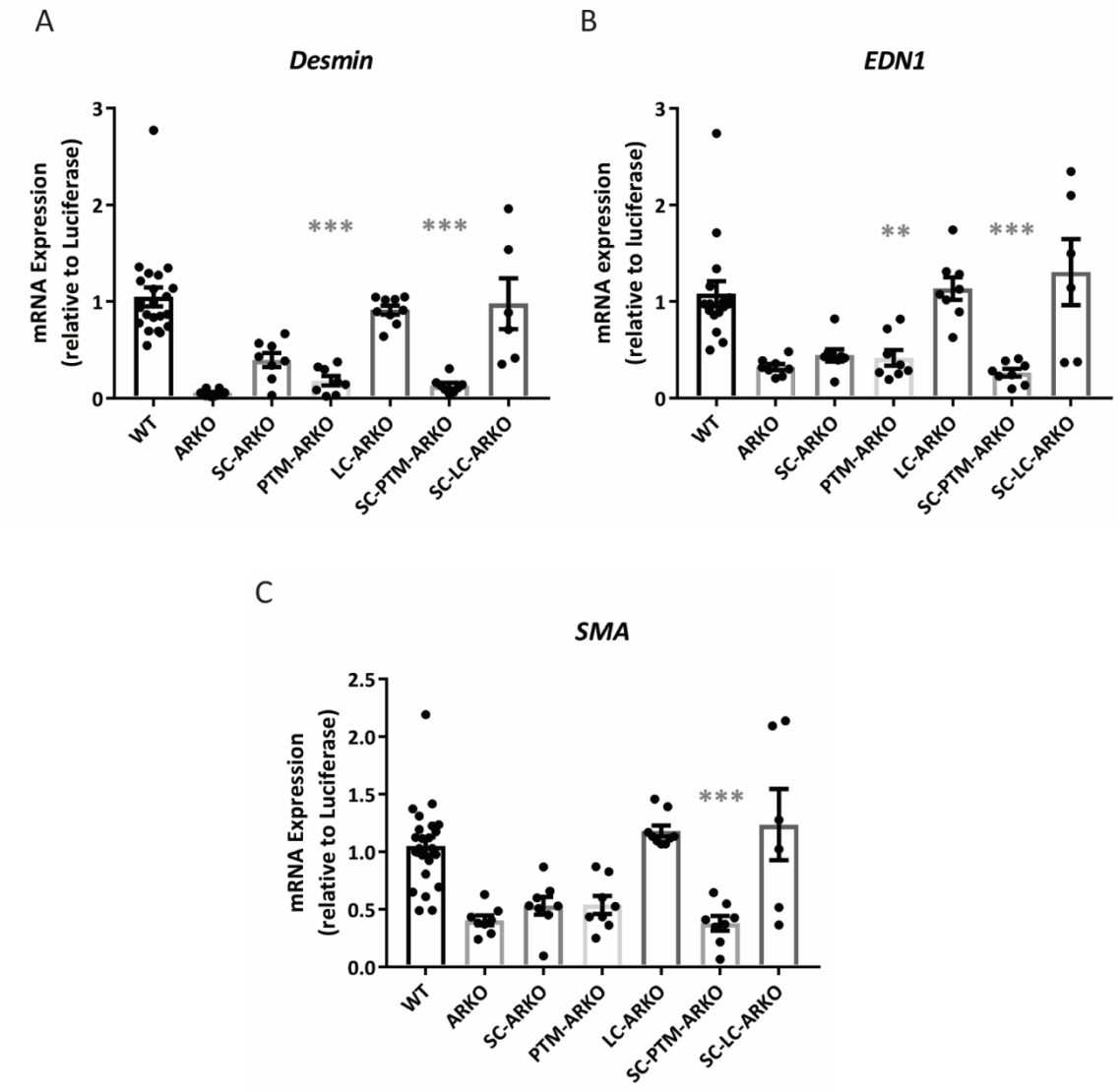


Figure 4.8 Impacts of single and double somatic cell ARKO on PTM cell function. Expression of PTM cell specific transcripts; (A) *Desmin,* (B) *END1* and (C) *SMA*. Kruskal-Wallis one-way ANOVAs were performed followed by a Dunn's multiple comparison test to determine differences between groups. T-tests were used for WT vs LC-ARKO and WT vs SC-LC-ARKO measurements. (** denotes p=<0.01 and *** denotes p=<0.001). Bars represent mean ±SEM. Between 6 and 22 animals for each of the groups were used in each of the animals. qRT-PCR was performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and technical staff from Professor Lee Smiths group (SC-LC-ARKO). Analysis was performed by Annalucia Darbey.

4.3.3.5 Impacts of double cell AR knockout on Leydig Cells in Adult Testis

The development of a complete adult Leydig cell population in the testis has been suggested to be dependent upon androgen signalling through Sertoli cell AR, with a reduction in adult Leydig cell numbers being reported in a SC-ARKO model [101]. In the present study, Leydig cell numbers were also all found to be significantly reduced in SC-LC-ARKO testis but surprisingly not in SC-PTM-ARKO testis (Figure 4.9). Quantification of Leydig cell AR revealed that the percentage of Leydig cells expressing AR was significantly reduced in SC-PTM-ARKO and SC-LC-ARKO testis, suggesting there may be some regulation of AR expression by other somatic cells of the testis (Figure 4.9). Surprisingly, despite the Aromatase-iCre targeting up to 24.78% of Leydig cells at postnatal d100 (as described in section 4.3.2.1), a 45% reduction in Leydig cell AR was observed compared to WT littermate controls, demonstrating that Leydig cell AR may also be regulating its own expression in an autocrine manner.

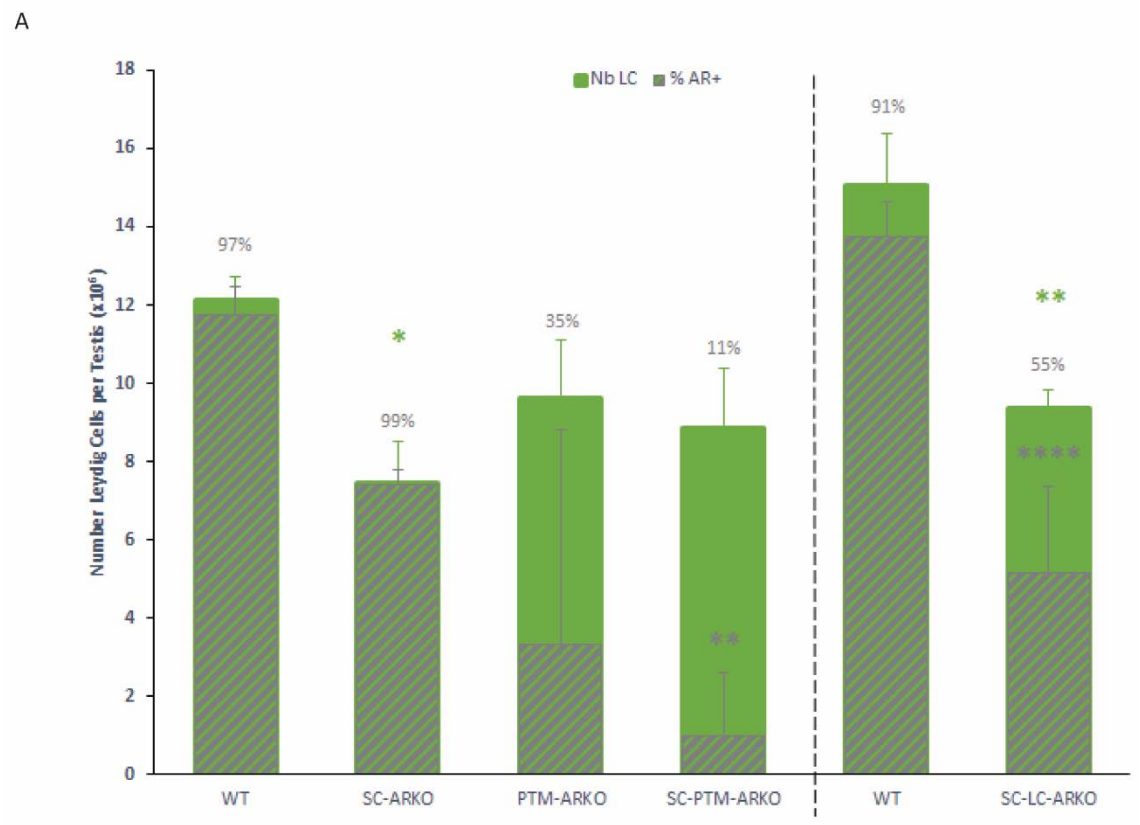


Figure 4.9 Impact of single and double somatic cell ARKO on Leydig cell number and LC-AR expression (full caption on following page).

Figure 4.9 Impact of single and double somatic cell ARKO on Leydig cell number and LC-AR expression (full caption). Leydig cell numbers (Green bars) in SC-ARKO (n= 5), PTM-ARKO (n= 5) and SC-PTM-ARKO (n= 5) compared to WT controls (n= 5) (Kruskal-Wallis one-way ANOVA followed by a Dunn's multiple comparison test) and SC-LC-ARKO (n= 7) compared to WT controls (n= 7) (Unpaired t-test) and Leydig cell AR expression (Grey/Green striped bars) in SC-ARKO (n= 4), PTM-ARKO (n= 10) and SC-PTM-ARKO (n= 5) compared to WT controls (n= 5) (One-way ANOVA followed by Dunnet's multiple comparisons test) and SC-LC-ARKO (n= 7) compared to WT controls (n= 7) (Unpaired t-test). Bars represent mean ±SEM. (* denotes p<0.05, ** denotes p<0.01 and **** denotes p=<0.0001). Quantification was performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and Annalucia Darbey (SC-LC-ARKO). Analysis was performed by Annalucia Darbey.

The adult Leydig cell are primarily responsible for the production of testosterone from its precursor cholesterol. Therefore, to gain insight into the functioning of the adult Leydig cells in the double somatic cell ARKO testes, steroidogenic enzyme mRNA expression was analysed (Figure 4.10). For the conversion of cholesterol to testosterone, cholesterol must first be synthesised. The rate limiting enzyme for this process is encoded by the gene *Hmgcr1*. This enzyme was significantly reduced in SC-PTM-ARKO testis indicating that in these testes there may be a reduction in the production of starting product required for steroidogenesis (Figure 4.10A). Transcript levels of *Star* were found to be significantly increased in LC-ARKO only, indicating that Leydig cell AR regulates this in an autocrine manner (Figure 4.10B). No significant differences were found in *Cyp11a1* transcript expressions, indicating that AR signalling may not be required for the expression of this steroidogenic enzyme (Figure 4.10C).

Interestingly, mRNA expression of *Hsd3b1*, the gene encoding 3beta-hydroxysteroid dehydrogenase-1 (HSD3B1); the enzyme responsible for conversion of pregnenolone to progesterone and marker of foetal Leydig cells, was significantly increased in both single and double somatic cell ARKOs involving peritubular myoid cell and Leydig cell AR. In particular, expression of *Hsd3b1* mRNA in PTM-ARKO and SC-PTM-ARKO testis was over 40 and 38 fold respectively when compared to wild-type litter mate control testis (Figure 4.10D). This would suggest that Leydig and peritubular myoid cell AR could be negatively regulating *Hsd3b1* mRNA expression in the adult WT testis. Cytochrome P450 17a1 (CYP17A1) is the enzyme responsible for the conversion of progesterone and subsequently 17α-hydroxyprogesterone to androstenedione. In the double somatic cell ARKO; SC-PTM-ARKO, expression of *Cyp17a1* mRNA is significantly reduced in the testis when compared to wild type littermate control

testis (Figure 4.10E). The gene encoding the enzyme responsible for the final conversion of androstenedione to testosterone, *Hsd17b3* was also significantly lower in SC-PTM-ARKO testis compared to WT littermate control testis (Figure 4.10F). Therefore, despite a potential increase of progesterone (like that reported in LC-ARKO testis following increased expression of *Hsd3b1),* this may not result in an increased conversion of steroidogenic compounds downstream as a result of the requirement of AR signalling to regulate the expression of genes encoding downstream steroidogenic enzymes.

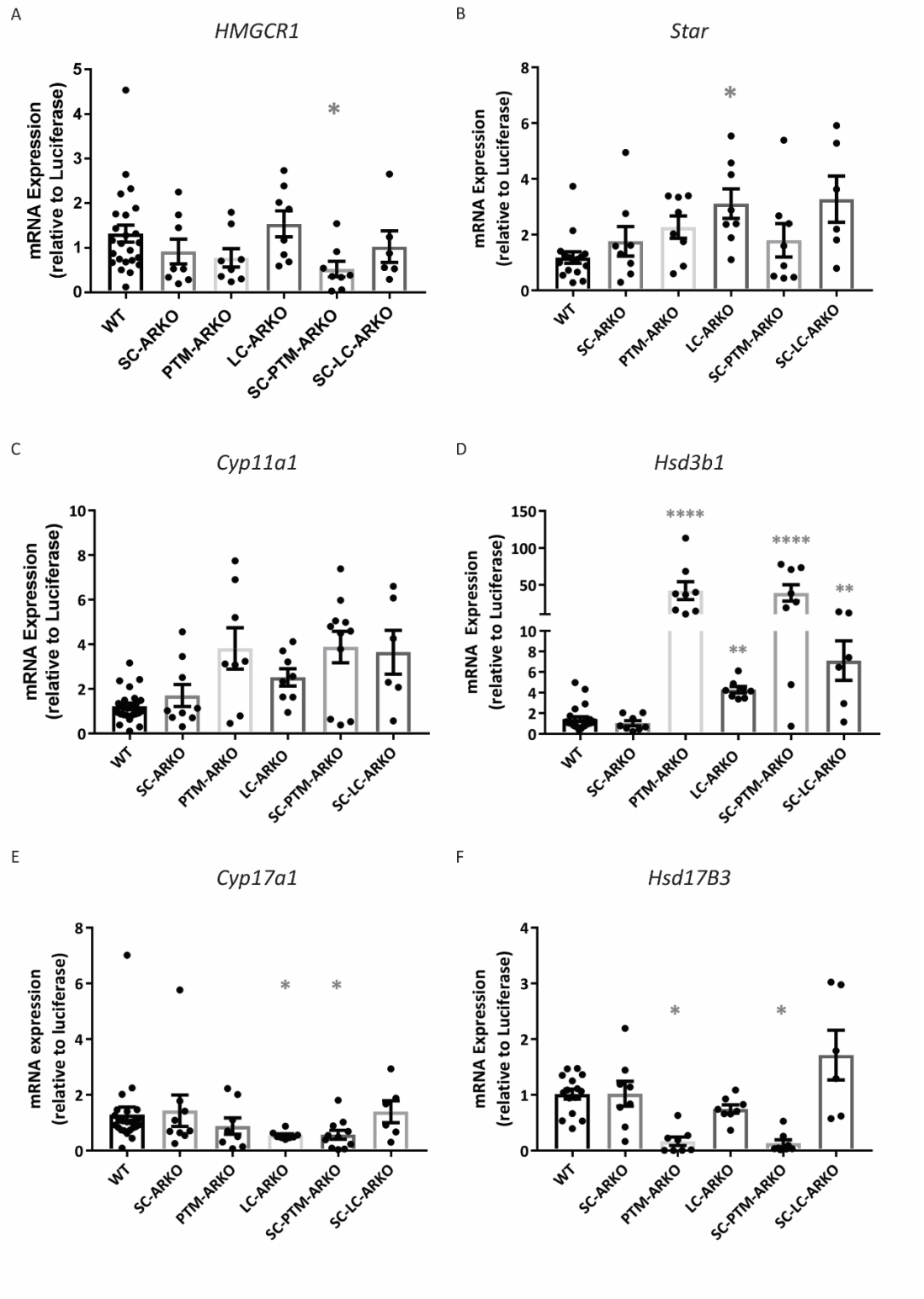

A
HMGCR1
mRNA Expression (relative to Luciferase)
B
Star
mRNA Expression (relative to Luciferase)
C
Cyp11a1
mRNA Expression (relative to Luciferase)
D
Hsd3b1
mRNA Expression (relative to Luciferase)
E
Cyp17a1
mRNA expression (relative to luciferase)
F
Hsd17B3
mRNA Expression (relative to Luciferase)
WT
SC-ARKO
PTM-ARKO
LC-ARKO
SC-PTM-ARKO
SC-LC-ARKO

Figure 4.10 Expression of Steroidogenic enzyme transcripts in single and double somatic cell ARKOs. mRNA expression of (A) HMGCR1, (B) Star, (C) Cyp11a1, (D) Hsd3β1, (E) Cyp17a1 and (F) Hsd17B3 in SC-ARKO, PTM-ARKO, LC-ARKO, SC-PTM-ARKO and SC-LC-ARKO adult testis. A, B, D and E were analysed for statistical differences using a one-way ANOVA followed by a Tukey's multiple comparisons test. D and F we analysed for statistical differences using a Kruskal-Wallis one-way ANOVA followed by a Dunn's multiple comparison test. Bars represent mean ±SEM. (* denotes $p<0.05$, ** denotes $p<0.01$ and **** denotes $p=<0.0001$). Between 6 and 24 animals for each of the groups were used in each of the animals. qRT-PCR was performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and technical staff from Professor Lee Smiths group (SC-LC-ARKO). Analysis was performed by Annalucia Darbey.

With a disturbance at some key stages of the steroidogenic pathway in SC-PTM-ARKO testis, it could be expected that circulating serum testosterone levels may differ in comparison to WT littermate controls. The seminal vesicle weight (a biomarker of system androgen action) was significantly reduced in SC-PTM-ARKO, indicative of circulating low androgens/androgen signalling (Figure 4.11A). However, analysis of serum hormone levels revealed that this did not seem to be the case with both circulating testosterone and circulating LH levels in SC-PTM-ARKO and SC-LC-ARKO animals being comparable to controls (Figure 4.11B&C). The reduction in seminal vesicle weight in PTM-ARKO mice has previously been reported in the literature and was attributed to the smMHC-eGFP-Cre also targeting the smooth muscle located within the seminal vesicle, impacting the secretory function of the seminal vesicle as well as its response to androgens [465]. As the SC-PTM-ARKO was also generated using the smMHC-eGFP-Cre, this reasoning can transferred when considering the reduction in seminal vesicle weight seen in these animals.

Interestingly, the intratesticular testosterone (iTT) levels in SC-PTM-ARKO and SC-LC-ARKO testis was significantly greater than their respective WT controls (Figure 4.11D), suggesting that there may be an overproduction and/or accumulation of testosterone within these double somatic cell ARKO testis. The reasoning behind this is currently unclear, though observations in a previously published PTM-ARKO model revealed an accumulation of lipids within 'abnormal' Leydig cells,. In our double somatic cell ARKOs, the greater impact on AR signalling (particularly as the SC-PTM-ARKO also has significant reductions in Leydig cell AR expression) may result in this build-up of testosterone within the testis, though this still remains to be fully explored.

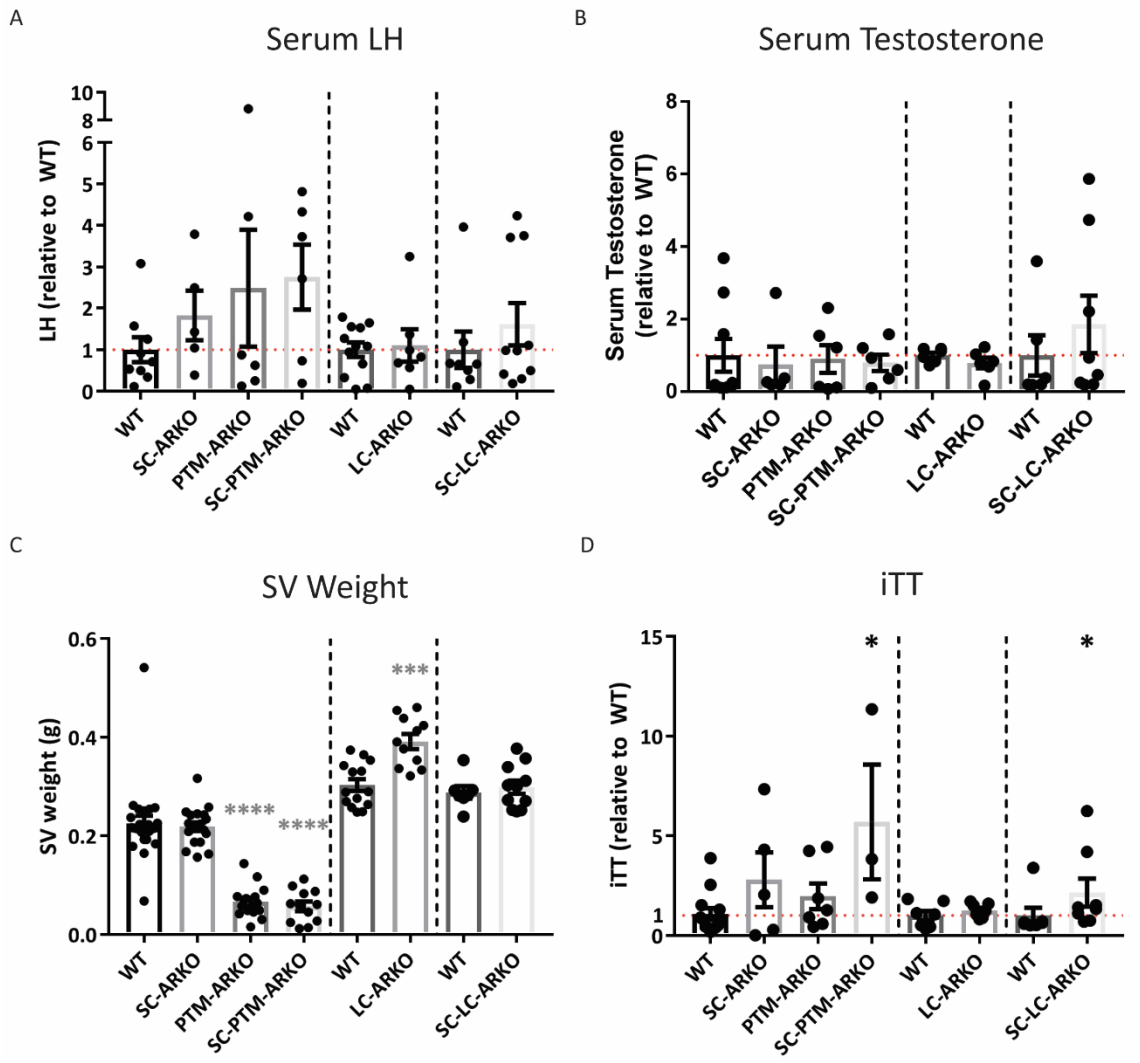


Figure 4.11 Impacts of single and double somatic cell ARKO on Circulating and Intratesticular Hormone Parameters in Adulthood. (A) Serum Luteinising Hormone (LH) and (B) Serum testosterone levels in adult SC-ARKO (n= 5), PTM-ARKO (n= 6-7), SC-PTM-ARKO (n= 6), LC-ARKO (n= 6-7) and SC-LC-ARKO (n= 8-10) normalised to respective WT controls (n= 9-12). (C) Seminal Vesicle (SV) weight in WT (n= 7-23), SC-ARKO (n= 19), PTM-ARKO (n= 15), SC-PTM-ARKO (n= 12), LC-ARKO (n= 14) and SC-LC-ARKO (n= 11) (A, B & C: SC-ARKO, PTM-ARKO and SC-PTM-ARKO were analysed for statistical differences compared to WT littermate controls using a Kruskal-Wallis one-way ANOVA followed by a Dunn's multiple comparisons test, LC-ARKO and SC-LC-ARKO were analysed for statistical differences compared to WT controls using unpaired t-testis). (D) Intratesticular testosterone (iTT) levels in adult SC-ARKO (n= 5), PTM-ARKO (n= 7), SC-PTM-ARKO (n= 3), LC-ARKO (n= 7) and SC-LC-ARKO (n= 8) normalised to respective WT (n= 7-13) controls. (SC-ARKO, PTM-ARKO and SC-LC-ARKO testis were analysed for statistical differences compared to WT littermates using a one-way ANOVA followed by a Dunnet's multiple comparisons test, LC-ARKO testis; using an unpaired t-test and SC-LC-ARKO testis;

using a Mann Whitney *U*-test). Bars represent mean ±SEM. (* denotes p<0.05 and ** denotes p<0.01). Dotted red line is representative of 1/WT values to which each model has been normalised.

Finally, in previous LC-ARKO studies, Leydig cell AR was found to be essential for Leydig cell maturation and functioning. Expression of mRNA and detection using immunohistochemistry revealed that Insulin like 3 (Insl3); a marker for Leydig cell maturation, was significantly reduced in SC-PTM-ARKO testis comparable to that seen in previously published PTM-ARKO and LC-ARKO studies [108, 124] (Figure 4.12). Surprisingly, this wasn't the case in SC-LC-ARKO testis with a no reduction in *Insl3* mRNA expression and patchy expression of *Insl3* in SC-LC-ARKO testis sections (Figure 4.12A&C). This indicates that, despite a significant reduction in Leydig cell AR (Figure 4.9) the majority of Leydig cells underwent maturation as normal in the SC-LC-ARKO testis. Furthermore, mRNA expression of the gene encoding the steroidogenic enzyme 3beta-hydroxysteroid dehydrogenase-6 (HSD3β6) was significantly reduced in LC-ARKO testis as previously reported but not in SC-LC-ARKO testis when compared to wild type littermate controls (Figure 4.12B). Taken together with the results in Figure 4.9, these results suggest that a threshold of Leydig cell AR expression is required in order for proper Leydig cell maturation to occur.

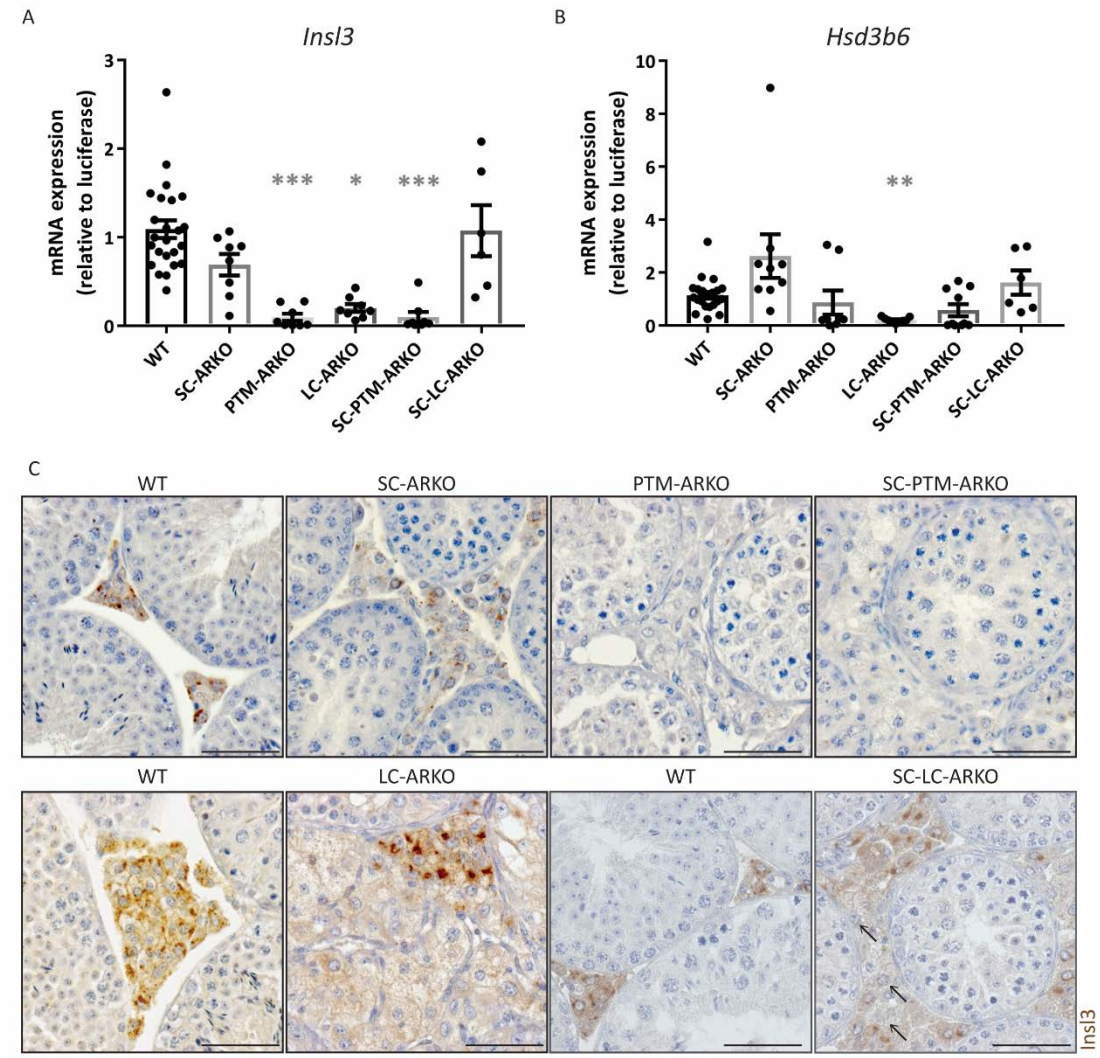


Figure 4.12 Leydig cell maturation in adult single and double somatic cell ARKO mice. mRNA transcript expression of Leydig cell maturation markers (A) *Insl3* and (B) *Hsd3b6* in WT, SC-ARKO, PTM-ARKO, LC-ARKO, SC-PTM-ARKO and SC-LC-ARKO testis. Kruskal-Wallis one-way ANOVAs were performed followed by a Dunn's multiple comparison testis to determine differences between groups. Bars represent mean ±SEM. (* denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p=<0.001$). (C) Immuno-staining of INSL3 in SC-ARKO, PTM-ARKO, SC-PTM-ARKO, LC-ARKO and SC-LC-ARKO testis alongside respective WT controls. (Arrows indicate INSL3 negative Leydig cells). Scale Bars = 100µm. qRT-PCR and staining's were performed by Mr Martin O'Neil (SC-ARKO, PTM-ARKO, SC-PTM-ARKO), Dr Laura O'Hara (LC-ARKO) and technical staff from Professor Lee Smiths group (SC-LC-ARKO). Analysis was performed by Annalucia Darbey.

4.3.3.6 Schematic of AR roles in Adult Testis Function

The generation of double somatic cell ARKO models have provided further insight into the roles of AR in both the steroidogenic and spermatogenic functions of the testis. The schematic denoted as Figure 4.13 illustrates some of the roles of somatic cell AR on testicular somatic cell functions including those in which more than one somatic cell AR may be involved in supporting that function, resulting in an 'additive' effect when knocked out from more than one cell type.

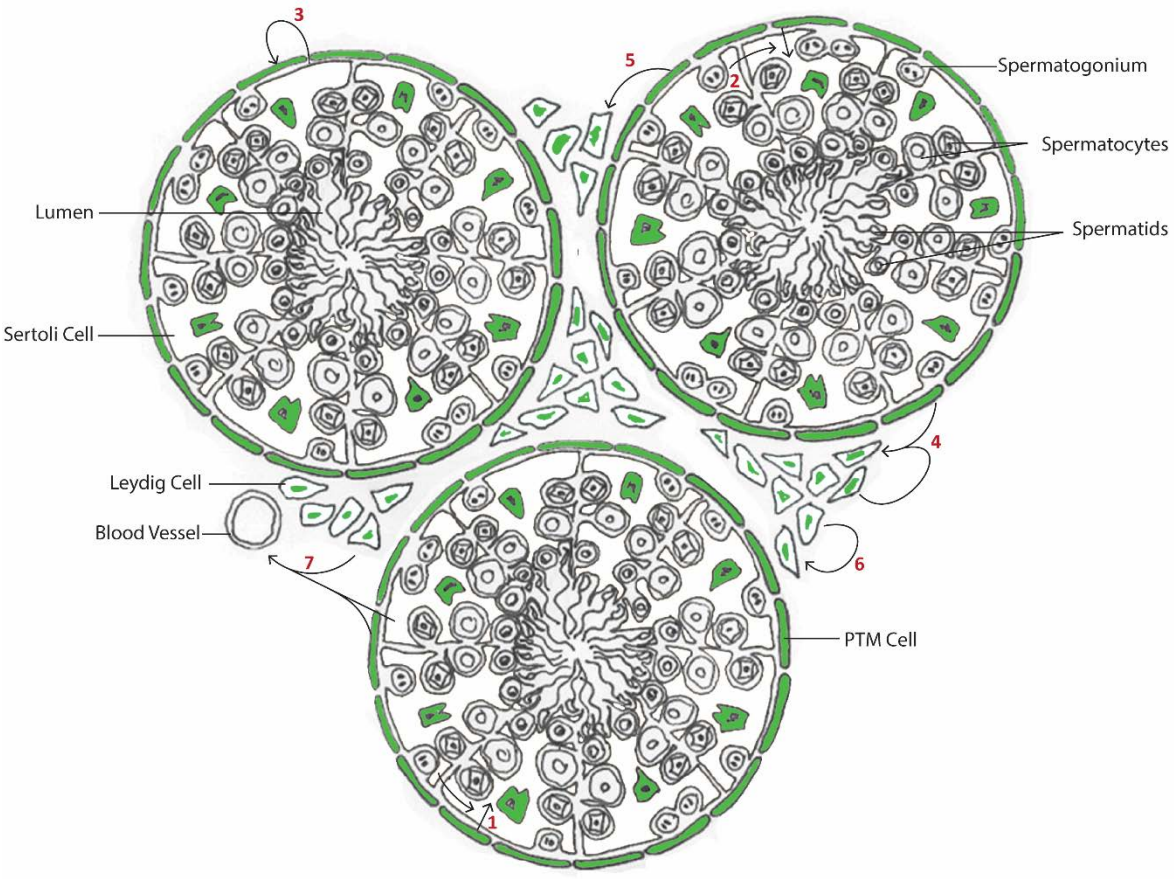


Figure 4.13 Androgen receptor mediated functions in the adult mouse testis. Schematic of AR medicating signalling in the adult testis and the roles of specific somatic cell AR in these processes. 1 – Seminiferous tubule lumen development and maintenance. 2 – SC polarity/ seminiferous tubule organisation. 3 – Peritubular myoid cell contractility. 4 – Leydig cell Maturation. 5 – Leydig cell AR expression. 6 – Steroidogenesis in Leydig cells. 7 – Secretion of testosterone from testis.

4.4 Discussion

Testicular somatic cell expression of AR has been shown to be crucial for proper functioning of the adult testis. The overarching aims of this chapter were to generate a model that could be used to determine the practical uses of the viral vector injection protocols established in the preceding chapter. Alongside this, the model would also function as a comparator to single and double somatic cell ARKOs that have previously been generated. Each of the single somatic cell ARKOs; SC-ARKO [6, 24, 48, 101], PTM-ARKO [52, 124] and LC-ARKO [108], have been published in the literature. A double somatic cell ARKO in Sertoli and peritubular myoid cells (SC-PTM-ARKO) was previously generated but not yet published in Professor Lee Smith's group (by Martin O'Neill, Centre for Reproductive Health, Edinburgh).

To generate an additional ARKO model specifically from the Sertoli and Leydig cells, a recently published Aromatase-iCre was obtained from Fumel *et al* (published in 2015) and first bred to a YFP reporter line to determine location and timing of iCre expression in the testis [17]. Analysis of YFP expression in the testis revealed YFP expression from post-natal day (pnd) 0 within Sertoli cells located in the tubular compartment of the testis. From pnd18, YFP expression could be observed in HSD3β positive cells in the interstitial compartment, indicating Aromatase-iCre expression in the immature Leydig cells. Quantification of YFP expression in the Leydig cells revealed that Aromatase-iCre expression increased as mouse age increased up to d100, suggesting either that Aromatase-iCre was being activated as the Leydig cells were undergoing maturation or that the Aromatase-iCre was being expressed by d18 and subsequent proliferation of the targeted cells (and subsequent inheritance of YFP expression in daughter cells) resulted in 24.78% of the Leydig cell population expressing the reporter.

To generate the SC-LC-ARKO animals, Aromatase-iCre males were mated with female mice homozygous for a floxed AR, producing animals with AR knocked out in both Sertoli and Leydig cells alongside wild type (Cre negative) littermates. Though Leydig cell targeting in this model may be relatively low, a SC-LC-ARKO model, generated using the Aromatase-iCre, would permit the comparison of adjacent Leydig cells, exposed to the same endocrine and paracrine environment (similar to the method practiced in the previously published LC-ARKO article), with the added factor of absence of SC-ARKO signalling. Alongside this, archived tissue from SC-ARKO, PTM-ARKO and SC-PTM-ARKO (another novel double somatic cell ARKO

previously generated, but not yet published), previously collected in the lab, was utilised for a side by side comparison, with the goal of providing a more complete picture of AR signalling in the adult testis.

Analysis of the gross phenotype of the double somatic cell ARKO testis revealed similarities between the single somatic cell ARKO models with testis weight being significantly reduced in comparison to each models respective WT controls. A reduction in testis weight and tubule dimeter may signify a reduction in sperm production and testis function [468-471], H&E staining of testis sections revealed striking differences in testicular histology, with both double somatic cell ARKO models being comparable to SC-ARKO and PTM-ARKO testis, both of which exhibited disrupted spermatogenesis.

The PTM-ARKO model (generated using the smMHC-eGFP-Cre) and the impacts of PTM-ARKO on Leydig cell function has previously been published and reports a weak AR signal in Leydig cells considered 'abnormal' due to impaired development as a result of PTM-ARKO [124]. In both PTM-ARKO and SC-PTM-ARKO testis, a high proportion of interstitial cells appeared to lack AR expression, suggesting that either peritubular myoid cell AR regulates expression of AR in Leydig cells or that the smMHC-eGFP-Amh-Cre was active in some Leydig cells and this was something that was taken into consideration when interpreting the subsequent data produced.

Sertoli cells are crucial for the maintenance of testis function in the adult testis with ablation of Sertoli cells being shown to impact peritubular myoid cell activity, Leydig cell numbers and function, germ cell numbers and in supporting the vasculature system in the testis [5, 7-9]. As expected, Sertoli cell AR expression was significantly reduced in SC-ARKO, SC-PTM-ARKO and SC-LC-ARKO but not in PTM-ARKO testis, confirming that expression (or lack of) of AR on Sertoli cells has no impact on AR expression in other testicular cell types. Androgen signalling has been shown to have an impact of final Sertoli cell number, with the total ARKO testis having a significant reduction in Sertoli cell nuclear volumes and SC-ARKO testis having a small but significant reduction in Sertoli cell nuclear volume at pnd140 [24]. In WT testis, Sertoli cell nuclei usually migrates towards the basal region of the seminiferous tubule and loss of AR in SC-ARKO, PTM-ARKO, SC-PTM-ARKO and SC-LC-ARKO appeared to impact this, as evident from the presence of Sox9+ immunostaining within the centre of seminiferous

tubules as opposed to it being restricted to the periphery. The disruption in the organisation of the tubules is similar to that seen in the previously published SC-ARKO and PTM-ARKO studies and was attributed to altered signalling in peritubular myoid cells and a loss of Sertoli cell function with this study also suggesting a role of the paracrine signalling in the testis [472]. Similar disruptions to the polarity of Sertoli cells have also been seen in testis with Wilms tumour-1 (*Wt1*) -deficient Sertoli cells. *Wt1* and its product WT1 is specifically expressed in Sertoli cells and has been shown to be involved in the regulation of *Dhh* expression in the testis [473] and *Wt1* deficiency resulted in a down regulation of Wnt signalling genes, cell polarity genes, blood testis barrier (BTB) integrity and subsequent germ cell loss [474]. The role of the BTB in sustaining Sertoli cell polarity in the seminiferous tubules has also been implicated given the loss of Sertoli cell polarity and subsequent loss of the Sertoli cell epithelial phenotype seen in mice deficient of Claudin 11, a key junctional protein of the BTB [28]

Desert hedgehog is a intercellular signal transducer secreted by the Sertoli cells with receptors (patched 1) found to be located on late primary spermatocytes, secondary spermatocytes, round spermatids, peritubular myoid cells and Leydig cells in the adult mouse testis and known to have a role in germ cell survival and in steroidogenesis in the adult mouse testis [473, 475]. Dhh is significantly reduced in SC-PTM-ARKO testis, but not in SC-ARKO or PTM-ARKO testis. This would suggest that Sertoli and peritubular myoid cell may be functioning in a synergistic manner through AR signalling. WT1 has also been shown to bind to multiple sites in the AR promoter, resulting in transcriptional repression of AR promoter and downregulating androgen responsive genes suggesting that there may be some association between *WT1, DHH* and the paracrine mechanisms involved in AR signalling in the adult testis.

Aquaporin 8, a water channel protein, has been shown to be expressed by Sertoli cells and is thought to be involved in the movement of water from the interstitial space into the lumen of the seminiferous tubules [467, 476, 477]. Analysis of *Aqp8* mRNA expression in all testicular cell specific ARKO models, excluding LC-ARKO, revealed a significant reduction of expression. This would also suggest that Leydig cells have no influence on the fluid production of Sertoli cells, unlike the peritubular myoid cells which appear to be crucial for the movement of fluid/water from the interstitial compartment to the luminal compartment.

Considering lack of expression of Aqp8 in peritubular myoid cells, is indicative of the androgen mediated interactions between Sertoli and peritubular myoid cells.

Peritubular myoid cells are smooth muscle cells surrounding the seminiferous tubules within the testis and are responsible for the contractility and movement of fluid and spermatozoa through the testis as well as being crucial for regulating spermatogenesis and steroidogenesis [52, 124, 478, 479]. Desmin and SMA are peritubular myoid cell and blood vessel specific proteins that are known to play critical roles in the structure and functioning of the contractile apparatus in the peritubular myoid cells whereas EDN1 is a protein known to be secreted by Sertoli cell with the ability to stimulate peritubular myoid cell contraction [122, 128, 478, 480]. As previously reported, significant reduction of *Desmin* was found in PTM-ARKO testis and in SC-PTM-ARKO revealing that peritubular myoid cells alone are regulating the expression of this gene through AR signalling. *SMA* expression was significantly reduced in SC-PTM-ARKO in comparison to wildtype litter mate controls. *EDN1* was also significantly decreased in both PTM-ARKO and SC-PTM-ARKO testis, indicating that, despite EDN1 being a Sertoli cell secreted protein, the expression of *EDN1* is still under androgen mediated peritubular myoid cell control. The relationship between Sertoli cell and peritubular myoid cell functionality has been well documented as peritubular myoid cell functioning is lost in the absence of Sertoli cells [7, 52, 481]. The reduced expression of *SMA* in SC-PTM-ARKO testis only is also testament to this, proposing that in SC-ARKO and PTM-ARKO testis, there may be some compensation for the loss of AR in one somatic cell type, compensation that can't take place with knock out of AR in both.

Androgen signalling via Sertoli cells has previously been shown to be essential for the development of a full complement of Leydig cells in the testis with the complete ARKO and SC-ARKO testis having a significant reduction in Leydig cell number [101, 109] and this reduction in Leydig cell was mirrored in SC-LC-ARKO testis. AR expression was found to be further reduced than expected in SC-LC-ARKO suggesting that Leydig cell AR may regulate its own expression in an autocrine manner.

The main function of Adult Leydig cell is to produce testosterone from cholesterol through the process of steroidogenesis. The loss of AR from both Sertoli and peritubular myoid cells results in a reduction of *Hmgcr1*, *Cyp17a1* and *17bhsd3* in the adult testis. Interestingly,

expression of *HSD3β1*, is significantly increased in SC-PTM-ARKO and SC-LC-ARKO; similar to the increase also seen in the single somatic cell ARKOS; PTM-ARKO and LC-ARKO. This would suggest an increase in the levels of progesterone in the testis; something that was confirmed in LC-ARKO testis [108] but would need confirming in the present study. The data could also benefit through reporting to the number of Leydig cells reported in the testis of each of the knock out models; permitting the examination of enzymatic gene expression per Leydig cell. A disruption in steroidogenic enzyme expression, in addition to the reduction in seminal vesicle weight in PTM-ARKO and SC-PTM-ARKO animals, would suggest an impact on testosterone production. However, this was not the case in any of the ARKO models, with serum testosterone and LH levels being comparable to controls in each. One reason for this may be, as suggested in O'Hara *et al* (2015) that the proposed increase in bioavailability of progesterone within the testis may serve as a reservoir from which the residing populations of Leydig cells that retain AR expression can utilise for testosterone production. The potential relationship between the testicular vasculature and testicular somatic cell types such as the Sertoli and peritubular myoid cells has also been described in the literature [8]. With the smMHC-eGFP-Cre also reported to target the smooth muscle cells surrounding the vasculature [52], coupled with the influence of Sertoli cells on vascular function and retention [8], it is possible that AR mediated interactions between these cell types may function in the control of secretion of testosterone from the testis through the vasculature, explaining the increase in intra-testicular testosterone in SC-PTM-ARKO and SC-LC-ARKO testis though this would benefit from further investigation.

Insl3 expression was significantly downregulated at both the level of mRNA transcript and the protein level in PTM-ARKO, LC-ARKO and SC-PTM-ARKO testis revealing a compromise in the maturation of Leydig cells in these testis. Whether this is due to the reduction in Leydig cell AR or due to paracrine signals from peritubular myoid cells is still unclear. In the study by Welsh *et al* (2011), this was partly attributed to a reduction in SF-1 expression [124]. Further study into the mechanisms controlling SF-1 originating in peritubular myoid cells and potentially elsewhere (given the immature Leydig cells present in the LC-ARKO model despite peritubular myoid cell function remaining intact) would be beneficial alongside investigation into the roles of IGF-1 (given its role in Leydig cell development and maturation) [85, 86, 482]. The roles of FSHR in Leydig cell maturation in cell specific ARKO models may also prove interesting due to the link between FSHR signalling and Leydig cell development alongside

the potential AR mediated expression of metastasis-associated protein 2 (MTA2) in Sertoli cells and its role in FSHR expression in Sertoli cells [102]. Interestingly, Leydig cell maturation did not appear to be impacted in SC-LC-ARKO testis as expression levels of *Insl3* in the testis were comparable to WT controls. This may be attributed to the numbers of AR retaining Leydig cells remaining in the testis, suggesting there may be some compensatory mechanism or threshold mechanism involved in Leydig cell maturation in which Leydig cells may contribute to their own maturation in an autocrine manner.

The generation of double somatic cell ARKO models has provided further insight into the androgen mediated interactions between the somatic cells in the adult testis. Some of the cellular functions perturbed by AR ablation include Sertoli cell fluid secretion, influencing lumen development and maintenance, Leydig cell steroidogenesis and autocrine control of AR expression and maturation, peritubular myoid cell contractility and even potential secretion of testosterone from the testis. Given the results published previously describing the impacts of cell specific single cell ARKO in the testis, impacts on steroidogenic and spermatogenic functionality of the testis were expected, however, the androgen mediated paracrine manner in which all somatic cell types contribute the functions of the testis is described in more detail here using analysis of double somatic cell ARKOs within the context of single somatic cell ARKOs.

The subsequent chapter will combine the methodology and techniques optimised in chapter 3 along with the knowledge gained in the present chapter to present the practicalities of viral vector delivery to the adult testis. The cell-specific ARKO model chosen to determine the potential of gene repair or rescue with lentiviral vectors was the SC-ARKO model. Given the vast knowledge of the Sertoli cell functions in the testis and a recent study highlighting the importance of AR signalling for post-meiotic germ cell development specifically in adulthood [45], using the SC-ARKO testis would be an ideal model for repair through the re-delivery of mouse AR cDNA using lentiviral vectors.

Chapter 5 Demonstrating Practical Uses of Lentiviral Vector Delivery with the Replacement of Androgen Receptor in Sertoli Cell Androgen Receptor Knock out Testis.

5.1 Introduction

In Chapter 3, techniques for the targeting and delivery of viral vectors to the somatic cells of the adult testis were described, demonstrating some evidence for targeting Leydig cells with viral vectors and an improved methodology for targeting adult Sertoli cells with lentiviral vectors. As discussed earlier, a handful of studies have demonstrated successful targeting of Sertoli cells and subsequent delivery of transgenes and/or rescue of male infertility phenotypes using adenoviral [334, 335], adeno-associated viral [348] and Lentiviral vectors [45, 397, 399]. However, in the three studies that have demonstrated repair of a phenotype using gene delivery, the treatment was delivered to the testis during the pre-pubertal stages of life [396, 397] and are therefore, could be considered as demonstrating a prevention of infertility as opposed to a rescue of infertility in adulthood.

Currently, most cases of male factor infertility and hypogonadism remain idiopathic, in part due to the limited genetics testing into men with these conditions at the clinic. With the majority of males presenting at clinic in adulthood, and the increasing incidence of age-related male infertility and hypogonadism, the importance of developing a technique that can be utilised in the adult male is increasingly important. Alongside this, gene editing technology is currently developing at an exponential rate and there are a number of gaps and contradictions remaining in the literature surrounding their potential for use in the testis. Therefore, the present study sought to confirm the potential applications of lentiviral delivered transgenes to the adult testis.

First, a mouse model exhibiting testicular dysfunction was identified for gene repair or replacement (as described in Chapter 4). The models generated were cell specific knockouts of Androgen Receptor (AR) from single or double testicular somatic cells using Cre/*LoxP* technology, providing a tool from which both methods optimised for delivery of viral vectors to either of these cell types in the adult testis could be utilised. Other uses for the model were that they could also be used to gain further insight into some of the synergistic and

additive roles of the somatic cell types through AR mediated signalling in the adult testis. The well characterised Sertoli cell ARKO model was identified as the most suitable model to be used to demonstrate the potential of transgene delivery through Sertoli cell targeting with lentiviral vectors.

It is well described in the literature that the AR expression Sertoli cells is known to have a number of key roles in both testis function and development [7-9]. As evident from previous studies and through the results described in Chapter 3, it is through AR that some of these crucial functions are mediated; in particular, the Sertoli cells support of complete spermatogenesis with spermatogenesis being halted before meiosis in the SC-ARKO testis [48]. An acute model of Sertoli cell AR ablation has been generated using the delivery of lentiviral vectors, demonstrating the importance of Sertoli cell AR for completion of post-meiotic spermatogenesis [45]. However, it is not currently known whether the absence of AR through development (as is the case for the SCARKO mice studied) has a permanent impact on the Sertoli cells ability to function and consequently whether this is a reversible phenotype.

Using lentiviral vector delivered mouse Androgen receptor cDNA and the low impact methodologies described in Chapter 3, it may be possible to address the unknown developmental impacts of Sertoli cell AR loss on testis function. Alongside this the practical uses of the methodology optimised and described in Chapter 3 can be established as a suitable technique for delivery of transgenics with the potential for restoration of a gene and subsequent repair of a testicular phenotype.

5.2 Hypothesis & Aims

5.2.1 Hypothesis

It is hypothesised that delivery of lentiviral vectors carrying mouse AR cDNA will target Sertoli cells of the adult SC-ARKO testis, restore Sertoli cell AR expression and subsequently restore spermatogenesis in the rescued tubules.

5.2.2 Aims

From this hypothesis, the aims of the experiments described in this chapter were established:

Aim 1: To deliver an exogenous transgene (mAR) to the Sertoli cells of adult SC-ARKO testis following lentiviral injection

Aim 2: To determine whether lentiviral delivered mouse AR is capable of restoring AR expression in Sertoli cells of SC-ARKOs and subsequently restoring spermatogenesis in SC-ARKO testis.

5.2.3 Approach

To achieve these aims, Sertoli cell specific Androgen Receptor knock out (SC-ARKO) mice were generated as described in 2.1.3.3. Testis were injected at pnd100 (as described in 5.3.1), collected 50 days later and analysed in comparison to testis receiving no injection, injection of vehicle or injection of a mAR negative (control) lentiviral vector.

5.3 Results

5.3.1 Generation of SC-ARKO mice and Injection of Lentiviral vectors carrying Mouse Androgen Receptor cDNA

Sertoli cell specific AR knock out mice were generated as described in 2.1.3.3 and as depicted in the schematic in Figure 5.1A. PCR amplification of genomic DNA isolated from ear clips of SC-ARKO animals demonstrate the inheritance of Cre recombinase with a band present at 239bp alongside the internal positive control gene Interleukin 2 (330bp band) (Figure 5.1B). At pnd100, lentiviral vectors carrying mouse AR and monomeric GFP (LV-mAR-moeGFP), or monomeric GFP alone (LV-moeGFP) downstream of a CMV promoter (lentiviral constructs depicted in Figure 5.1C) were introduced into the rete compartment of SC-ARKO testis (or those of WT littermates) as described in section 2.3.3. Figure 5.1D illustrates the plan used for injection in that one testis was used for LV-mAR-moeGFP injection and the contralateral was injected with LC-moeGFP control virus, serving as an internal control from which direct comparisons were able to be made. The same methodology also applied for vehicle injections where the contralateral testis was used as a non-injection control.

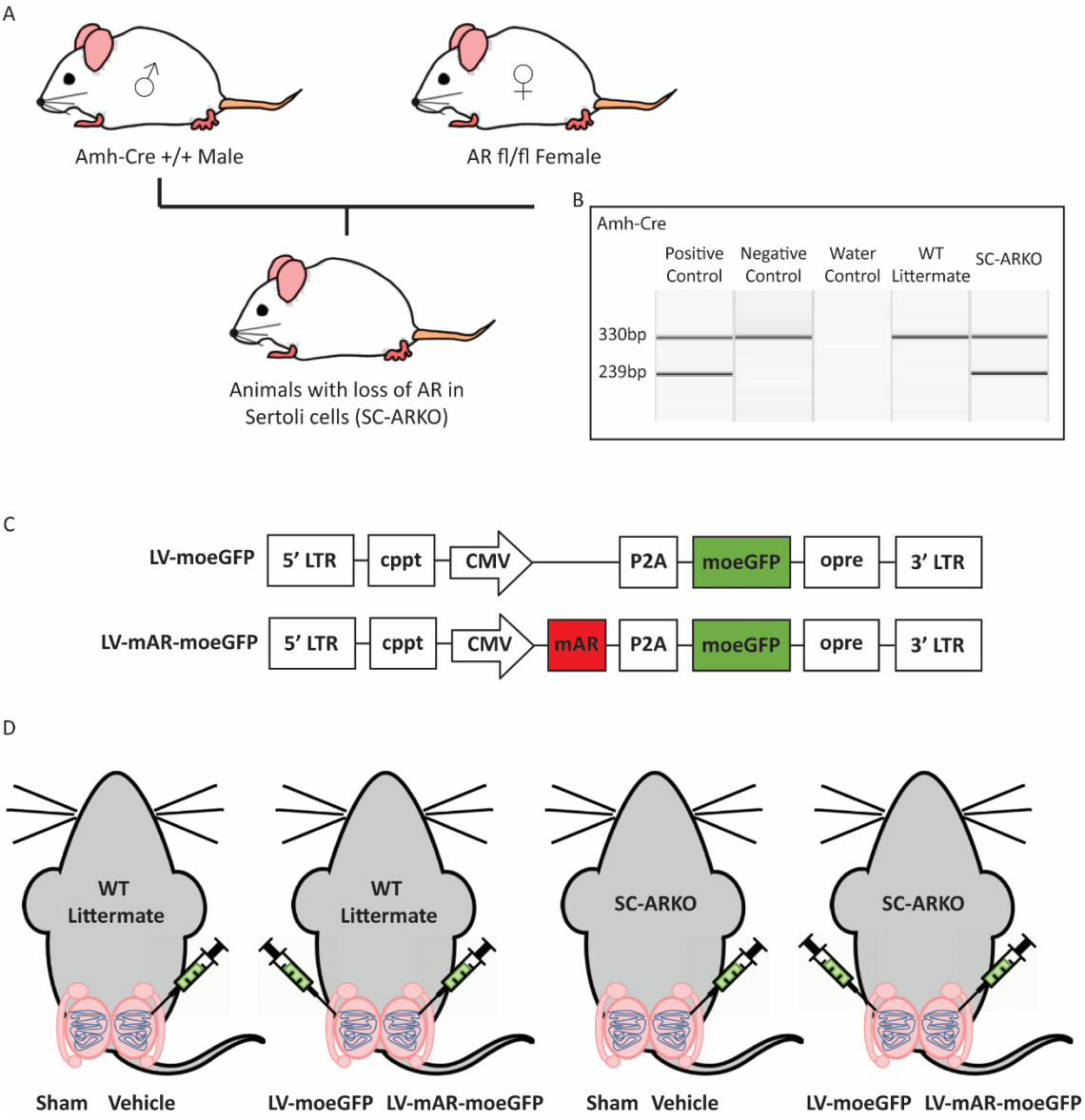


Figure 5.1 Generation of SC-ARKO animals for the injection of lentiviral vectors carrying mAR. (A) Generation of animals with a loss of AR in Sertoli cells using Amh-Cre positive males and females with a floxed AR. (B) PCR analysis of genomic DNA isolated from WT littermate controls and SC-ARKO ear clips. Bands at 330bp are representative of the Interleukin-2 internal positive control. Bands at 239bp indicate inheritance of Amh-Cre. No inheritance of Amh-Cre was detected in WT littermate and Negative controls. No amplification of DNA was detected in Water controls. (C) Schematic diagrams of the lentiviral vectors used; LV-moeGFP – a VSVG pseudotyped lentiviral vector carrying moeGFP, LV-mAR-moeGFP - VSVG pseudotyped Lentiviral vector expressing mouse androgen receptor and a monomeric enhanced GFP downstream of the CMV promoter and separated with a P2A cleavage site.

(D) Schematic of plan for the injection of viruses described into adult WT and SC-ARKO testis. Mice were injected at pnd100 and collected 50dpi.

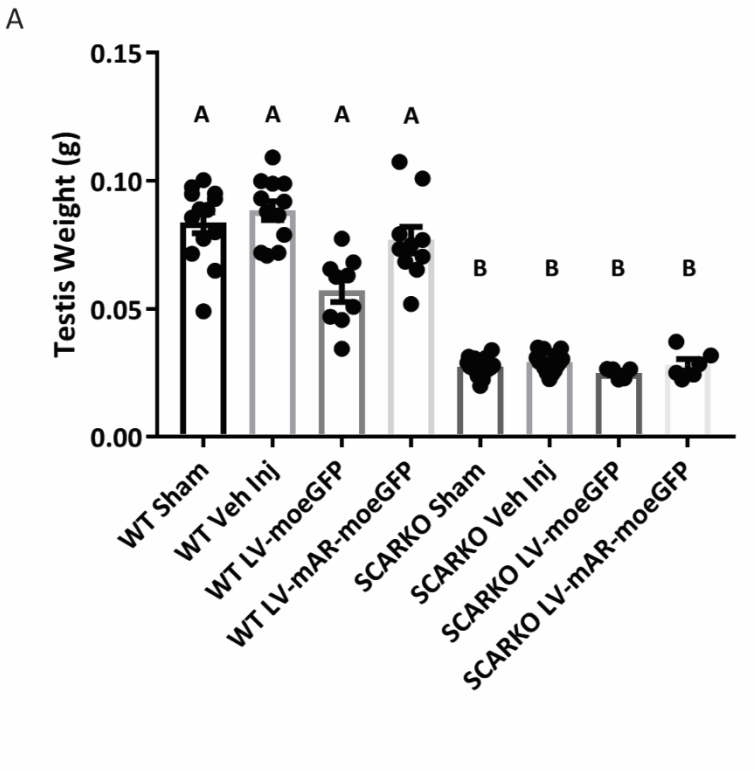
5.3.2 Gross Morphology & Histology of WT and SC-ARKO Testis following Injection of Lentiviral Vectors carrying mAR and/or monomeric GFP

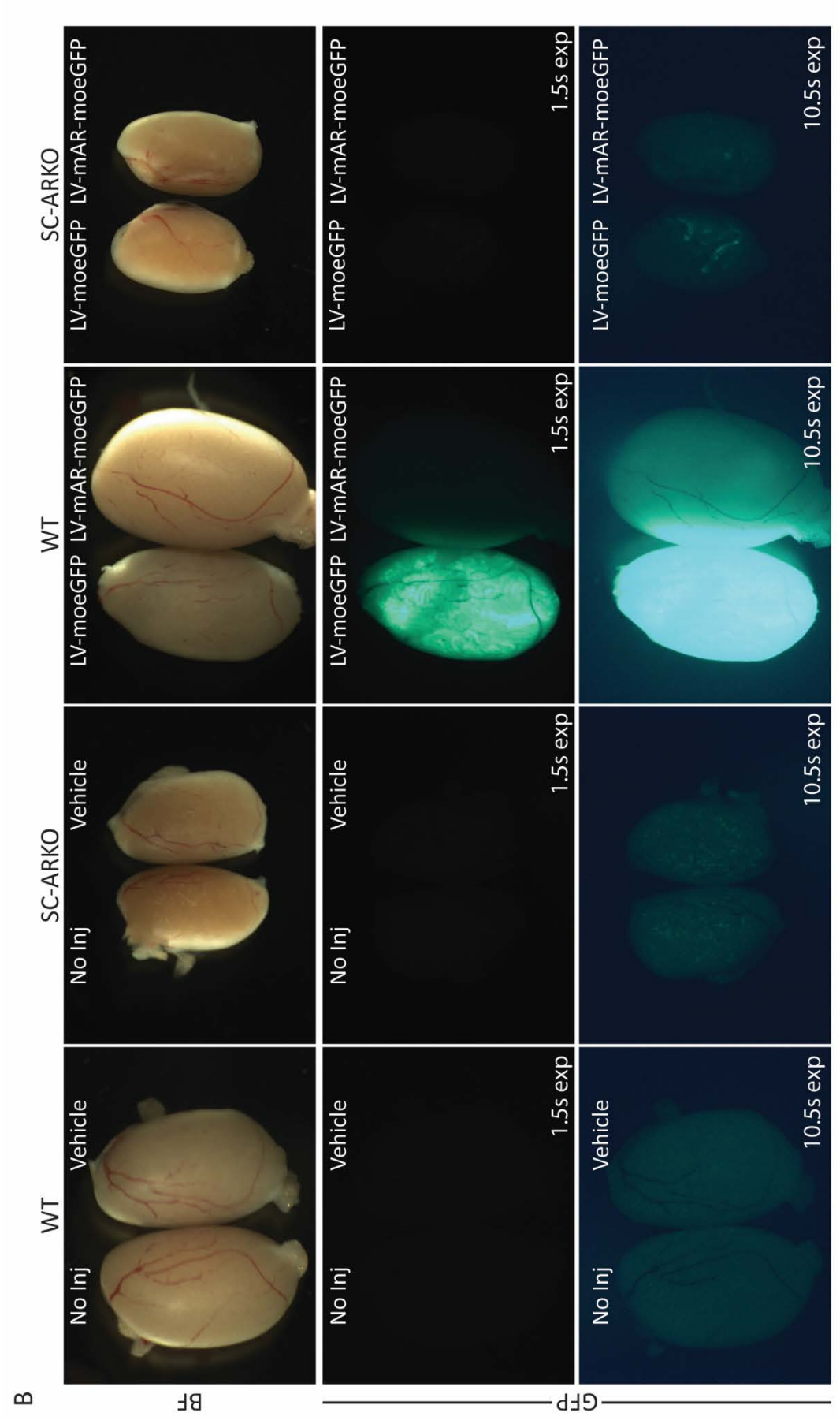
To determine whether procedures had an impact on testis function, gross morphology of the reproductive system was observed on collection of the tissue. As expected, SC-ARKO testis exhibited a significant reduction in testis weight compared to WT control testis, regardless of treatment. There were no significant differences in weight between treated testis of the same genotype (Figure 5.2A). This would suggest that delivery of lentiviral vectors to the tubular compartment has no impact on testis weight and therefore, testicular cell survival and function.

Both lentiviral vectors used in the present experiments included a monomeric GFP reporter within the construct, permitting the visualisation of transduced cells/tissue using a fluoroscope or with antibodies in fixed transduced cells/tissue. Visualisation of non-injected and vehicle injected using a fluoroscope with GFP filter revealed no GFP fluorescence, as expected. Strong GFP fluorescence was observed within the tubular compartment of LV-moeGFP injected WT testis (Figure 5.2B), indicating integration and expression of lentiviral vectors into cells within the tubular compartment. GFP fluorescence was also detected in LV-moeGFP injected SC-ARKO testis, though this appeared to be much weaker than that of signal in WT testis as it was only more clearly visible in image captures taken after an increased exposure to the GFP fluorescent light (Figure 5.2B). Surprisingly, no GFP expression was observed in LV-mAR-moeGFP injected WT and SC-ARKO testis (Figure 5.2B).

There were no striking differences in the appearance of WT testicular histology, regardless of treatment, with spermatogenesis seemingly occurring as normal (Figure 5.2B). Control treated (No injection, vehicle injected and LV-moeGFP injected) SC-ARKO testis were comparable to one another, each with smaller tubules (compared to WT controls), a lack of lumen and no elongated spermatids present within the tubules (Figure 5.2B). This indicated that, despite a lack of lumen in SC-ARKO testis, that the introduction of substances into the tubules, via the rete, has no negative impacts on testis histology. SC-ARKO testis injected with LC-mAR-moeGFP, however, appeared to have a lumen present within the seminiferous

tubules, suggesting the tubular fluid producing mechanism, mediated by Sertoli cell AR, may have been restored (Figure 5.2C).




B
BF
GFP
WT
SC-ARKO
WT
SC-ARKO
No Inj
Vehicle
No Inj
Vehicle
LV-moeGFP
LV-mAR-moeGFP
LV-moeGFP
LV-mAR-moeGFP
1.5s exp
10.5s exp

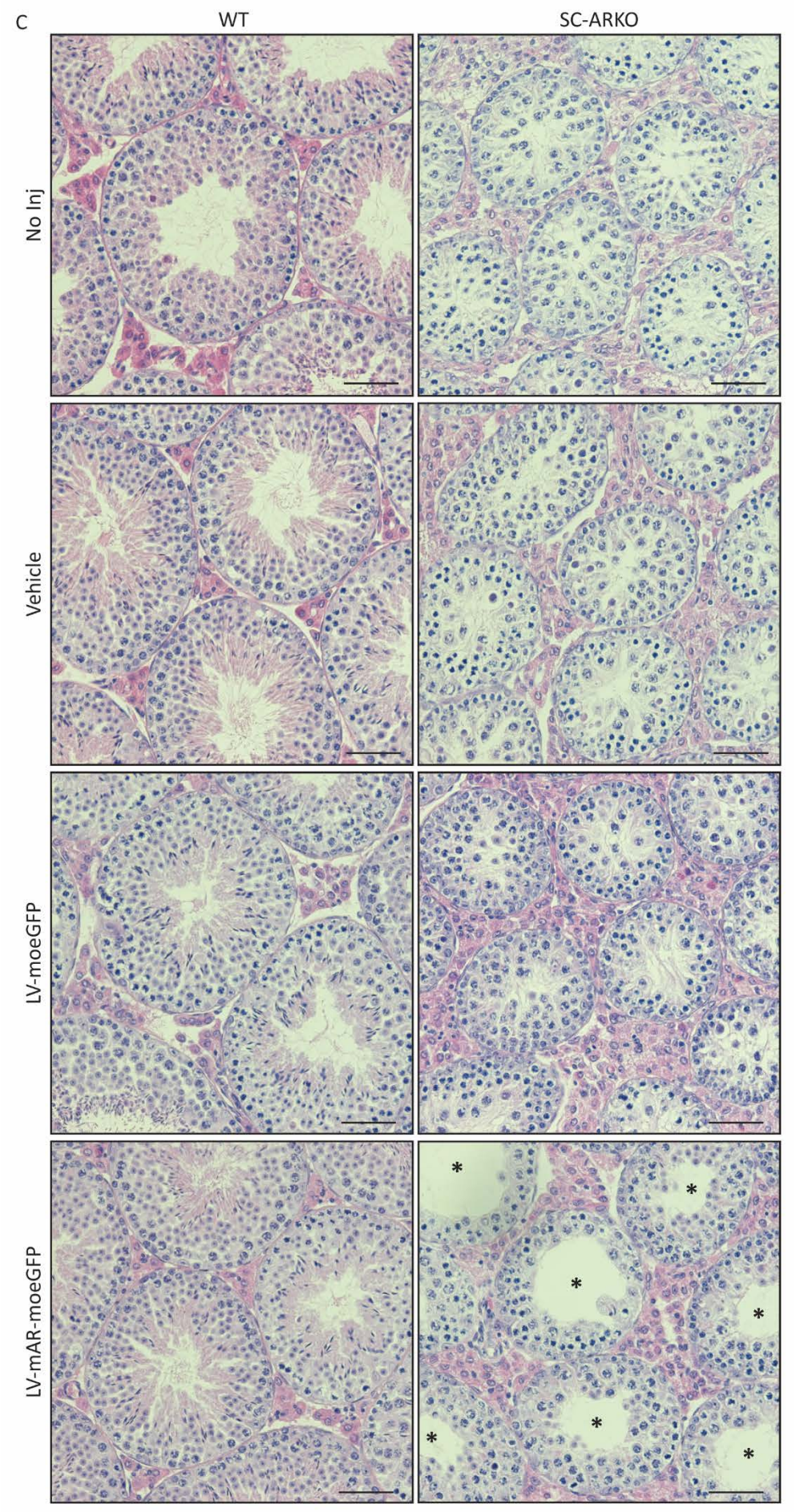
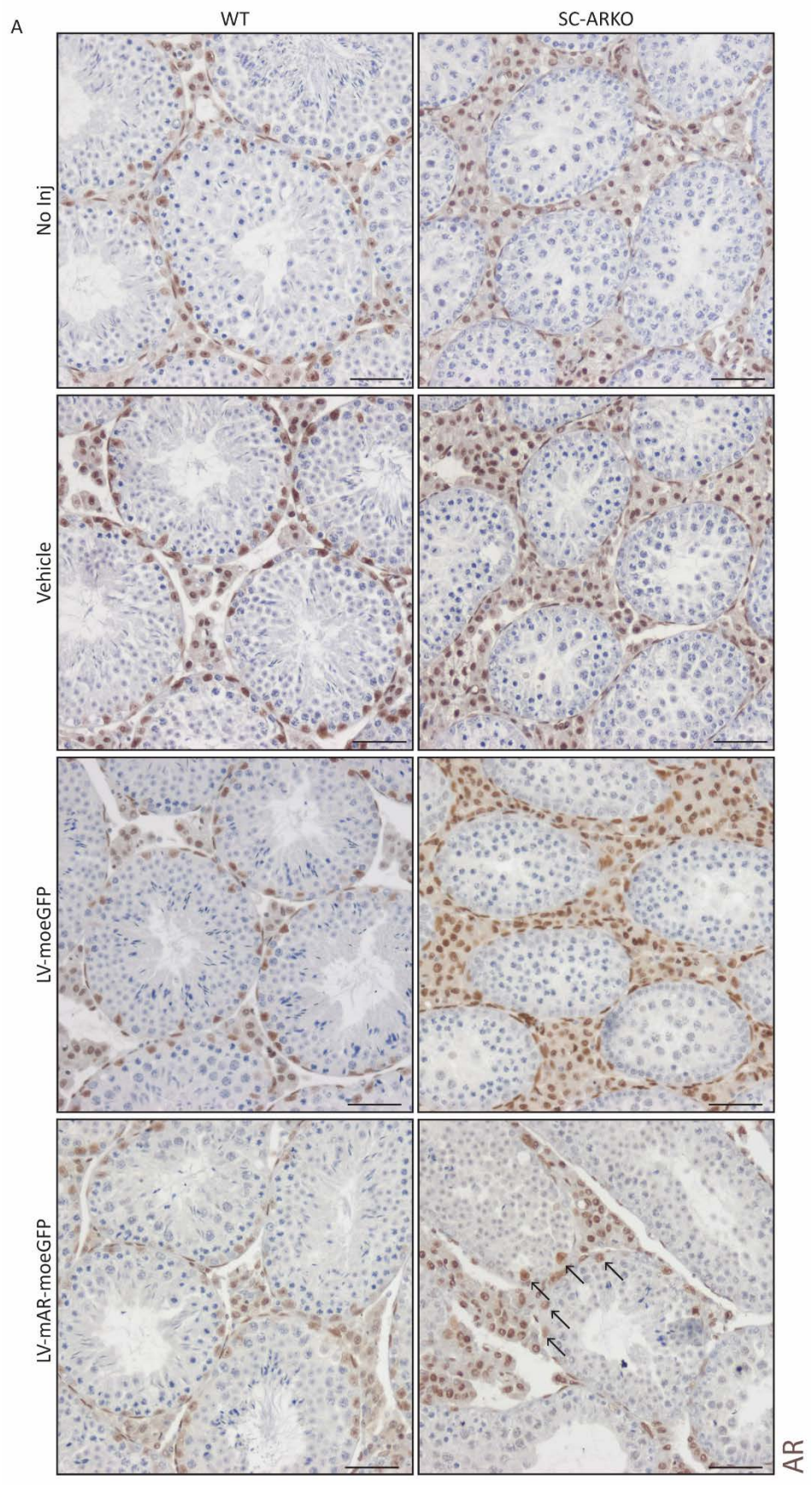

C
WT
SC-ARKO
No Inj
Vehicle
LV-moeGFP
LV-mAR-moeGFP

Figure 5.2 Gross Morphology and Histology of Testis following no injection or injection of vehicle or Lentiviral Vectors carrying mAR and/or monomeric GFP demonstrates no disruption of testicular histology in WT or SC-ARKO testis as a result of the procedure or introduction of a solution into the tubules. (A) Testis weights of LV-moeGFP or LV-mAR-moeGFP injected WT or SC-ARKO testis at collection. Shared letters above columns indicate no significant difference (Kruskal Wallis; *p*=<0.0001). Bars are representative or averages ± SEM (B) Images of freshly dissected uninjected, vehicle, LV-moeGFP and LV-mAR-moeGFP injected WT and SC-ARKO testis under brightfield (BF) or green fluorescent light (after 1.5 seconds and 10.5 seconds exposure). (C) Representative Haematoxylin and Eosin stained sections of uninjected, vehicle, LV-moeGFP and LV-mAR-moeGFP injected WT and SC-ARKO testis. * indicate presence of a lumen in LV-mAR-moeGFP SC-ARKO testis. Scale bars = 20µm

5.3.3 Adult SC-ARKO Testis injected with LV-mAR-moeGFP exhibit restoration of expression of AR in Sertoli Cells

Due to the lack of moeGFP fluorescence observed in LV-mAR-moeGFP injected testis, detection of the GFP reporter in the testis was attempted but not successful in these samples. Therefore to determine whether the delivered AR transgene is being expressed by the targeted Sertoli cells, chromogenic immunostaining for AR was performed on both WT and SC-ARKO testis sections. AR expression in WT testis, regardless of treatment, was located as expected in Sertoli cells, peritubular myoid cells and Leydig cells (Figure 5.3A). In SC-ARKO control treated testis (no injection, vehicle injected, LV-moeGFP injected), AR expression was limited to peritubular myoid cells and Leydig cells and completely absent from Sertoli cells. Detection of AR staining within seminiferous tubules of SC-ARKO + LV-mAR-moeGFP testis indicates successful delivery and integration of the lentiviral vectors into the targeted Sertoli cell DNA (Figure 5.3A). Closer inspection of the AR expression in LV-mAR-moeGFP injected SC-ARKO testis further confirmed the localisation of the AR staining within the Sertoli cells, and also revealed what appeared to be elongated spermatids in the tubular compartment, in direct contact with Sertoli cells exhibiting restored AR expression (Figure 5.3B). Quantification of SC-ARKO testis injected with LV-mAR-moeGFP found that 100% of injected testis have some evidence of Sertoli cells expressing AR, and that SC-ARKO testis injected with LV-moeGFP exhibited no evidence of Sertoli cell AR expression (Figure 5.3C). Chi-squared analysis confirmed that there was a significant association between genotype and treatment and whether presence of AR was observed (p=<0.0001).


A
WT
SC-ARKO
No Inj
Vehicle
LV-moeGFP
LV-mAR-moeGFP
AR

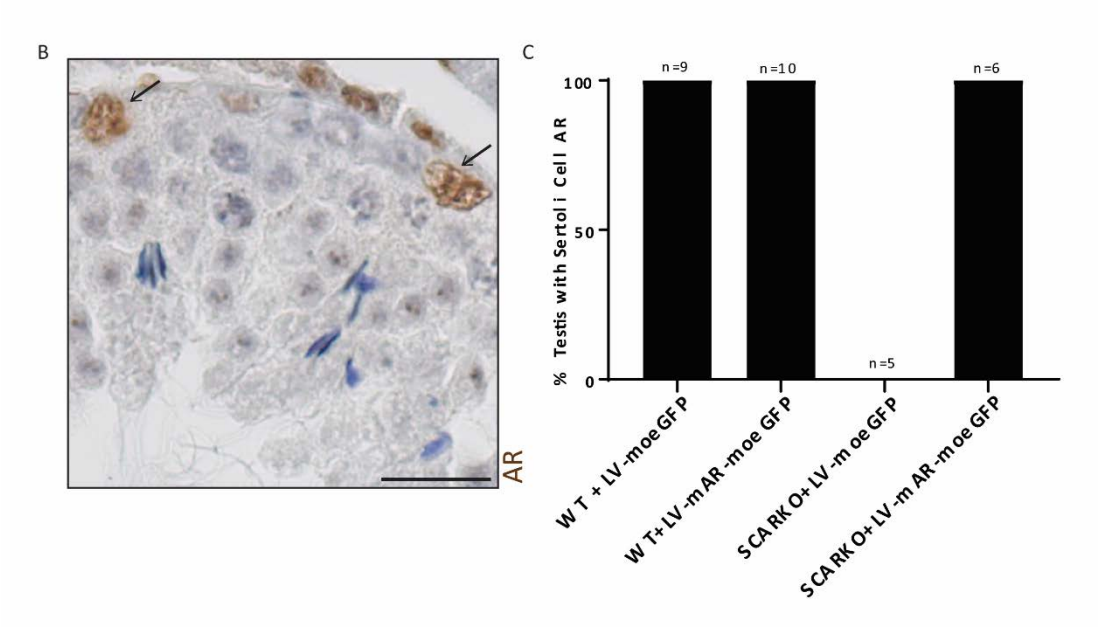
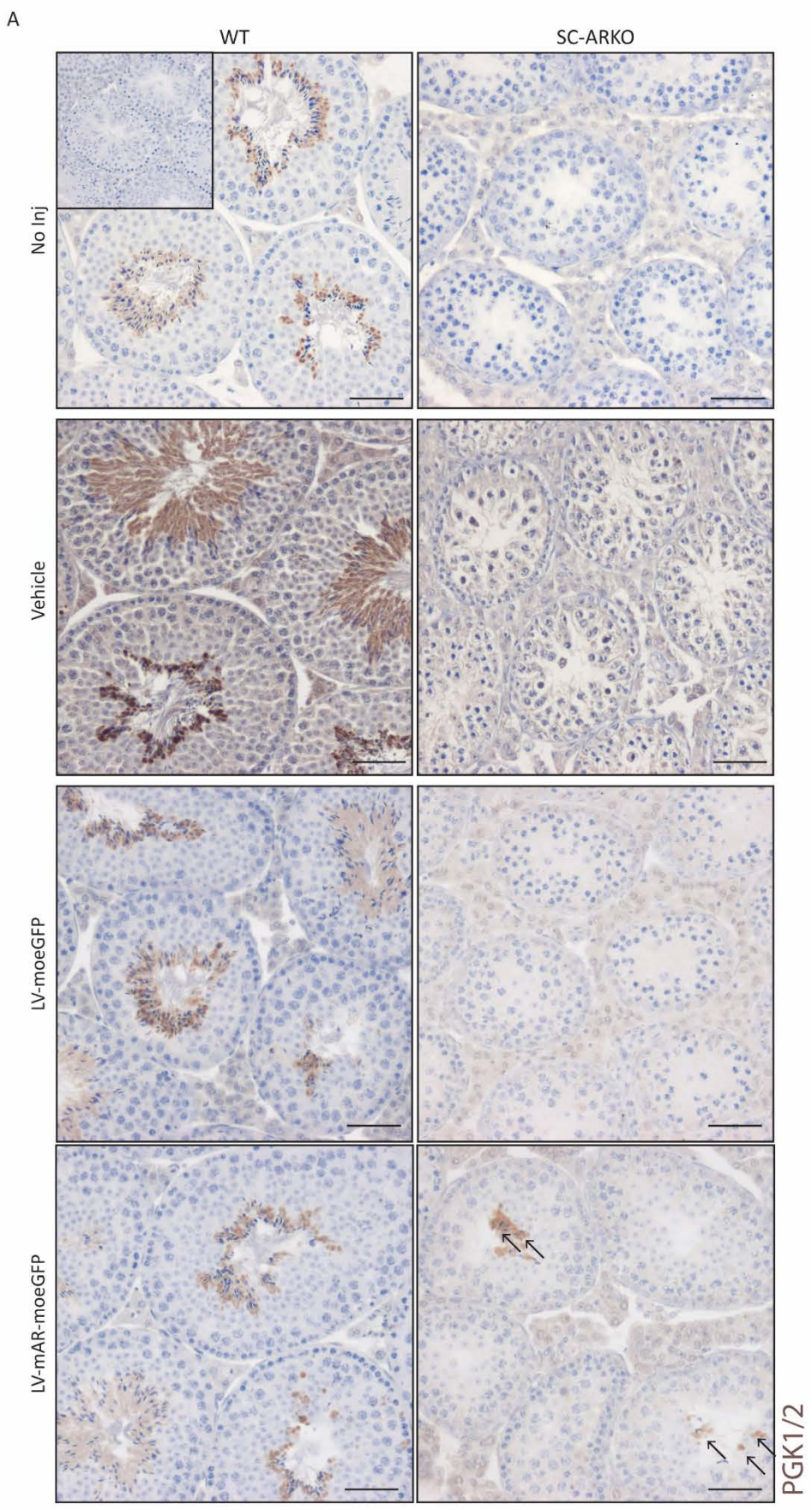


Figure 5.3 AR Expression in LV-moeGFP or LV-mAR-moeGFP injected WT and SC-ARKO testis. (A) AR immunostaining in uninjected, vehicle, LV-moeGFP or LV-mAR-moeGFP injected WT and SC-ARKO testis. Arrows depict AR positive Sertoli cells in LV-mAR-moeGFP injected SC-ARKO testis. Scale bars = 20µm. (B) A close up image of a SCARKO+LV-mAR-moeGFP testis demonstrates restoration of Sertoli cell AR expression. Arrows depict AR positive Sertoli cells. Scale bars = 8.3µm. (C) Visual representation of number of WT and SCARKO testis with evidence (through immunostaining) of Sertoli Cell AR. As expected all WT testis, regardless of injection, had evidence of Sertoli cell AR whereas LV-moeGFP injected SCARKO testis lacked any Sertoli Cell AR. 100% of the six SCARKO testis injected with LV-mAR-moeGFP, had evidence of AR expression in Sertoli cells. Chi-squared analysis confirmed that there was a significant association between genotype and treatment and whether presence of AR was detected ($p=<0.0001$).

5.3.4 Restoration of Sertoli Cell AR expression in SC-ARKO testis results in repair of spermatogenic function of the testis.

In addition to the AR staining visible in SC-ARKO + LV-mAR-moeGFP testis, morphologically mature spermatids were also visible in SC-ARKO+LV-mAR-moeGFP testis. To confirm this, chromogenic immunostaining of spermatid marker; PGK1/2 was carried out on both WT and SC-ARKO testis sections. SC-ARKO+LV-mAR-moeGFP testis exhibit a number of tubules with positively stained spermatids in comparison to SC-ARKO+ LV-moeGFP, which exhibit no PGK1/2 stained spermatids (Figure 5.4A). Male SC-ARKO mice have previously been reported to lack of PGK1/2 positive spermatids (indicating a halt of spermatogenesis post meiosis) [5], therefore, positive staining noted in these studies indicates a restoration of spermatogenesis as a result of mAR transgene delivery by lentiviral vector. The characteristic shape of the 'hook-headed' mouse elongated spermatids are easily identifiable in LV-mAR-moeGFP injected testis (Figure 5.4B) further confirming a rescue of spermatogenic function. Elongated spermatids were detected in 66% of SC-ARKO testis injected with LV-mAR-moeGFP and in none of the SC-ARKO testis injected with LV-moeGFP (Figure 5.4C). Chi-squared analysis confirmed that there was a significant association between genotype and treatment and whether presence of elongated spermatids was observed ($p=<0.0001$).


A
WT
SC-ARKO
No Inj
Vehicle
LV-moeGFP
LV-mAR-moeGFP
PGK1/2

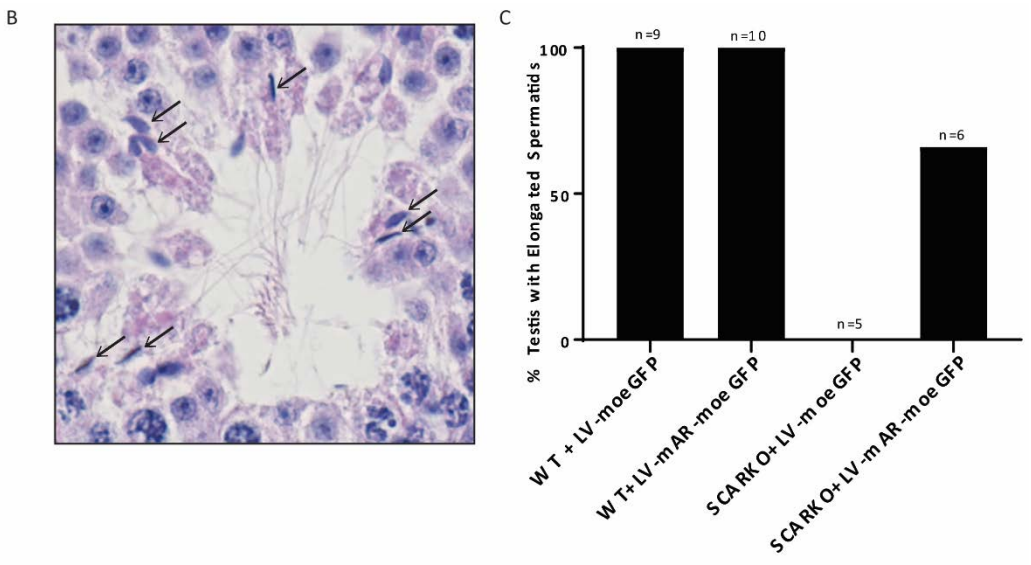


Figure 5.4 Quantifying success of using lentiviral delivered mAR to rescue SCARKO testicular phenotype. (A) Representative immunostaining for Androgen Receptor (AR) and Phosphoglycerate Kinase 1+2(PGK1/2) in WT and SCARKO testis 50 days post procedure with either no injection or injection of LV-moeGFP or LV-mAR-moeGFP. Primary antibody negative controls are included (inset left). Scale bars = 10µm. (B) A close up of image of a testis section from a SC-ARKO testis injected with LV-mAR-moeGFP demonstrating restoration of spermatogenesis. (C) Visual representation of number of WT and SCARKO testis with/without presence of spermatids following injection with either LV-moeGFP or LV-mAR-moeGFP. As expected, all WT testis exhibited presence of mature elongated spermatids regardless of lentiviral injection and LV-moe-GFP injected SCARKO testis lacked presence of mature elongated spermatids. Of the six SCARKO testis injected with LV-mAR-moeGFP, four testis (66.6%) exhibited evidence of mature elongated spermatids. Chi-squared analysis confirmed that there was a significant association between genotype and treatment and whether presence of mature spermatids was observed ($p=<0.0001$).

5.4 Discussion

The overarching aim of the experiments described in this chapter was to demonstrate the practical uses of lentiviral delivery to the testis, in particular to demonstrate delivery/restoration of a transgene in a mouse model of testicular dysfunction.

There has previously been two published studies demonstrating the rescue of gene using lentiviral vector delivered transgenes and as a consequence, fertility. The first by Ikawa and colleagues in 2002, in which full spermatogenesis was detected in Sl/Sl^{d} mutant mice resulted in the generation of offspring via microinsemination [397]. The second to do so was Li *et al* in 2013 [396]. However, this group were unable to produce progeny from their lentiviral injected PICK1 null testis. One reason for this may be that, unlike the study by Ikawa *et al*, Li *et al* did not attempt to generate offspring using assisted reproductive techniques such as intra cytoplasmic sperm injection (ICSI) and instead allowed the mice to mate naturally. This may be a due to an insufficient number of mature spermatids being produced due to relatively low Sertoli cell targeting of the lentiviral vector and/or due to an insufficient number of maturing spermatozoa reaching the epididymis for maturation. However, in both of these studies, mice were injected prior to the completion of testis maturation/puberty, with Ikawa *et al* injecting testis at between pnd 5-10 and Li *et al* injected testis between pnd 21 and 42 [396, 397], whereas the aim of the experiments described in the present study were to demonstrate the potential of lentiviral vector delivery to the Sertoli cells in the adult testis.

To determine whether it would be possible to rescue a phenotype in the adult testis, SC-ARKO (and WT) testis were injected with a lentiviral vector carrying mAR and moeGFP (or control viruses carrying moeGFP alone). It was found that, despite control virus injected testis emitting signal under a GFP fluorescent lamp, this was not the case for those injected with lentiviral vector carrying mAR and moeGFP. The co-expression of multiple transgenes from a single Lentiviral vector was permitted with the incorporation of 2A “self-cleaving” peptide between the two transgenes of interest (Figure 5.1C – denoted as “P2A”). These self-cleaving peptides allow ribosomes to skip the formation of a glycel-prolyl peptide bond at the C-terminus of the 2A peptide [483, 484]. Successful skipping of the ribosome allows the translation and cleavage co-translationally of two proteins. Therefore, failure of the

expression of the protein downstream of the P2A site may be due to the ribosome "falling off" the viral mRNA, discontinuing the translation of the gene downstream of the p2A cleavage site [484]. If this is the case, this would imply that the gene upstream of the p2a cleavage site; mAR, would still have been translated to protein successfully but not that downstream of the p2a cleavage site; GFP, affording explanation to the lack of GFP expression in LV-mAR-moeGFP injected testis.

The lack of moeGFP expression in LV-mAR-moeGFP injected testis meant that visualisation of the lentiviral vectors was not possible using immunostaining methods with an antibody specific to moeGFP. However, in SC-ARKO testis, AR protein was detected in SCs using immunohistochemistry in all LV-mAR-moeGFP injected SC-ARKO testis, confirming successful delivery of the exogenous mAR transgene to the Sertoli cells in the adult SC-ARKO testis and, as a result, successful rescue of AR expression.

As discussed in Chapter 4, one important AR mediated role of the Sertoli cells in the adult testis is the production and movement of water and fluids within the seminiferous tubules [467, 476, 477]. In LV-mAR-moeGFP injected SC-ARKO testis, there appeared to be a lumen developed within the seminiferous tubules, indicative of some rescue of Sertoli cell function, though this is yet to be quantified. The function of the luminal fluid, produced by the Sertoli cells, is not only for the transport of elongated spermatids towards the epididymis, but it also provides a favourable microenvironment for spermatogenesis to occur [485, 486]. Elongated spermatids were found to be present in 66% of LV-mAR-moeGFP injected SC-ARKO testis, a stage of spermatogenesis not previously seen in untreated SC-ARKO testis [48]. This demonstrates the rescue of spermatogenesis in the SC-ARKO testis following the restoration of mAR expression in the Sertoli cells. The results presented in this study also demonstrate the reversal of the SCARKO testicular phenotype in tubules targeted by the mAR expressing lentiviral vector. This suggests that absence Sertoli cell AR throughout development in SC-ARKO testis does not appear to have a permanent impact on the Sertoli cells capacity to support spermatogenesis in adulthood following the restoration of mAR.

The introduction of lentiviral vectors to the seminiferous tubules of the testis has been shown to be useful for the delivery of exogenous transgenes with adult knock out of genes [45] a rescue of gene knockout being demonstrated in the literature [396, 397]. Delivery of

lentiviral particles carrying mAR was shown to restore AR expression in SCs lacking AR and, consequently, spermatogenesis with evidence of elongated spermatids in tubules with rescue of AR expression. Furthermore, by delivering mAR to SCs in adulthood with lentiviral vectors, it has been shown that SC-ARKO does not impact SCs capacity to support spermatogenesis in adulthood. This is supportive of an adult SC-ARKO in which lentiviral delivered ARmiRNA induced the halt in post meiotic spermatogenesis seen in Cre/loxP derived SC-ARKO models, confirming the key role of SC-AR in the adult testis.

This provides further evidence indicative of the potential of using lentiviral vectors for the targeting of SCs in the adult testis. Furthermore, the results discussed give some insight into the capacity of delivering transgenes into the testis with the purpose of either generating testis specific and/or rescuing transgenic models of testicular dysfunction. Some evidence is also provided considering the scope of lentiviral transgene delivery for therapeutic purposes in male reproductive disorders.

Chapter 6 General Discussion & Future Perspectives

The causes of male reproductive disorders are yet to be fully defined and our understanding of the genetics behind male hypogonadism and infertility remains limited. Despite significant advances in the management of infertility (with IVF) and hypogonadism (with androgen replacement) over the past two decades, treatments continue to focus upon addressing the symptoms presented at clinic as opposed to the underlying causes of these conditions.

The advancements of gene delivery vectors over the past two decades has rejuvenated the prospects for the developments of clinical gene therapies. With the variety of DNA delivery vectors and the overlapping capacities of each of these vectors, the potential to develop treatments for a large scope of genetic disorders is becoming increasingly conceivable. Alongside this, the rapid expansion of gene editing technologies such as CRISPR/Cas9 has made the manipulation of genes *in vivo* more accessible to researchers, both for furthering knowledge of biological systems and for discovering potential routes to therapies. The use of these technologies for gene therapy is something that is already becoming a reality for some disorders with the number of successful clinical trials that are currently under assessment.

Both DNA vector delivery systems and gene modification technologies have been demonstrated in the mammalian testis with varying rates of success. With the potential to target the germ line, use of these tools in the human testis remains controversial, therefore it is essential that further exploration of these techniques is carried out to fully define the potential they have for use in this system. A number of studies have also demonstrated targeting of the somatic cells in testis; those responsible for both the production of testosterone and those responsible for the support of spermatogenesis. With intricate combinations of these tools, researchers could identify and thus discover a solution for the treatment of these clinically prevalent conditions potentially originating in these somatic cell types of the testis.

The overarching hypothesis of the work described was that, following optimisation of viral vector delivery to the testis, targeting of the adult testicular somatic cell would be permitted *in vivo*, providing a tool from which (i) further insights into testicular function can be

determined and (ii) the development of therapeutic techniques to treat male reproductive disorders could be established. This was then addressed in three stages of experiments; first an optimisation and validation of somatic cell targeting with viral vectors with the aim of delivering an exogenous transgene to specific cell types, taking care to avoid disturbance of the testicular micro-environment as a result of the delivery procedure. Secondly, a model was required for the purpose of repair or replacement of a gene. Given the broad knowledge base already available in the field of androgen signalling in the testis, this gene was selected for replacement within an ARKO model. Generation of single and double somatic cell ARKO models permitted both the identification of a suitable model for repair and allowed a systematic comparison of models for assessment of androgen receptor mediated paracrine signalling in the adult testis. Finally, on the selection of a model for repair, the potential of lentiviral vector delivered transgenes to rescue a model of testicular dysfunction and infertility was determined.

6.1 Establishment and validation of viral vector delivery to target the adult testicular somatic cells.

The use of gene editing systems and the technology developed to deliver them is becoming increasingly common within scientific research. With regards to targeting the cell populations residing in the testis, the majority of studies have focussed on specifically targeting the germ cell population with aim of generating transgenic animals [363, 394-396, 487]. However, few studies have demonstrated a robust system for targeting the germ cell population *in vivo* to produce transgenic lines with a number of studies failing to demonstrate targeting of the germ cell population *in vivo* [361, 488, 489]. First attempted in 2004 using a Maloney leukaemia retrovirus, Shinohara *et al* demonstrated the transduction of SSC and subsequent generation of transgenic offspring following injection into juvenile testis (5 to 10 days old) with an average success rate of 2.8% [363]. The reasoning behind injecting juvenile testis in this study was due to the lack of tight junctions present in the seminiferous tubules at this age point; theoretically allowing easier access of the viral particles to the SSC population. Similarly, juvenile testis injections into both the seminiferous tubules [395] and into the interstitial compartment [394, 395] were utilised in later studies investigating lentiviral vector delivered transgenes for the generation of transgenic offspring and promoters driving delivered transgene expression [394, 395]. Both studies provided contrasting evidence for the generation of transgenic offspring; with Sehgal et al [394] reporting failure using a CMV

driven transgene and success using an EF-1 driven transgene and Qin et al [395] reporting the contrary attributing this to transgene silencing through DNA methylation.

Given the ambiguity of the published data using lentiviral vectors, we sought to repeat the studies to determine the likelihood of delivered transgenes being transmitted to offspring in future studies utilising lentiviral interstitial injections. Analysis of pups produced from 'Pre-Founders' injected with lentiviral vectors expressing GFP under either the CMV or the EF-1 promoter revealed that there had been no transmission of GFP to offspring. This would suggest that either the spermatogonial stem cell population is not being targeted or that, if they are targeted, lentiviral vectors are not able to integrate into the spermatogonial stem cell DNA, preventing the transmission of exogenous/foreign transgenes to progeny. This has previously been suggested by Takehashi (2007) in that male germ cells may acquire resistance to viral infection as they mature. Furthermore a number of studies have demonstrated the resistance or repression of viral DNA expression in different types of viral vector infected stem cells (embryonic, haematopoietic and germ line stem cells), indicating that using these viral vectors to induce stable integration, expression and, as a consequence, to generate transgenic animals, may come with great difficulty [490-492].

For the purpose of developing an alternative therapeutic for the treatment of male reproductive disorders, the inability of the viral vector used for transgene delivery would be beneficial, particularly as editing of the human germ line is currently prohibited in 29 countries [303]. Therefore, further in-depth investigation into the potential for germline transmission following delivery of these viral vectors *in vivo,* both interstitially and into the seminiferous tubules would be beneficial.

Delivery of lentiviral vectors into the interstitial compartment resulted in uptake of the virus particles specifically by the Leydig cells. However, cells targeted with the lentiviral vector ultimately underwent apoptosis, potentially as a result of viral integration into the Leydig cell DNA involving the activation of DNA dependent protein kinases [493]. Previously published studies demonstrating Leydig cell lentiviral uptake have either collected 4dpi (prior to detection of apoptosis in the present study) or utilised a lentiviral vector delivered transgene under a different promoter; the EF1α promoter, the latter of which reporting a drop (but not complete loss) in transgene expression at 9dpi [188, 494].

Apoptosis was not seen in testis injected interstitially with adenoviral vectors, potentially due to adenoviral vectors being incapable of integration, though why apoptosis occurs in Leydig cells but not in Sertoli cells is unclear as yet.

The relative success of adenoviral vector targeting of Leydig cells seen in the experiments described in this thesis are a validation of those seen in a study by Penny *et al* (2017), published during the time-frame of these experiments [175]. This would suggest that adenoviral vectors are more suitable than lentiviral vectors for Leydig cell targeting, though given the transient nature of the delivered transgene expression, and the inability to deliver more than once due to the high immunogenicity characteristics, they may only be suitable for delivery of gene modification and editing technologies within a research setting, for example for the delivery of Cre recombinase.

Therefore, further study, into alternative vector types such adeno-associated viral vectors may be beneficial. Recently, a study has highlighted the capacity of these vectors to integrate into numerous somatic cell types in the testis, depending on the serotype used, but also demonstrated lack of germ line transmission. Given the reduced immunogenicity and the capacity for integration of adeno-associated viral vectors, further exploration into their uses in the testis would be valuable, particularly for the development of therapeutic strategies.

Previous investigations into lentiviral delivery to the seminiferous tubules have utilised injections into the efferent duct of the testis. As the efferent duct is a fragile structure, crucial for the movement of mature spermatids from the testis into the epididymis, damage can incur catastrophic effects to the testicular function. This was evident in a number of testis following injection into the efferent duct and, as a result, prompted an optimisation of the injections into a different area of the testis. This area was chosen to be the rete testis, given the access of this area to the seminiferous tubules. Injections at lower pressures into this compartment were found to be less likely to induce negative impacts like those seen in previous studies in the group.

Delivery of lentiviral vectors to the tubular compartment has been shown to specifically target the Sertoli cells with next to no consequences of lentiviral integration, as demonstrated in a number of published studies [45, 396, 397]. Given the apparent success of this, future prospects of this technique are deemed to be promising, in both research and in clinic, as evident from the number of lentiviral vector based therapies currently reaching

the stages of clinical trials (196 at the time of writing) (http://www.abedia.com/wiley/vectors.php). Further investigation to increase targeting efficiencies in the testis, and to establish methods of targeting peritubular myoid cells (potentially through adeno-associated viral vectors as suggested in the aforementioned study [348]) would provide options for targeting all somatic cell types, in singular or in combination, permitting both investigation and study of the primary somatic cell types at any time point in the adult testis.

6.2 Identifying a model of testicular dysfunction and gaining further insight into androgen signalling in the adult testis

Having successfully optimised a technique to target and deliver exogenous transgenes to the Sertoli cells, a tool in which this technique could be tested and validated was required. Given the extensive knowledge of the roles of AR available, a cell specific ARKO was chosen for rescue. Single and double cell specific ARKOS were generated to identify the most appropriate model for gene rescue with lentiviral vectors. This also permitted an in parallel characterisation of the double somatic cell ARKO models alongside the previously published single cell specific ARKOs, affording further insight into the androgen receptor mediated autocrine and paracrine networks that may be at play in the somatic cell in the adult testis.

In the adult testis, the proper functioning and positioning of both the Sertoli cells and peritubular myoid cells is key for providing support of spermatogenesis and for formation of the BTB, implicating the close relationship these two somatic cells have. This partnership between these two somatic cells was evident following analysis of SMA in single and double somatic cell ARKOs. Transcript expression of SMA was significantly reduced in SC-PTM-ARKO testis but not in any single cell specific ARKO testis and thus emphasising the collaboration between Sertoli cell and peritubular myoid cells. As a result of AR loss in peritubular myoid and/or Sertoli cells, Sertoli cells also begin to lose their polarity, evident from movement of the Sertoli cell nucleus away from the periphery of the seminiferous tubule. One reason for this could be a disruption in the production of extracellular matrix components, a key role of both Sertoli and peritubular myoid cells and for the cell to cell association patterns and polarity maintenance in these cell types [120, 121, 495]. Therefore, analysis of these components, through immuno-staining and through transcript analysis, along with the

apparent associations with AR signalling, using the single and double somatic cell ARKO testis would merit further investigation.

Signalling through Sertoli cell AR is known to be essential for the integrity of the BTB. It has been suggested that WT1 and AR, both of which are expressed by Sertoli cells, may functionally interact during testis development [31, 42, 496]. In mice deficient of WT1, AR expression is not impacted, but integrity of the BTB is lost with reduction in expression of tight junction proteins [497]. The same researchers also demonstrated that the loss of the WT1 in adulthood results in a reduction in the expression of DHH and subsequently a reduction in steroidogenic gene expression [473]. In SC-PTM-ARKO testis, but not in single ARKO knockouts, transcript expression of DHH was significantly reduced, as were a number of steroidogenic enzyme transcripts. This suggests an androgen mediated role for the peritubular myoid cells in paracrine signalling associated with Dhh between Sertoli cells and Leydig cells, though further investigation into this and the status of the BTB would be beneficial for a further understanding of the mechanisms involved.

Each of the cell specific ARKO models revealed no significant differences in serum testosterone levels, suggesting the lack of spermatogenesis in all models (excluding LC-ARKO), is a either a result of lack of Sertoli cell AR or signalling through the peritubular myoid cells and not through any additional factors produced by Leydig cells. Alongside this, for SC-ARKO and SC-LC-ARKO testis, a reduction in LC numbers and few changes in steroidogenic enzymes may signify that the Leydig cells remaining in the testis are each expressing steroidogenic genes and producing testosterone at an increased rate compared to those in other cell specific ARKO models and to WT testis, though serum LH levels are not significantly increased in any of the cell specific ARKO models. This could be examined by stimulating a maximal output from Leydig cells using human chorionic gonadotrophin, with a lack of additional response being indicative of the Leydig cells already functioning to their maximum output.

Another observation made was the reduction of AR expression in Leydig cells in the double somatic cells ARKO testes. For the SC-PTM-ARKO testis, it is not yet clear whether the significant reduction of Leydig cell AR is a result of the smMHC-eGFP targeting Leydig cell progenitors early in testis development or whether it is a true impact of reduced androgen signalling in Sertoli and peritubular myoid cells on the expression of AR in Leydig cells. Indeed,

in the PTM-ARKO testis there is not a significant reduction in AR positive Leydig cells, though any true significant reduction may be masked by the large variances seen in this model.

Leydig cell AR expression was also significantly reduced in SC-LC-ARKO testis, but not in SC-ARKO testis. Though a reduction in Leydig cell AR expression was expected, it was not expected to be as great a reduction as the one observed. This could suggest that expression of AR in Leydig cells may be influenced by Leydig cell AR expression in an autocrine manner.

Interestingly, despite this reduction in AR, the expression of Leydig cell maturation markers; *Insl3* and *Hsd3β6* were not impacted in SC-LC-ARKO like that seen in a published LC-ARKO study [108]. Immunostaining of INSL3 revealed a lack of expression in a small majority of Leydig cells, suggesting that these cells have not undergone full maturation. Given the lack of Leydig cell AR expression in the SC-LC-ARKO, this was surprising and could indicate that a threshold of Leydig cell AR may be required for maturation of the Leydig cell population.

The experiments described were able to provide some additional information permitting a wider perspective as to the autocrine and paracrine signalling networks present in the testis in adulthood with the use of double somatic cell ARKO models.

Development of cell specific targeting systems like those described in chapter one and those published in the literature to allow knock out of AR in single and multiple cell types in the adult testis specifically would allow further dissection of the signalling networks at play in the testis in adulthood [45]. This would permit researchers to distinguish between cell specific roles in development and those in adulthood.

Considering the wealth of knowledge available for the role of Sertoli cell androgen receptor in development and in adulthood testicular function and the improved methodology for targeting Sertoli cells with lentiviral vectors, the Sertoli cell ARKO testis was chosen to be utilised for gene rescue using lentiviral vector delivered transgenes.

6.3 Determining the potential of viral vectors to rescue fertility in the adult SC-ARKO testis

Delivery of viral vectors to testicular somatic cells has been demonstrated previously with the purpose of restoring gene expression. However, each of the studies injected the viral vectors into the testis of juvenile mutant mice [348, 396, 397]. Though gene expression

appeared to be successfully restored in these experiments, the delivery of viral vectors to pre-pubertal testis may give a false impression as to the capability of a lentiviral vector to target an adult somatic cell population and deliver an exogenous transgene. Theoretically, targeting pre-pubertal cells still undergoing proliferation, could result in what appears to be an increased proportion of the adult somatic cell population compared to targeting later in life. Alongside this, testicular boundaries such as the BTB is yet to have developed in the pre-pubertal testis, potentially making it appear as if lentiviral vectors can breach the barrier in adulthood. It is therefore important to determine the practicalities of using this technology in the adult testis.

The experiments described in chapter five were carried out to determine the potential of lentiviral vector delivered transgenes to repair or rescue a SC-ARKO testis in adulthood. To do so, lentiviral vectors carrying mouse AR cDNA were delivered to the rete compartment in the testis, using the low pressure injections optimised in chapter three. Inspection of sections of SC-ARKO testis injected with lentivirus carrying AR revealed what appeared to be the development of lumen within the seminiferous tubules. As demonstrated in chapter four, signalling via Sertoli cell AR is essential for the production of tubular fluids and for thus the development of a lumen.

Targeting and integration of the lentiviral vector into the Sertoli cells was confirmed with the re-expression of AR in all LV-mAR-moeGFP injected SC-ARKO testis but not in those injected with AR negative control lentivirus. As a result of this re-expression of AR in the Sertoli cells, spermatogenesis appeared to continue past the final stages of meiosis with the presence of elongated spermatids being observed in LV-mAR-moeGFP injected SC-ARKO testis and not in those injected with mAR negative lentiviral vectors. The presence of elongated spermatids was noted in four out of six of the LV-mAR-moeGFP injected SC-ARKO testis and was noted in tubules in which AR expression had been restored.

Further study using this system of rescuing AR expression in Sertoli cells and intracytoplasmic sperm injection would be beneficial to determine whether the resultant mature spermatids are capable of producing viable offspring. Regardless, further investigation of control virus injected WT testis would also be beneficial to determine the potential of germ line transmission to offspring; something of particular importance if the technique were to be considered for use as an option for human therapeutics.

The re-introduction of Sertoli cell AR to SC-ARKO testis has confirmed that the absence of Sertoli cell AR throughout development does not appear to have an impact on Sertoli cell function and that its key role is the support of spermatogenesis through androgen signalling. It also endorses the results seen in an adult knock down of AR, produced using lentiviral vector delivered Cre in AR floxed testis, revealing that ablation of Sertoli cell AR in adulthood resulted in a lack of progression of spermatogenesis past meiosis [45]. Additional experiments into the impacts of Sertoli cell loss and rescue on Leydig cell function would be interesting to perform and could be done so following optimisations to increase targeting efficiency and by injecting the viral vectors into both testis (thereby avoiding compensatory mechanisms from the contralateral testis).

Furthermore, restoration of the fertility phenotype in the adult testis confirms the great potential of lentiviral vector transgene delivery for both the development of adult specific transgenic mouse models and for the potential of therapeutics for the treatment of male reproductive disorders. Though this study has demonstrated that only a small number of SC need to express AR to produce mature elongated spermatids, further investigation to increase the targeting efficiency would be advantageous from both the perspective of research uses and for the development of therapeutics. Additionally, further analysis into the potential for transgene transmission to the germ line is crucial following injection of viral vectors into the seminiferous tubules.

Further development of viral and non-viral targeting of somatic cells could permit the generation of adult or age specific (following optimisation of injection techniques in younger testis) mouse models of testis dysfunction. Alongside this, combination of the vector delivery techniques with the rapidly expanding tools for gene editing technology, including the CRISPR/Cas9 system, will allow tailored strategies for knock down and re-expression of one or multiple genes within one organ. Improvements in both the diagnosis and the development of possibilities for bespoke treatment options, like those described here, would be beneficial for the millions of men afflicted with reproductive disorders such as infertility and/or hypogonadism.

6.4 Conclusions

The hypothesis of the experiments described is that the optimisation of viral vector delivery into the testis will permit targeting the adult testicular somatic cells *in vivo,* providing a tool from which (i) further insights into testicular function can be determined and (ii) the development of therapeutic techniques to treat male reproductive disorders could be established.

Novel observations to support this hypothesis include:

1. Injection of substances at low pressure into the rete compartment of the testis reduce the negative side effects seen in testis injected at high pressure into the efferent duct, resulting in a technique more suited to the purpose of gene delivery and repair.

2. Delivery of androgen receptor to Sertoli cells in the adult SC-ARKO testis results in re-expression of androgen receptor and subsequently the production of elongated spermatids; a stage of spermatogenesis not previously seen in a SC-ARKO testis.

3. Delivery of androgen receptor to Sertoli cells in the adult SC-ARKO testis also revealed that the loss of AR during development has no permanent impact on the functioning of the Sertoli cell following reintroduction of AR.

Additional novel observations reported in thesis include:

1. The detection of markers of apoptosis and subsequent loss of targeted Leydig cells could suggest that the use lentiviral vectors to target Leydig cells may be unsuitable.

2. There may be an androgen mediated role for the peritubular myoid cells in paracrine signalling associated with Dhh between Sertoli cells and Leydig cells in adulthood.

3. The expression of AR in Leydig cells may be regulated in an autocrine manner in adulthood.

Bibliography

1. Punab, M., et al., *Causes Of Severe Male Factor Infertility: A 9-Year Prospective Monocenter Study On 1,737 Patients With Suboptimal Total Sperm Counts.* Submitted, 2016.
2. Tajar, A., et al., *Characteristics of secondary, primary, and compensated hypogonadism in aging men: evidence from the European Male Ageing Study.* J.Clin.Endocrinol.Metab, 2010. **95**(4): p. 1810-1818.
3. Wu, F.C., et al., *Identification of late-onset hypogonadism in middle-aged and elderly men.* N Engl J Med, 2010. **363**(2): p. 123-35.
4. Skakkebaek, N.E., et al., *Male Reproductive Disorders and Fertility Trends: Influences of Environment and Genetic Susceptibility.* Physiol Rev, 2016. **96**(1): p. 55-97.
5. Rebourcet, D., et al., *Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis.* Development, 2014. **141**(10): p. 2139-49.
6. O'Shaughnessy, P.J., et al., *Direct action through the sertoli cells is essential for androgen stimulation of spermatogenesis.* Endocrinology, 2010. **151**(5): p. 2343-8.
7. Rebourcet, D., et al., *Sertoli cells maintain Leydig cell number and peritubular myoid cell activity in the adult mouse testis.* PLoS One, 2014. **9**(8): p. e105687.
8. Rebourcet, D., et al., *Sertoli Cells Modulate Testicular Vascular Network Development, Structure, and Function to Influence Circulating Testosterone Concentrations in Adult Male Mice.* Endocrinology, 2016. **157**(6): p. 2479-88.
9. Rebourcet, D., et al., *Sertoli Cell Number Defines and Predicts Germ and Leydig Cell Population Sizes in the Adult Mouse Testis.* Endocrinology, 2017. **158**(9): p. 2955-2969.
10. Karl, J. and B. Capel, *Sertoli cells of the mouse testis originate from the coelomic epithelium.* Dev Biol, 1998. **203**(2): p. 323-33.
11. Behringer, R.R., M.J. Finegold, and R.L. Cate, *Mullerian-inhibiting substance function during mammalian sexual development.* Cell, 1994. **79**(3): p. 415-25.
12. Orth, J.M., G.L. Gunsalus, and A.A. Lamperti, *Evidence From Sertoli Cell-Depleted Rats Indicates That Spermatid Number in Adults Depends on Numbers of Sertoli Cells Produced During Perinatal Development*.* Endocrinology, 1988. **122**(3): p. 787-794.
13. Vergouwen, R.P., et al., *Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice.* J Reprod Fertil, 1991. **93**(1): p. 233-43.
14. Baker, P.J. and P.J. O'Shaughnessy, *Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice.* Reproduction, 2001. **122**(2): p. 227-34.
15. Johnston, H., et al., *Regulation of Sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse.* Endocrinology, 2004. **145**(1): p. 318-29.
16. Pitetti, J.-L., et al., *An Essential Role for Insulin and IGF1 Receptors in Regulating Sertoli Cell Proliferation, Testis Size, and FSH Action in Mice.* Molecular Endocrinology, 2013. **27**(5): p. 814-827.

17. Fumel, B., et al., *Expression of dominant-negative thyroid hormone receptor alpha1 in Leydig and Sertoli cells demonstrates no additional defect compared with expression in Sertoli cells only.* PLoS One, 2015. **10**(3): p. e0119392.
18. van Haaster, L.H., et al., *High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels.* Endocrinology, 1993. **133**(2): p. 755-60.
19. Cooke, P.S. and E. Meisami, *Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels.* Endocrinology, 1991. **129**(1): p. 237-43.
20. Kirby, J.D., et al., *Developmental hormonal profiles accompanying the neonatal hypothyroidism-induced increase in adult testicular size and sperm production in the rat.* Endocrinology, 1992. **131**(2): p. 559-65.
21. Auharek, S.A. and L.R. de Franca, *Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL/6J mice made transiently hypo- and hyperthyroidic during the neonatal period.* J Anat, 2010. **216**(5): p. 577-88.
22. Lara, N.L.M. and L.R. Franca, *Neonatal hypothyroidism does not increase Sertoli cell proliferation in iNOS(-/-) mice.* Reproduction, 2017. **154**(1): p. 13-22.
23. Abel, M.H., et al., *Spermatogenesis and Sertoli Cell Activity in Mice Lacking Sertoli Cell Receptors for Follicle-Stimulating Hormone and Androgen.* Endocrinology, 2008. **149**(7): p. 3279-3285.
24. Tan, K.A., et al., *The role of androgens in sertoli cell proliferation and functional maturation: studies in mice with total or Sertoli cell-selective ablation of the androgen receptor.* Endocrinology, 2005. **146**(6): p. 2674-83.
25. Willems, A., et al., *Selective Ablation of the Androgen Receptor in Mouse Sertoli Cells Affects Sertoli Cell Maturation, Barrier Formation and Cytoskeletal Development.* PLOS ONE, 2010. **5**(11): p. e14168.
26. McCabe, M.J., et al., *Androgen initiates Sertoli cell tight junction formation in the hypogonadal (hpg) mouse.* Biol Reprod, 2012. **87**(2): p. 38.
27. Saitou, M., et al., *Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands.* Molecular Biology of the Cell, 2000. **11**(12): p. 4131-4142.
28. Mazaud-Guittot, S., et al., *Claudin 11 Deficiency in Mice Results in Loss of the Sertoli Cell Epithelial Phenotype in the Testis.* Biology of Reproduction, 2010. **82**(1): p. 202-213.
29. Smith, B.E. and R.E. Braun, *Germ cell migration across Sertoli cell tight junctions.* Science, 2012. **338**(6108): p. 798-802.
30. Janecki, A., A. Jakubowiak, and A. Steinberger, *Regulation of transepithelial electrical resistance in two-compartment Sertoli cell cultures: in vitro model of the blood-testis barrier.* Endocrinology, 1991. **129**(3): p. 1489-96.
31. Meng, J., et al., *Androgens regulate the permeability of the blood-testis barrier.* Proc Natl Acad Sci U S A, 2005. **102**(46): p. 16696-700.
32. Chakraborty, P., et al., *Androgen-dependent sertoli cell tight junction remodeling is mediated by multiple tight junction components.* Mol Endocrinol, 2014. **28**(7): p. 1055-72.

33. Lie, P.P., C.Y. Cheng, and D.D. Mruk, *Interleukin-1alpha is a regulator of the blood-testis barrier.* Faseb j, 2011. **25**(4): p. 1244-53.
34. Lui, W.Y., W.M. Lee, and C.Y. Cheng, *TGF-betas: their role in testicular function and Sertoli cell tight junction dynamics.* Int J Androl, 2003. **26**(3): p. 147-60.
35. Skinner, M.K., *Cell-cell interactions in the testis.* Endocr Rev, 1991. **12**(1): p. 45-77.
36. Mruk, D.D. and C.Y. Cheng, *The Mammalian Blood-Testis Barrier: Its Biology and Regulation.* Endocr Rev, 2015. **36**(5): p. 564-91.
37. O'Shaughnessy, P.J., *Hormonal control of germ cell development and spermatogenesis.* Semin Cell Dev Biol, 2014. **29**: p. 55-65.
38. Dimitriadis, F., et al., *The Sertoli cell as the orchestra conductor of spermatogenesis: spermatogenic cells dance to the tune of testosterone.* Hormones (Athens), 2015. **14**(4): p. 479-503.
39. Lyon, M.F., P.H. Glenister, and M. Lynn Lamoreux, *Normal spermatozoa from androgen-resistant germ cells of chimaeric mice and the role of androgen in spermatogenesis.* Nature, 1975. **258**: p. 620.
40. Johnston, D.S., et al., *Murine germ cells do not require functional androgen receptors to complete spermatogenesis following spermatogonial stem cell transplantation.* Endocrinology, 2001. **142**(6): p. 2405-8.
41. Kerr, J.B., et al., *Stage-Dependent changes in spermatogenesis and sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone.* The Anatomical Record, 1993. **235**(4): p. 547-559.
42. Wang, R.-S., et al., *Androgen Receptor in Sertoli Cell Is Essential for Germ Cell Nursery and Junctional Complex Formation in Mouse Testes.* Endocrinology, 2006. **147**(12): p. 5624-5633.
43. Hazra, R., et al., *Temporal role of Sertoli cell androgen receptor expression in spermatogenic development.* Mol Endocrinol, 2013. **27**(1): p. 12-24.
44. Willems, A., et al., *The development of an inducible androgen receptor knockout model in mouse to study the post-meiotic effects of androgens on germ cell development.* Spermatogenesis, 2011. **1**(4): p. 341-353.
45. Willems, A., et al., *Sertoli cell androgen receptor signalling in adulthood is essential for post-meiotic germ cell development.* Mol Reprod Dev, 2015. **82**(9): p. 626-7.
46. O'Shaughnessy, P.J., et al., *Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors.* Reproduction, 2010. **139**(1): p. 177-84.
47. Abel, M.H., et al., *Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen.* Endocrinology, 2008. **149**(7): p. 3279-85.
48. De Gendt, K., et al., *A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1327-32.
49. Lim, P., et al., *Sertoli cell androgen receptor DNA binding domain is essential for the completion of spermatogenesis.* Endocrinology, 2009. **150**(10): p. 4755-65.

50. Holdcraft, R.W. and R.E. Braun, *Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids.* Development, 2004. **131**(2): p. 459-67.
51. Tan, K.A., et al., *Androgen regulation of stage-dependent cyclin D2 expression in Sertoli cells suggests a role in modulating androgen action on spermatogenesis.* Biol Reprod, 2005. **72**(5): p. 1151-60.
52. Welsh, M., et al., *Androgen action via testicular peritubular myoid cells is essential for male fertility.* Faseb j, 2009. **23**(12): p. 4218-30.
53. Singh, J., C. O'Neill, and D.J. Handelsman, *Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice.* Endocrinology, 1995. **136**(12): p. 5311-21.
54. Meachem, S.J., et al., *Androgen action on the restoration of spermatogenesis in adult rats: effects of human chorionic gonadotrophin, testosterone and flutamide administration on germ cell number.* Int J Androl, 1997. **20**(2): p. 70-9.
55. McLachlan, R.I., et al., *The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH.* J Endocrinol, 1996. **148**(1): p. 1-9.
56. Spaliviero, J.A., et al., *Luteinizing hormone receptor-mediated effects on initiation of spermatogenesis in gonadotropin-deficient (hpg) mice are replicated by testosterone.* Biol Reprod, 2004. **70**(1): p. 32-8.
57. Hazra, R., et al., *Elevated expression of the Sertoli cell androgen receptor disrupts male fertility.* Am J Physiol Endocrinol Metab, 2016. **311**(2): p. E396-404.
58. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.* Nat Genet, 1997. **15**(2): p. 201-4.
59. Wreford, N.G., et al., *Analysis of the Testicular Phenotype of the Follicle-Stimulating Hormone β-Subunit Knockout and the Activin Type II Receptor Knockout Mice by Stereological Analysis*.* Endocrinology, 2001. **142**(7): p. 2916-2920.
60. O'Shaughnessy, P.J., A. Monteiro, and M. Abel, *Testicular development in mice lacking receptors for follicle stimulating hormone and androgen.* PLoS One, 2012. **7**(4): p. e35136.
61. Kanatsu-Shinohara, M., et al., *Regulation of Mouse Spermatogonial Stem Cell Self-Renewing Division by the Pituitary Gland1*. Biology of Reproduction, 2004. **70**(6): p. 1731-1737.
62. Chen, S.R. and Y.X. Liu, *Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling.* Reproduction, 2015. **149**(4): p. R159-67.
63. Chen, L.-Y., W.D. Willis, and E.M. Eddy, *Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development.* Proceedings of the National Academy of Sciences, 2016. **113**(7): p. 1829-1834.
64. Tanaka, T., et al., *The Luteinizing Hormone-Testosterone Pathway Regulates Mouse Spermatogonial Stem Cell Self-Renewal by Suppressing WNT5A Expression in Sertoli Cells.* Stem Cell Reports, 2016. **7**(2): p. 279-91.

65. Huang, Z., B. Rivas, and A.I. Agoulnik, *Insulin-like 3 signaling is important for testicular descent but dispensable for spermatogenesis and germ cell survival in adult mice.* Biol Reprod, 2012. **87**(6): p. 143.
66. Nef, S. and L.F. Parada, *Cryptorchidism in mice mutant for Insl3.* Nat Genet, 1999. **22**(3): p. 295-9.
67. van den Driesche, S., et al., *Inter-relationship between testicular dysgenesis and Leydig cell function in the masculinization programming window in the rat.* PLoS One, 2012. **7**(1): p. e30111.
68. van den Driesche, S., et al., *Proposed role for COUP-TFII in regulating fetal Leydig cell steroidogenesis, perturbation of which leads to masculinization disorders in rodents.* PLoS One, 2012. **7**(5): p. e37064.
69. Park, S.Y., M. Tong, and J.L. Jameson, *Distinct roles for steroidogenic factor 1 and desert hedgehog pathways in fetal and adult Leydig cell development.* Endocrinology, 2007. **148**(8): p. 3704-10.
70. El-Gehani, F., et al., *Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis.* Biol Reprod, 1998. **58**(1): p. 116-23.
71. O'Shaughnessy, P.J., P.J. Baker, and H. Johnston, *The foetal Leydig cell--differentiation, function and regulation.* Int J Androl, 2006. **29**(1): p. 90-5; discussion 105-8.
72. Ge, R.S., et al., *In search of rat stem Leydig cells: identification, isolation, and lineage-specific development.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2719-24.
73. P., P.F., *Ultrastructural evidence of mature Leydig cells and Leydig cell regression in the neonatal human testis.* The Anatomical Record, 1990. **228**(4): p. 405-417.
74. Faria, M.J.S., et al., *Apoptosis Process in Mouse Leydig Cells during Postnatal Development.* Microscopy and Microanalysis, 2003. **9**(1): p. 68-73.
75. Kerr, J.B. and C.M. Knell, *The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis.* Development, 1988. **103**(3): p. 535-544.
76. Ariyaratne, H.B.S. and S.M.L. Chamindrani Mendis-Handagama, *Changes in the Testis Interstitium of Sprague Dawley Rats from Birth to Sexual Maturity1*. Biology of Reproduction, 2000. **62**(3): p. 680-690.
77. Shima, Y., et al., *Fetal Leydig Cells Persist as an Androgen-Independent Subpopulation in the Postnatal Testis.* Molecular Endocrinology, 2015. **29**(11): p. 1581-1593.
78. Kaftanovskaya, E.M., et al., *Genetic ablation of androgen receptor signaling in fetal Leydig cell lineage affects Leydig cell functions in adult testis.* Faseb j, 2015. **29**(6): p. 2327-37.
79. Stanley, E., et al., *Identification, Proliferation, and Differentiation of Adult Leydig Stem Cells.* Endocrinology, 2012. **153**(10): p. 5002-5010.
80. Teerds, K.J., et al., *Development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels.* Biol Reprod, 1998. **59**(2): p. 344-50.
81. Davidoff, M.S., et al., *Progenitor cells of the testosterone-producing Leydig cells revealed.* J Cell Biol, 2004. **167**(5): p. 935-44.
82. Kilcoyne, K.R., et al., *Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells.* Proceedings of the National

Academy of Sciences of the United States of America, 2014. **111**(18): p. E1924-E1932.
83. Gnessi, L., et al., *Testicular development involves the spatiotemporal control of PDGFs and PDGF receptors gene expression and action.* J Cell Biol, 1995. **131**(4): p. 1105-21.
84. Baker, J., et al., *Effects of an Igf1 gene null mutation on mouse reproduction.* Mol Endocrinol, 1996. **10**(7): p. 903-18.
85. Hu, G.X., et al., *Deletion of the Igf1 gene: suppressive effects on adult Leydig cell development.* J Androl, 2010. **31**(4): p. 379-87.
86. Wang, G. and M.P. Hardy, *Development of leydig cells in the insulin-like growth factor-I (igf-I) knockout mouse: effects of igf-I replacement and gonadotropic stimulation.* Biol Reprod, 2004. **70**(3): p. 632-9.
87. Zhang, F.P., et al., *Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice.* Mol Endocrinol, 2001. **15**(1): p. 172-83.
88. Zhang, F.P., et al., *Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice.* Endocrinology, 2004. **145**(3): p. 1453-63.
89. Lei, Z.M., et al., *Testicular Phenotype in Luteinizing Hormone Receptor Knockout Animals and the Effect of Testosterone Replacement Therapy1.* Biology of Reproduction, 2004. **71**(5): p. 1605-1613.
90. Ma, X., et al., *Targeted disruption of luteinizing hormone β-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(49): p. 17294-17299.
91. Rijntjes, E., et al., *Prenatal induced chronic dietary hypothyroidism delays but does not block adult-type Leydig cell development.* Am J Physiol Endocrinol Metab, 2009. **296**(2): p. E305-14.
92. Kobayashi, K., et al., *Dose-dependent effects of perinatal hypothyroidism on postnatal testicular development in rat offspring.* The Journal of Toxicological Sciences, 2014. **39**(6): p. 867-874.
93. Hardy, M.P., et al., *Differentiation of Leydig Cell Precursors In Vitro: A Role for Androgen.* Endocrinology, 1990. **127**(1): p. 488-490.
94. Shan, L.X. and M.P. Hardy, *Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells.* Endocrinology, 1992. **131**(3): p. 1107-1114.
95. Siril Ariyaratne, H.B., et al., *Studies on the onset of Leydig precursor cell differentiation in the prepubertal rat testis.* Biol Reprod, 2000. **63**(1): p. 165-71.
96. Hardy, M.P., B.R. Zirkin, and L.L. Ewing, *Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat.* Endocrinology, 1989. **124**(2): p. 762-70.
97. Sriraman, V., et al., *Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1.* Mol Cell Endocrinol, 2000. **162**(1-2): p. 113-20.
98. Karpova, T., et al., *Steroidogenic factor 1 differentially regulates fetal and adult leydig cell development in male mice.* Biol Reprod, 2015. **93**(4): p. 83.

99. Teerds, K.J. and J.H. Dorrington, *Localization of transforming growth factor beta 1 and beta 2 during testicular development in the rat.* Biol Reprod, 1993. **48**(1): p. 40-5.
100. Özgüden-Akkoç, C.G. and A. Özer, *Immunohistochemical localization of transforming growth factor β1 and β2 in mouse testes during postnatal development.* Biotechnic & Histochemistry, 2012. **87**(2): p. 154-159.
101. De Gendt, K., et al., *Development and function of the adult generation of Leydig cells in mice with Sertoli cell-selective or total ablation of the androgen receptor.* Endocrinology, 2005. **146**(9): p. 4117-26.
102. Baker, P.J., et al., *Failure of normal Leydig cell development in follicle-stimulating hormone (FSH) receptor-deficient mice, but not FSHbeta-deficient mice: role for constitutive FSH receptor activity.* Endocrinology, 2003. **144**(1): p. 138-45.
103. Wu, X., et al., *Pubertal and Adult Leydig Cell Function in Mullerian Inhibiting Substance-Deficient Mice.* Endocrinology, 2005. **146**(2): p. 589-595.
104. Rouiller-Fabre, V., et al., *Effect of anti-Mullerian hormone on Sertoli and Leydig cell functions in fetal and immature rats.* Endocrinology, 1998. **139**(3): p. 1213-20.
105. Salva, A., et al., *Mullerian-inhibiting substance inhibits rat Leydig cell regeneration after ethylene dimethanesulphonate ablation.* Biol Reprod, 2004. **70**(3): p. 600-7.
106. Qin, J., M.J. Tsai, and S.Y. Tsai, *Essential roles of COUP-TFII in Leydig cell differentiation and male fertility.* PLoS One, 2008. **3**(9): p. e3285.
107. Benton, L., L.X. Shan, and M.P. Hardy, *Differentiation of adult Leydig cells.* J Steroid Biochem Mol Biol, 1995. **53**(1-6): p. 61-8.
108. O'Hara, L., et al., *Autocrine androgen action is essential for Leydig cell maturation and function, and protects against late-onset Leydig cell apoptosis in both mice and men.* Faseb j, 2015. **29**(3): p. 894-910.
109. O'Shaughnessy, P.J., et al., *Failure of normal adult Leydig cell development in androgen-receptor-deficient mice.* J Cell Sci, 2002. **115**(Pt 17): p. 3491-6.
110. Shan, L.X., et al., *Differential regulation of steroidogenic enzymes during differentiation optimizes testosterone production by adult rat Leydig cells.* Endocrinology, 1993. **133**(5): p. 2277-83.
111. Zirkin, B.R. and L.L. Ewing, *Leydig cell differentiation during maturation of the rat testis: a stereological study of cell number and ultrastructure.* Anat Rec, 1987. **219**(2): p. 157-63.
112. Teerds, K.J., et al., *Turnover time of Leydig cells and other interstitial cells in testes of adult rats.* Arch Androl, 1989. **23**(2): p. 105-11.
113. Clark, A.M., K.K. Garland, and L.D. Russell, *Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules.* Biol Reprod, 2000. **63**(6): p. 1825-38.
114. Hazra, R., et al., *Sertoli Cell Androgen Receptor Expression Regulates Temporal Fetal and Adult Leydig Cell Differentiation, Function, and Population Size.* Endocrinology, 2013. **154**(9): p. 3410-3422.
115. Fecteau, K.A., et al., *Detection of platelet-derived growth factor-alpha (PDGF-A) protein in cells of Leydig lineage in the postnatal rat testis.* Histol Histopathol, 2006. **21**(12): p. 1295-302.

116. O'Shaughnessy, P.J., I.D. Morris, and P.J. Baker, *Leydig cell re-generation and expression of cell signaling molecules in the germ cell-free testis.* Reproduction, 2008. **135**(6): p. 851-8.
117. Kerr, J.B. and R.M. Sharpe, *Stimulatory effect of follicle-stimulating hormone on rat Leydig cells. A morphometric and ultrastructural study.* Cell Tissue Res, 1985. **239**(2): p. 405-15.
118. Chen, H., et al., *Leydig Cell Stem Cells: Identification, Proliferation and Differentiation.* Molecular and cellular endocrinology, 2017. **445**: p. 65-73.
119. Clermont, Y., *Contractile elements in the limiting membrane of the seminiferous tubules of the rat.* Experimental cell research, 1958. **15**(2): p. 438-440.
120. Skinner, M.K., Tung, P. S., Fritz, I. B., *Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components.* The Journal of Cell Biology, 1985. **100**(6): p. 1941-1947.
121. Tung, P.S. and I.B. Fritz, *Extracellular matrix components and testicular peritubular cells influence the rate and pattern of Sertoli cell migration in vitro.* Dev Biol, 1986. **113**(1): p. 119-34.
122. Maekawa, M., K. Kamimura, and T. Nagano, *Peritubular myoid cells in the testis: their structure and function.* Arch Histol Cytol, 1996. **59**(1): p. 1-13.
123. De Gendt, K. and G. Verhoeven, *Tissue- and cell-specific functions of the androgen receptor revealed through conditional knockout models in mice.* Molecular and Cellular Endocrinology, 2012. **352**(1): p. 13-25.
124. Welsh, M., et al., *Androgen receptor signalling in peritubular myoid cells is essential for normal differentiation and function of adult Leydig cells.* Int J Androl, 2012. **35**(1): p. 25-40.
125. Fernández, D., et al., *Identification and Characterization of Myosin from Rat Testicular Peritubular Myoid Cells.* Biology of Reproduction, 2008. **79**(6): p. 1210-1218.
126. Farr, C.H. and L.C. Ellis, *In-vitro contractility of rat seminiferous tubules in response to prostaglandins, cyclic GMP, testosterone and 2,4'-dibromoacetophenone.* J Reprod Fertil, 1980. **58**(1): p. 37-42.
127. Rossi, F., et al., *Angiotensin II stimulates contraction and growth of testicular peritubular myoid cells in vitro.* Endocrinology, 2002. **143**(8): p. 3096-104.
128. Santiemma, V., et al., *Endothelin-1 stimulates deoxyribonucleic acid synthesis and contraction in testicular peritubular myoid cells.* Biol Reprod, 1996. **54**(3): p. 583-90.
129. Santiemma, V., et al., *Adrenomedullin inhibits the contraction of cultured rat testicular peritubular myoid cells induced by endothelin-1.* Biol Reprod, 2001. **64**(2): p. 619-24.
130. Jain, S., et al., *Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis.* Development, 2004. **131**(21): p. 5503-5513.
131. Naughton, C.K., et al., *Glial Cell-Line Derived Neurotrophic Factor-Mediated RET Signaling Regulates Spermatogonial Stem Cell Fate1.* Biology of Reproduction, 2006. **74**(2): p. 314-321.
132. Meng, X., et al., *Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF.* Science, 2000. **287**(5457): p. 1489-1493.

133. Jijiwa, M., et al., *GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells.* Genes to Cells, 2008. **13**(4): p. 365-374.
134. Chen, L.Y., et al., *Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance.* Endocrinology, 2014. **155**(12): p. 4964-74.
135. Oatley, J.M., et al., *Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal.* Development, 2009. **136**(7): p. 1191-1199.
136. DeFalco, T., et al., *Macrophages Contribute to the Spermatogonial Niche in the Adult Testis.* Cell Reports, 2015. **12**(7): p. 1107-1119.
137. Nurmio, M., et al., *Peritubular myoid cells have a role in postnatal testicular growth.* Spermatogenesis, 2012. **2**(2): p. 79-87.
138. Nurmio, M., et al., *Inhibition of tyrosine kinases PDGFR and C-Kit by imatinib mesylate interferes with postnatal testicular development in the rat.* Int J Androl, 2007. **30**(4): p. 366-76; discussion 376.
139. Moreno, S.G., et al., *TGFβ signaling in male germ cells regulates gonocyte quiescence and fertility in mice.* Developmental Biology, 2010. **342**(1): p. 74-84.
140. Bowles, J., et al., *Retinoid signaling determines germ cell fate in mice.* Science, 2006. **312**(5773): p. 596-600.
141. Suzuki, A. and Y. Saga, *Nanos2 suppresses meiosis and promotes male germ cell differentiation.* Genes Dev, 2008. **22**(4): p. 430-5.
142. van Pelt, A.M. and D.G. de Rooij, *Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells.* Endocrinology, 1991. **128**(2): p. 697-704.
143. Li, H., et al., *Vitamin A deficiency results in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis.* Biol Reprod, 2011. **84**(2): p. 336-41.
144. Travers, A., et al., *Effects of Vitamin A on In Vitro Maturation of Pre-Pubertal Mouse Spermatogonial Stem Cells.* PLOS ONE, 2013. **8**(12): p. e82819.
145. Raverdeau, M., et al., *Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis.* Proceedings of the National Academy of Sciences, 2012. **109**(41): p. 16582-16587.
146. Arkoun, B., et al., *Retinol Improves In Vitro Differentiation of Pre-Pubertal Mouse Spermatogonial Stem Cells into Sperm during the First Wave of Spermatogenesis.* PLOS ONE, 2015. **10**(2): p. e0116660.
147. DiNapoli, L., J. Batchvarov, and B. Capel, *FGF9 promotes survival of germ cells in the fetal testis.* Development, 2006. **133**(8): p. 1519-27.
148. Ostrer, H., et al., *A cellular study of human testis development.* Sex Dev, 2007. **1**(5): p. 286-92.
149. Bowles, J., et al., *FGF9 suppresses meiosis and promotes male germ cell fate in mice.* Dev Cell, 2010. **19**(3): p. 440-9.
150. Jahnukainen, K., et al., *Increased Apoptosis Occurring During the First Wave of Spermatogenesis Is Stage-Specific and Primarily Affects Midpachytene*

Spermatocytes in the Rat Testis1. Biology of Reproduction, 2004. **70**(2): p. 290-296.
151. Rodriguez, I., et al., *An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis.* The EMBO Journal, 1997. **16**(9): p. 2262-2270.
152. Billig, H., et al., *Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages.* Endocrinology, 1995. **136**(1): p. 5-12.
153. Singh, J. and D.J. Handelsman, *The effects of recombinant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice.* J Androl, 1996. **17**(4): p. 382-93.
154. Haywood, M., et al., *Sertoli and Germ Cell Development in Hypogonadal (hpg) Mice Expressing Transgenic Follicle-Stimulating Hormone Alone or in Combination with Testosterone.* Endocrinology, 2003. **144**(2): p. 509-517.
155. Matikainen, T., et al., *Effects of recombinant human FSH in immature hypophysectomized male rats: evidence for Leydig cell-mediated action on spermatogenesis.* J Endocrinol, 1994. **141**(3): p. 449-57.
156. de Rooij, D.G., *The nature and dynamics of spermatogonial stem cells.* Development, 2017. **144**(17): p. 3022-3030.
157. Huckins, C., *The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation.* The Anatomical Record, 1971. **169**(3): p. 533-557.
158. Oakberg, E.F., *A new concept of spermatogonial stem-cell renewal in the mouse and its relationship to genetic effects.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1971. **11**(1): p. 1-7.
159. Oakberg, E.F., *Spermatogonial stem-cell renewal in the mouse.* Anat Rec, 1971. **169**(3): p. 515-31.
160. Braun, R.E., et al., *Genetically haploid spermatids are phenotypically diploid.* Nature, 1989. **337**(6205): p. 373-6.
161. Hara, K., et al., *Mouse Spermatogenic Stem Cells Continually Interconvert between Equipotent Singly Isolated and Syncytial States.* Cell Stem Cell, 2014. **14**(5): p. 658-672.
162. Nakagawa, T., et al., *Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment.* Science, 2010. **328**(5974): p. 62-7.
163. Oatley, M.J., et al., *Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice.* Biol Reprod, 2011. **85**(2): p. 347-56.
164. Helsel, A.R., et al., *ID4 levels dictate the stem cell state in mouse spermatogonia.* Development (Cambridge, England), 2017. **144**(4): p. 624-634.
165. Bremner, W.J., et al., *Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens.* Endocrinology, 1994. **135**(3): p. 1227-34.
166. Meistrich, M.L. and R.A. Hess, *Assessment of spermatogenesis through staging of seminiferous tubules.* Methods Mol Biol, 2013. **927**: p. 299-307.
167. Meng, J., et al., *Sertoli cell-specific deletion of the androgen receptor compromises testicular immune privilege in mice.* Biol Reprod, 2011. **85**(2): p. 254-60.

168. Govin, J., et al., *The role of histones in chromatin remodelling during mammalian spermiogenesis.* Eur J Biochem, 2004. **271**(17): p. 3459-69.
169. Krishnamurthy, H., et al., *Qualitative and Quantitative Decline in Spermatogenesis of the Follicle-Stimulating Hormone Receptor Knockout (FORKO) Mouse1.* Biology of Reproduction, 2000. **62**(5): p. 1146-1159.
170. O'Donnell, L., et al., *Testosterone Withdrawal Promotes Stage-Specific Detachment of Round Spermatids from the Rat Seminiferous Epithelium1.* Biology of Reproduction, 1996. **55**(4): p. 895-901.
171. Gottsch, M.L., et al., *A role for kisspeptins in the regulation of gonadotropin secretion in the mouse.* Endocrinology, 2004. **145**(9): p. 4073-7.
172. Messager, S., et al., *Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1761-6.
173. Irwig, M.S., et al., *Kisspeptin Activation of Gonadotropin Releasing Hormone Neurons and Regulation of KiSS-1 mRNA in the Male Rat.* Neuroendocrinology, 2004. **80**(4): p. 264-272.
174. Clem, B.F., E.A. Hudson, and B.J. Clark, *Cyclic Adenosine 3′,5′-Monophosphate (cAMP) Enhances cAMP-Responsive Element Binding (CREB) Protein Phosphorylation and Phospho-CREB Interaction with the Mouse Steroidogenic Acute Regulatory Protein Gene Promoter.* Endocrinology, 2005. **146**(3): p. 1348-1356.
175. Penny, G.M., et al., *Probing GATA factor function in mouse Leydig cells via testicular injection of adenoviral vectors.* Reproduction, 2017. **154**(4): p. 455-467.
176. Schrade, A., et al., *GATA4 Is a Key Regulator of Steroidogenesis and Glycolysis in Mouse Leydig Cells.* Endocrinology, 2015. **156**(5): p. 1860-1872.
177. Hatano, O., et al., *Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development.* Development, 1994. **120**(10): p. 2787-2797.
178. Stocco, D.M., et al., *Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought.* Molecular Endocrinology, 2005. **19**(11): p. 2647-2659.
179. Narayan, P., *Genetic Models for the Study of Luteinizing Hormone Receptor Function.* Front Endocrinol (Lausanne), 2015. **6**: p. 152.
180. Pignataro, O.P., et al., *Mitochondrial biosynthesis of cholesterol in Leydig cells from rat testis.* Mol Cell Endocrinol, 1983. **33**(1): p. 53-67.
181. Rone, M.B., J. Fan, and V. Papadopoulos, *Cholesterol transport in steroid biosynthesis: role of protein–protein interactions and implications in disease states.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2009. **1791**(7): p. 646-658.
182. Miller, W.L., *Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2007. **1771**(6): p. 663-676.
183. Hasegawa, T., et al., *Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice.* Mol Endocrinol, 2000. **14**(9): p. 1462-71.
184. Lin, D., et al., *Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis.* Science, 1995. **267**(5205): p. 1828-1831.

185. Hu, M.C., et al., *Steroid deficiency syndromes in mice with targeted disruption of Cyp11a1.* Mol Endocrinol, 2002. **16**(8): p. 1943-50.
186. Tajima, T., et al., *Heterozygous mutation in the cholesterol side chain cleavage enzyme (p450scc) gene in a patient with 46,XY sex reversal and adrenal insufficiency.* J Clin Endocrinol Metab, 2001. **86**(8): p. 3820-5.
187. Hiort, O., et al., *Homozygous disruption of P450 side-chain cleavage (CYP11A1) is associated with prematurity, complete 46,XY sex reversal, and severe adrenal failure.* J Clin Endocrinol Metab, 2005. **90**(1): p. 538-41.
188. Kim, C.J., et al., *Severe Combined Adrenal and Gonadal Deficiency Caused by Novel Mutations in the Cholesterol Side Chain Cleavage Enzyme, P450scc.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(3): p. 696-702.
189. Miller, W.L. and R.J. Auchus, *The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders.* Endocrine Reviews, 2011. **32**(1): p. 81-151.
190. Brock, B.J. and M.R. Waterman, *Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species.* Biochemistry, 1999. **38**(5): p. 1598-606.
191. Sheffield, J.W. and P.J. O'Shaughnessy, *Testicular steroid metabolism during development in the normal and hypogonadal mouse.* J Endocrinol, 1988. **119**(2): p. 257-64.
192. Ikeda, Y., et al., *Androgen receptor gene knockout male mice exhibit impaired cardiac growth and exacerbation of angiotensin II-induced cardiac fibrosis.* J Biol Chem, 2005. **280**(33): p. 29661-6.
193. Zhu, D., et al., *Ablation of the androgen receptor from vascular smooth muscle cells demonstrates a role for testosterone in vascular calcification.* Sci Rep, 2016. **6**: p. 24807.
194. Huang, C.K., et al., *New therapy via targeting androgen receptor in monocytes/macrophages to battle atherosclerosis.* Hypertension, 2014. **63**(6): p. 1345-53.
195. Ashcroft, G.S. and S.J. Mills, *Androgen receptor–mediated inhibition of cutaneous wound healing.* The Journal of Clinical Investigation, 2002. **110**(5): p. 615-624.
196. Huang, C.K., et al., *Suppression of androgen receptor enhances the self-renewal of mesenchymal stem cells through elevated expression of EGFR.* Biochim Biophys Acta, 2013. **1833**(5): p. 1222-34.
197. Wilhelmson, A.S., et al., *Androgens regulate bone marrow B lymphopoiesis in male mice by targeting osteoblast-lineage cells.* Endocrinology, 2015. **156**(4): p. 1228-36.
198. Tsai, M.Y., et al., *The reduced trabecular bone mass of adult ARKO male mice results from the decreased osteogenic differentiation of bone marrow stroma cells.* Biochem Biophys Res Commun, 2011. **411**(3): p. 477-82.
199. Saita, Y., et al., *Combinatory effects of androgen receptor deficiency and hind limb unloading on bone.* Horm Metab Res, 2009. **41**(11): p. 822-8.
200. Cheng, B.H., et al., *Testosterone delivered with a scaffold is as effective as bone morphologic protein-2 in promoting the repair of critical-size segmental defect of femoral bone in mice.* PLoS One, 2013. **8**(8): p. e70234.

201. Sinnesael, M., et al., *Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity: evidence from targeted AR disruption in mouse osteocytes.* J Bone Miner Res, 2012. **27**(12): p. 2535-43.
202. Venken, K., et al., *Relative impact of androgen and estrogen receptor activation in the effects of androgens on trabecular and cortical bone in growing male mice: a study in the androgen receptor knockout mouse model.* J Bone Miner Res, 2006. **21**(4): p. 576-85.
203. Kaftanovskaya, E.M., et al., *Cryptorchidism in mice with an androgen receptor ablation in gubernaculum testis.* Mol Endocrinol, 2012. **26**(4): p. 598-607.
204. O'Hara, L., et al., *Androgen receptor expression in the caput epididymal epithelium is essential for development of the initial segment and epididymal spermatozoa transit.* Endocrinology, 2011. **152**(2): p. 718-29.
205. MacLean, H.E., et al., *Impaired skeletal muscle development and function in male, but not female, genomic androgen receptor knockout mice.* Faseb j, 2008. **22**(8): p. 2676-89.
206. Altuwaijri, S., et al., *Androgen receptor regulates expression of skeletal muscle-specific proteins and muscle cell types.* Endocrine, 2004. **25**(1): p. 27-32.
207. Karlsson, S.A., et al., *Neural Androgen Receptors Modulate Gene Expression and Social Recognition But Not Social Investigation.* Front Behav Neurosci, 2016. **10**: p. 41.
208. Studer, E., et al., *Serotonin depletion-induced maladaptive aggression requires the presence of androgens.* PLoS One, 2015. **10**(5): p. e0126462.
209. Sato, T., et al., *Brain masculinization requires androgen receptor function.* Proc Natl Acad Sci U S A, 2004. **101**(6): p. 1673-8.
210. Navarro, G., et al., *Extranuclear Actions of the Androgen Receptor Enhance Glucose-Stimulated Insulin Secretion in the Male.* Cell Metab, 2016. **23**(5): p. 837-51.
211. Rubinow, K.B., et al., *Hematopoietic androgen receptor deficiency promotes visceral fat deposition in male mice without impairing glucose homeostasis.* Andrology, 2015. **3**(4): p. 787-96.
212. McInnes, K.J., et al., *Deletion of the androgen receptor in adipose tissue in male mice elevates retinol binding protein 4 and reveals independent effects on visceral fat mass and on glucose homeostasis.* Diabetes, 2012. **61**(5): p. 1072-81.
213. Bennett, N.C., et al., *Molecular cell biology of androgen receptor signalling.* Int J Biochem Cell Biol, 2010. **42**(6): p. 813-27.
214. McEwan, I.J., et al., *Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors.* Nucl Recept Signal, 2007. **5**: p. e001.
215. Jenster, G., et al., *Identification of two transcription activation units in the N-terminal domain of the human androgen receptor.* J Biol Chem, 1995. **270**(13): p. 7341-6.
216. Freedman, L.P., *Anatomy of the steroid receptor zinc finger region.* Endocr Rev, 1992. **13**(2): p. 129-45.
217. Schoenmakers, E., et al., *Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal*

extension of the DNA-binding domains. Biochem J, 1999. **341 (Pt 3)**: p. 515-21.
218. Clinckemalie, L., et al., *The hinge region in androgen receptor control.* Molecular and Cellular Endocrinology, 2012. **358**(1): p. 1-8.
219. Heery, D.M., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**(6634): p. 733-6.
220. van de Wijngaart, D.J., et al., *Androgen receptor coregulators: recruitment via the coactivator binding groove.* Mol Cell Endocrinol, 2012. **352**(1-2): p. 57-69.
221. Pratt, W.B., *Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts.* Molecular and Cellular Endocrinology, 1990. **74**(1): p. C69-C76.
222. Picard, D., et al., *Reduced levels of hsp90 compromise steroid receptor action in vivo.* Nature, 1990. **348**(6297): p. 166-8.
223. Cutress, M.L., et al., *Structural basis for the nuclear import of the human androgen receptor.* Journal of Cell Science, 2008. **121**(7): p. 957-968.
224. Bennett, N.C., et al., *Molecular cell biology of androgen receptor signalling.* The International Journal of Biochemistry & Cell Biology, 2010. **42**(6): p. 813-827.
225. Estrada, M., et al., *Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells.* Endocrinology, 2003. **144**(8): p. 3586-97.
226. Kang, H.Y., et al., *Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts.* J Bone Miner Res, 2004. **19**(7): p. 1181-90.
227. Deng, Q., et al., *Non-Genomic Action of Androgens is Mediated by Rapid Phosphorylation and Regulation of Androgen Receptor Trafficking.* Cell Physiol Biochem, 2017. **43**(1): p. 223-236.
228. Morris, P.D. and K.S. Channer, *Testosterone and cardiovascular disease in men.* Asian J Androl, 2012. **14**(3): p. 428-35.
229. Saad, F. and L. Gooren, *The role of testosterone in the metabolic syndrome: a review.* J Steroid Biochem Mol Biol, 2009. **114**(1-2): p. 40-3.
230. Saad, F. and L.J. Gooren, *The role of testosterone in the etiology and treatment of obesity, the metabolic syndrome, and diabetes mellitus type 2.* J.Obes., 2011. **2011**.
231. Laughlin, G.A., E. Barrett-Connor, and J. Bergstrom, *Low serum testosterone and mortality in older men.* J.Clin.Endocrinol.Metab, 2008. **93**(1): p. 68-75.
232. Pye, S.R., et al., *Late-Onset Hypogonadism and Mortality in Aging Men.* J Clin Endocrinol Metab, 2013: p. jc20132052.
233. Svartberg, J., et al., *Waist circumference and testosterone levels in community dwelling men. The Tromsø study.* European journal of epidemiology, 2004. **19**(7): p. 657-663.
234. Laaksonen, D.E., et al., *Sex hormones, inflammation and the metabolic syndrome: a population-based study.* Eur J Endocrinol, 2003. **149**(6): p. 601-8.
235. Grosman, H., et al., *Association between testosterone levels and the metabolic syndrome in adult men.* Aging Male, 2014. **17**(3): p. 161-5.

236. Polari, L., et al., *Weight gain and inflammation regulate aromatase expression in male adipose tissue, as evidenced by reporter gene activity.* Mol Cell Endocrinol, 2015. **412**: p. 123-30.
237. De Pergola, G., *The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone.* International Journal Of Obesity, 2000. **24**: p. S59.
238. Krausz, C., A.R. Escamilla, and C. Chianese, *Genetics of male infertility: from research to clinic.* Reproduction, 2015. **150**(5): p. R159-74.
239. Song, S.H., et al., *Recent advances in the genetics of testicular failure.* Asian J Androl, 2016. **18**(3): p. 350-5.
240. Jungwirth, A., et al., *European Association of Urology guidelines on Male Infertility: the 2012 update.* Eur Urol, 2012. **62**(2): p. 324-32.
241. Archambeault, D.R. and M.M. Matzuk, *Disrupting the male germ line to find infertility and contraception targets.* Ann Endocrinol (Paris), 2014. **75**(2): p. 101-8.
242. Morris, J.M., *The syndrome of testicular feminization in male pseudohermaphrodites.* Am J Obstet Gynecol, 1953. **65**(6): p. 1192-1211.
243. Wu, F.C., et al., *Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study.* J Clin Endocrinol Metab, 2008. **93**(7): p. 2737-45.
244. Travison, T.G., et al., *The Relative Contributions of Aging, Health, and Lifestyle Factors to Serum Testosterone Decline in Men.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(2): p. 549-555.
245. Schaap, L.A., A. Koster, and M. Visser, *Adiposity, muscle mass, and muscle strength in relation to functional decline in older persons.* Epidemiol Rev, 2013. **35**: p. 51-65.
246. Sinha, I., et al., *Testosterone is essential for skeletal muscle growth in aged mice in a heterochronic parabiosis model.* Cell Tissue Res, 2014. **357**(3): p. 815-21.
247. Hsu, B., et al., *The longitudinal relationship of sexual function and androgen status in older men: the Concord Health and Ageing in Men Project.* J Clin Endocrinol Metab, 2015. **100**(4): p. 1350-8.
248. Bacon, C.G., et al., *Sexual function in men older than 50 years of age: results from the health professionals follow-up study.* Ann Intern Med, 2003. **139**(3): p. 161-8.
249. Drake, M.T., et al., *Clinical review. Risk factors for low bone mass-related fractures in men: a systematic review and meta-analysis.* J Clin Endocrinol Metab, 2012. **97**(6): p. 1861-70.
250. Rodriguez, A., et al., *Aging, Androgens, and the Metabolic Syndrome in a Longitudinal Study of Aging.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(9): p. 3568-3572.
251. Liu, P.Y., et al., *Age-Related Changes in Serum Testosterone and Sex Hormone Binding Globulin in Australian Men: Longitudinal Analyses of Two Geographically Separate Regional Cohorts.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(9): p. 3599-3603.
252. Travison, T.G., et al., *Harmonized Reference Ranges for Circulating Testosterone Levels in Men of Four Cohort Studies in the United States and Europe.* J Clin Endocrinol Metab, 2017. **102**(4): p. 1161-1173.

253. Ferrini, R.L. and E. Barrett-Connor, *Sex hormones and age: a cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men.* Am J Epidemiol, 1998. **147**(8): p. 750-4.
254. Zhao, Y., et al., *Mono-(2-ethylhexyl) phthalate affects the steroidogenesis in rat Leydig cells through provoking ROS perturbation.* Toxicol In Vitro, 2012. **26**(6): p. 950-5.
255. Zhou, L., et al., *Oxidative stress and phthalate-induced down-regulation of steroidogenesis in MA-10 Leydig cells.* Reprod Toxicol, 2013. **42**: p. 95-101.
256. Wang, X. and D.M. Stocco, *The decline in testosterone biosynthesis during male aging: A consequence of multiple alterations.* Molecular and Cellular Endocrinology, 2005. **238**(1): p. 1-7.
257. Neaves, W.B., et al., *Leydig cell numbers, daily sperm production, and serum gonadotropin levels in aging men.* J Clin Endocrinol Metab, 1984. **59**(4): p. 756-63.
258. Horstman, A.M., et al., *The role of androgens and estrogens on healthy aging and longevity.* J Gerontol A Biol Sci Med Sci, 2012. **67**(11): p. 1140-52.
259. Handelsman, D.J., *Global trends in testosterone prescribing, 2000-2011: expanding the spectrum of prescription drug misuse.* Med J Aust, 2013. **199**(8): p. 548-51.
260. Gan, E.H., et al., *A UK epidemic of testosterone prescribing, 2001-2010.* Clin Endocrinol (Oxf), 2013. **79**(4): p. 564-70.
261. Rice, D., et al., *Men's health, low testosterone, and diabetes: individualized treatment and a multidisciplinary approach.* Diabetes Educ, 2008. **34 Suppl 5**: p. 97S-112S; quiz 113S-4S.
262. Snyder, P.J., et al., *Effects of Testosterone Treatment in Older Men.* N Engl J Med, 2016. **374**(7): p. 611-24.
263. Cunningham, G.R., et al., *Testosterone Treatment and Sexual Function in Older Men With Low Testosterone Levels.* J Clin Endocrinol Metab, 2016. **101**(8): p. 3096-104.
264. Finkle, W.D., et al., *Increased Risk of Non-Fatal Myocardial Infarction Following Testosterone Therapy Prescription in Men.* PLOS ONE, 2014. **9**(1): p. e85805.
265. Vigen, R., et al., *Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels.* Jama, 2013. **310**(17): p. 1829-36.
266. Basaria, S., et al., *Adverse Events Associated with Testosterone Administration.* New England Journal of Medicine, 2010. **363**(2): p. 109-122.
267. Dalmasso, C., et al., *Cardiovascular and Metabolic Consequences of Testosterone Supplements in Young and Old Male Spontaneously Hypertensive Rats: Implications for Testosterone Supplements in Men.* J Am Heart Assoc, 2017. **6**(10).
268. Budoff, M.J., et al., *Testosterone Treatment and Coronary Artery Plaque Volume in Older Men With Low Testosterone.* Jama, 2017. **317**(7): p. 708-716.
269. Darbey, A. and L.B. Smith, *Deliverable transgenics & gene therapy possibilities for the testes.* Mol Cell Endocrinol, 2017.
270. Cohen, S.N., et al., *Construction of biologically functional bacterial plasmids in vitro.* Proc Natl Acad Sci U S A, 1973. **70**(11): p. 3240-4.

271. Jaenisch, R. and B. Mintz, *Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA.* Proc Natl Acad Sci U S A, 1974. **71**(4): p. 1250-4.
272. Sauer, B., *Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(6): p. 2087-96.
273. Sauer, B. and N. Henderson, *Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5166-70.
274. Sauer, B. and N. Henderson, *Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome.* Nucleic Acids Res, 1989. **17**(1): p. 147-61.
275. Wolff, J.A., et al., *Direct gene transfer into mouse muscle in vivo.* Science, 1990. **247**(4949 Pt 1): p. 1465-8.
276. Wolff, J.A. and V. Budker, *The mechanism of naked DNA uptake and expression.* Adv Genet, 2005. **54**: p. 3-20.
277. Lin, H., et al., *Expression of recombinant genes in myocardium in vivo after direct injection of DNA.* Circulation, 1990. **82**(6): p. 2217-21.
278. Chang, C.Y., et al., *Intratumoral delivery of IL-18 naked DNA induces T-cell activation and Th1 response in a mouse hepatic cancer model.* BMC Cancer, 2007. **7**: p. 87.
279. Wang, L.Y., et al., *Intramuscular injection of naked plasmid DNA encoding human preproinsulin gene in streptozotocin-diabetes mice results in a significant reduction of blood glucose level.* Sheng Li Xue Bao, 2003. **55**(6): p. 641-7.
280. Matsuno, Y., et al., *Nonviral gene gun mediated transfer into the beating heart.* Asaio j, 2003. **49**(6): p. 641-4.
281. Arsenault, J., et al., *Regioselective biolistic targeting in organotypic brain slices using a modified gene gun.* J Vis Exp, 2014(92): p. e52148.
282. Abe, A., et al., *Gene gun-mediated skin transfection with FL gene suppresses the growth of murine fibrosarcoma.* J Med Invest, 2011. **58**(1-2): p. 39-45.
283. Liu, F., Y. Song, and D. Liu, *Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA.* Gene Ther, 1999. **6**(7): p. 1258-66.
284. Zhang, G., V. Budker, and J.A. Wolff, *High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA.* Hum Gene Ther, 1999. **10**(10): p. 1735-7.
285. Neumann, E., et al., *Gene transfer into mouse lyoma cells by electroporation in high electric fields.* Embo j, 1982. **1**(7): p. 841-5.
286. Yokoo, T., et al., *Liver-targeted hydrodynamic gene therapy: Recent advances in the technique.* World J Gastroenterol, 2016. **22**(40): p. 8862-8868.
287. Yuan, X., et al., *IL-38 alleviates concanavalin A-induced liver injury in mice.* Int Immunopharmacol, 2016. **40**: p. 452-457.
288. Dul, M., et al., *Hydrodynamic gene delivery in human skin using a hollow microneedle device.* J Control Release, 2017.
289. Maruyama, H., et al., *Rat kidney-targeted naked plasmid DNA transfer by retrograde injection into the renal vein.* Molecular Biotechnology, 2004. **27**(1): p. 23-31.

290. Kameda, S., et al., *Kidney-targeted naked DNA transfer by retrograde injection into the renal vein in mice.* Biochemical and Biophysical Research Communications, 2004. **314**(2): p. 390-395.
291. Peng, B., et al., *Comparison of spermatogenic damage induced at 6 months after ligation of the vas deferens at proximal and distal locations in the rabbit.* Andrologia, 2011. **43**(2): p. 129-38.
292. Xiang, Y., et al., *Long-term effect of vasectomy on spermatogenesis in men: a morphometric study.* Asian J Androl, 2013. **15**(3): p. 434-6.
293. Ogawa, T., et al., *Transplantation of testis germinal cells into mouse seminiferous tubules.* Int J Dev Biol, 1997. **41**(1): p. 111-22.
294. Heller, R., et al., *In vivo gene electroinjection and expression in rat liver.* FEBS Lett, 1996. **389**(3): p. 225-8.
295. Nishi, T., et al., *High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation.* Cancer Res, 1996. **56**(5): p. 1050-5.
296. Orlowski, S., et al., *Transient electropermeabilization of cells in culture.* Biochemical Pharmacology, 1988. **37**(24): p. 4727-4733.
297. Daud, A.I., et al., *Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma.* J Clin Oncol, 2008. **26**(36): p. 5896-903.
298. Michaelis, M., A. Sobczak, and J.M. Weitzel, *In vivo microinjection and electroporation of mouse testis.* J Vis Exp, 2014(90).
299. Usmani, A., et al., *A non-surgical approach for male germ cell mediated gene transmission through transgenesis.* Sci Rep, 2013. **3**.
300. Pradhan, B.S. and S.S. Majumdar, *An Efficient Method for Generation of Transgenic Rats Avoiding Embryo Manipulation.* Mol Ther Nucleic Acids, 2016. **5**(3): p. e293-.
301. Usmani, A., et al., *Robust generation of transgenic mice by simple hypotonic solution mediated delivery of transgene in testicular germ cells.* Mol Ther Methods Clin Dev, 2016. **3**: p. 16076-.
302. Umemoto, Y., et al., *Gene transfer to mouse testes by electroporation and its influence on spermatogenesis.* J Androl, 2005. **26**(2): p. 264-71.
303. Araki, M. and T. Ishii, *International regulatory landscape and integration of corrective genome editing into in vitro fertilization.* Reproductive Biology and Endocrinology, 2014. **12**(1): p. 108.
304. Ye, J., et al., *Anionic solid lipid nanoparticles supported on protamine/DNA complexes.* Nanotechnology, 2008. **19**(28): p. 285708.
305. Zhi, D., et al., *Transfection efficiency of cationic lipids with different hydrophobic domains in gene delivery.* Bioconjug Chem, 2010. **21**(4): p. 563-77.
306. Bangham, A.D. and R.W. Horne, *NEGATIVE STAINING OF PHOSPHOLIPIDS AND THEIR STRUCTURAL MODIFICATION BY SURFACE-ACTIVE AGENTS AS OBSERVED IN THE ELECTRON MICROSCOPE.* J Mol Biol, 1964. **8**: p. 660-8.
307. Gregoriadis, G., P.D. Leathwood, and B.E. Ryman, *Enzyme entrapment in liposomes.* FEBS Lett, 1971. **14**(2): p. 95-99.
308. Weissman, G., C. Cohen, and S. Hoffstein, *The correction, in vitro, of lysosomal enzyme deficiencies by means of immunoglobulin-coated liposomes.* Adv Exp Med Biol, 1976. **68**: p. 509-521.

309. Felgner, P.L., et al., *Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure.* Proc Natl Acad Sci U S A, 1987. **84**(21): p. 7413-7.
310. Zhu, N., et al., *Systemic gene expression after intravenous DNA delivery into adult mice.* Science, 1993. **261**(5118): p. 209-11.
311. Malone, R.W., P.L. Felgner, and I.M. Verma, *Cationic liposome-mediated RNA transfection.* Proc Natl Acad Sci U S A, 1989. **86**(16): p. 6077-81.
312. zur Muhlen, A., C. Schwarz, and W. Mehnert, *Solid lipid nanoparticles (SLN) for controlled drug delivery--drug release and release mechanism.* Eur J Pharm Biopharm, 1998. **45**(2): p. 149-55.
313. Pedersen, N., et al., *Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands.* Eur J Pharm Biopharm, 2006. **62**(2): p. 155-62.
314. Wolfrum, C., et al., *Mechanisms and optimization of in vivo delivery of lipophilic siRNAs.* Nat Biotechnol, 2007. **25**(10): p. 1149-57.
315. Ding, Y., et al., *A biomimetic nanovector-mediated targeted cholesterol-conjugated siRNA delivery for tumor gene therapy.* Biomaterials, 2012. **33**(34): p. 8893-905.
316. Kuo, Y.C. and I.H. Wang, *Enhanced delivery of etoposide across the blood-brain barrier to restrain brain tumor growth using melanotransferrin antibody- and tamoxifen-conjugated solid lipid nanoparticles.* J Drug Target, 2016. **24**(7): p. 645-54.
317. Celebi, C., et al., *Transient transmission of a transgene in mouse offspring following in vivo transfection of male germ cells.* Mol Reprod Dev, 2002. **62**(4): p. 477-82.
318. Miao, X.Y. and X. Zhang, *Production of transgenic mice carrying the Thanatin gene by intratesticular injection.* Biochem Biophys Res Commun, 2011. **415**(3): p. 429-33.
319. Rowe, W.P., et al., *Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture.* Proc Soc Exp Biol Med, 1953. **84**(3): p. 570-3.
320. Sharma, A., et al., *Adenovirus receptors and their implications in gene delivery.* Virus Res, 2009. **143**(2): p. 184-94.
321. Mali, S., *Delivery systems for gene therapy.* Indian J Hum Genet, 2013. **19**(1): p. 3-8.
322. Gao, H., et al., *Targeted P2X7 R shRNA delivery attenuates sympathetic nerve sprouting and ameliorates cardiac dysfunction in rats with myocardial infarction.* Cardiovasc Ther, 2017. **35**(2).
323. Morsy, M.A., et al., *An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 7866-71.
324. Schiedner, G., et al., *Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity.* Nat Genet, 1998. **18**(2): p. 180-3.
325. Hiltunen, M.O., et al., *Biodistribution of adenoviral vector to nontarget tissues after local in vivo gene transfer to arterial wall using intravascular and periadventitial gene delivery methods.* Faseb j, 2000. **14**(14): p. 2230-6.

326. Thacker, E.E., et al., *A genetically engineered adenovirus vector targeted to CD40 mediates transduction of canine dendritic cells and promotes antigen-specific immune responses in vivo.* Vaccine, 2009. **27**(50): p. 7116-24.
327. Kojima, Y., et al., *No evidence of germ-line transmission by adenovirus-mediated gene transfer to mouse testes.* Fertil Steril, 2008. **89**(5 Suppl): p. 1448-54.
328. Peters, A.H., et al., *Absence of germline infection in male mice following intraventricular injection of adenovirus.* Mol Ther, 2001. **4**(6): p. 603-13.
329. Hall, S.J., et al., *Direct exposure of mouse spermatogenic cells to high doses of adenovirus gene therapy vector does not result in germ cell transduction.* Hum Gene Ther, 2000. **11**(12): p. 1705-12.
330. Kojima, Y., et al., *Effects of adenovirus mediated gene transfer to mouse testis in vivo on spermatogenesis and next generation.* J Urol, 2003. **170**(5): p. 2109-14.
331. Qamar, I., et al., *ARR19 (androgen receptor corepressor of 19 kDa), an antisteroidogenic factor, is regulated by GATA-1 in testicular Leydig cells.* J Biol Chem, 2009. **284**(27): p. 18021-32.
332. Tanimoto, R., et al., *REIC/Dkk-3 as a potential gene therapeutic agent against human testicular cancer.* Int J Mol Med, 2007. **19**(3): p. 363-8.
333. Levasseur, A., et al., *Yes-associated protein and WW-containing transcription regulator 1 regulate the expression of sex-determining genes in Sertoli cells, but their inactivation does not cause sex reversal.* Biol Reprod, 2017. **97**(1): p. 162-175.
334. Qamar, I., M.F. Ahmad, and A. Narayanasamy, *A time-course study of long term over-expression of ARR19 in mice.* Sci Rep, 2015. **5**: p. 13014.
335. Gordon, J.W., *Seminiferous tubule cannulation (STC): a new, sensitive technique for detecting gene transfer in developing sperm.* Gene Ther, 2003. **10**(1): p. 43-50.
336. Hooley, R.P., et al., *Intra-testicular injection of adenoviral constructs results in Sertoli cell-specific gene expression and disruption of the seminiferous epithelium.* Reproduction, 2009. **137**(2): p. 361-70.
337. Kivela, A.M., et al., *High Plasma Lipid Levels Reduce Efficacy of Adenovirus-Mediated Gene Therapy.* Sci Rep, 2017. **7**(1): p. 386.
338. Croyle, M.A., et al., *PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver.* Hum Gene Ther, 2002. **13**(15): p. 1887-900.
339. Muck-Hausl, M., et al., *Ad 2.0: a novel recombineering platform for high-throughput generation of tailored adenoviruses.* Nucleic Acids Res, 2015. **43**(8): p. e50.
340. Ruzsics, Z., F. Lemnitzer, and C. Thirion, *Engineering adenovirus genome by bacterial artificial chromosome (BAC) technology.* Methods Mol Biol, 2014. **1089**: p. 143-58.
341. Hermonat, P.L. and N. Muzyczka, *Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells.* Proc Natl Acad Sci U S A, 1984. **81**(20): p. 6466-70.
342. Maguire, A.M., et al., *Safety and efficacy of gene transfer for Leber's congenital amaurosis.* N Engl J Med, 2008. **358**(21): p. 2240-8.

343. Lenis, T.L., et al., *Complement modulation in the retinal pigment epithelium rescues photoreceptor degeneration in a mouse model of Stargardt disease.* Proc Natl Acad Sci U S A, 2017. **114**(15): p. 3987-3992.
344. Watanabe, M., et al., *Adeno-associated virus-mediated human IL-10 gene transfer suppresses the development of experimental autoimmune orchitis.* Gene Ther, 2005. **12**(14): p. 1126-32.
345. Jakob, M., et al., *No evidence for germ-line transmission following prenatal and early postnatal AAV-mediated gene delivery.* J Gene Med, 2005. **7**(5): p. 630-7.
346. Honaramooz, A., et al., *Adeno-associated virus (AAV)-mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation.* Faseb j, 2008. **22**(2): p. 374-82.
347. Watanabe, S., et al., *In Vivo Genetic Manipulation of Spermatogonial Stem Cells and Their Microenvironment by Adeno-Associated Viruses.* Stem Cell Reports, 2018.
348. Watanabe, S., et al., *In Vivo Genetic Manipulation of Spermatogonial Stem Cells and Their Microenvironment by Adeno-Associated Viruses.* Stem Cell Reports, 2018. **10**(5): p. 1551-1564.
349. Halbert, C.L., J.M. Allen, and A.D. Miller, *Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene.* Nat Biotechnol, 2002. **20**(7): p. 697-701.
350. Chamberlain, K., J.M. Riyad, and T. Weber, *Expressing Transgenes That Exceed the Packaging Capacity of Adeno-Associated Virus Capsids.* Hum Gene Ther Methods, 2016. **27**(1): p. 1-12.
351. Blaese, R.M., et al., *Treatment of severe combined immunodeficiency disease (SCID) due to adenosine deaminase deficiency with CD34+ selected autologous peripheral blood cells transduced with a human ADA gene. Amendment to clinical research project, Project 90-C-195, January 10, 1992.* Hum Gene Ther, 1993. **4**(4): p. 521-7.
352. Blaese, R.M., et al., *T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years.* Science, 1995. **270**(5235): p. 475-80.
353. Hacein-Bey-Abina, S., et al., *A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency.* N Engl J Med, 2003. **348**(3): p. 255-6.
354. Hacein-Bey-Abina, S., et al., *LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1.* Science, 2003. **302**(5644): p. 415-9.
355. Hacein-Bey-Abina, S., et al., *Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1.* J Clin Invest, 2008. **118**(9): p. 3132-42.
356. Cavazzana-Calvo, M., et al., *Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease.* Science, 2000. **288**(5466): p. 669-72.
357. Howe, S.J., et al., *Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients.* J Clin Invest, 2008. **118**(9): p. 3143-50.
358. Browning, D.L. and G.D. Trobridge, *Insulators to Improve the Safety of Retroviral Vectors for HIV Gene Therapy.* Biomedicines, 2016. **4**(1).

359. Groth, A.C., et al., *Identification and characterization of enhancer-blocking insulators to reduce retroviral vector genotoxicity.* PLoS One, 2013. **8**(10): p. e76528.
360. Panthier, J.J., et al., *Pattern of expression of ecotropic murine leukemia virus in gonads of inoculated SWR/J mice.* J Virol, 1989. **63**(5): p. 2134-42.
361. Nagano, M., et al., *Retrovirus-mediated gene delivery into male germ line stem cells.* FEBS Lett, 2000. **475**(1): p. 7-10.
362. Kanatsu-Shinohara, M., et al., *Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells.* Proc Natl Acad Sci U S A, 2006. **103**(21): p. 8018-23.
363. Kanatsu-Shinohara, M., S. Toyokuni, and T. Shinohara, *Transgenic mice produced by retroviral transduction of male germ line stem cells in vivo.* Biol Reprod, 2004. **71**(4): p. 1202-7.
364. Porada, C.D., et al., *Male germ-line cells are at risk following direct-injection retroviral-mediated gene transfer in utero.* Mol Ther, 2005. **12**(4): p. 754-62.
365. Park, P.J., et al., *Factors determining the risk of inadvertent retroviral transduction of male germ cells after in utero gene transfer in sheep.* Hum Gene Ther, 2009. **20**(3): p. 201-15.
366. Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. **272**(5259): p. 263-7.
367. Naldini, L., et al., *Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11382-8.
368. Schlimgen, R., et al., *Risks Associated With Lentiviral Vector Exposures and Prevention Strategies.* J Occup Environ Med, 2016. **58**(12): p. 1159-1166.
369. Wu, X., et al., *Transcription start regions in the human genome are favored targets for MLV integration.* Science, 2003. **300**(5626): p. 1749-51.
370. Huang, Z.M. and T.S. Yen, *Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcripts.* Mol Cell Biol, 1995. **15**(7): p. 3864-9.
371. Donello, J.E., J.E. Loeb, and T.J. Hope, *Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element.* J Virol, 1998. **72**(6): p. 5085-92.
372. Zufferey, R., et al., *Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors.* J Virol, 1999. **73**(4): p. 2886-92.
373. Kingsman, S.M., K. Mitrophanous, and J.C. Olsen, *Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE).* Gene Ther, 2004. **12**(1): p. 3-4.
374. Kim, H., H. Lee, and Y. Yun, *X-gene product of hepatitis B virus induces apoptosis in liver cells.* J Biol Chem, 1998. **273**(1): p. 381-5.
375. Hohne, M., et al., *Malignant transformation of immortalized transgenic hepatocytes after transfection with hepatitis B virus DNA.* Embo j, 1990. **9**(4): p. 1137-45.
376. Themis, M., et al., *Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice.* Mol Ther, 2005. **12**(4): p. 763-71.

377. Eddie, S.L., et al., *Prokineticin Ligands and Receptors Are Expressed in the Human Fetal Ovary and Regulate Germ Cell Expression of COX2.* J Clin Endocrinol Metab, 2015. **100**(9): p. E1197-205.
378. McCloskey, C., et al., *The inwardly rectifying K+ channel KIR7.1 controls uterine excitability throughout pregnancy.* EMBO Mol Med, 2014. **6**(9): p. 1161-74.
379. Smith, L.B., et al., *KATNAL1 regulation of sertoli cell microtubule dynamics is essential for spermiogenesis and male fertility.* PLoS Genet, 2012. **8**(5): p. e1002697.
380. Yang, S., et al., *A simple and effective method to generate lentiviral vectors for ex vivo gene delivery to mature human peripheral blood lymphocytes.* Hum Gene Ther Methods, 2012. **23**(2): p. 73-83.
381. Schambach, A., et al., *Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression.* Gene Ther, 2006. **13**(7): p. 641-5.
382. Levine, B.L., et al., *Gene transfer in humans using a conditionally replicating lentiviral vector.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17372-7.
383. McGarrity, G.J., et al., *Patient monitoring and follow-up in lentiviral clinical trials.* J Gene Med, 2013. **15**(2): p. 78-82.
384. Palfi, S., et al., *Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial.* Lancet, 2014. **383**(9923): p. 1138-46.
385. Chick, H.E., et al., *Integrase-deficient lentiviral vectors mediate efficient gene transfer to human vascular smooth muscle cells with minimal genotoxic risk.* Hum Gene Ther, 2012. **23**(12): p. 1247-57.
386. Peluffo, H., et al., *Efficient gene expression from integration-deficient lentiviral vectors in the spinal cord.* Gene Ther, 2013. **20**(6): p. 645-57.
387. Apolonia, L., et al., *Stable gene transfer to muscle using non-integrating lentiviral vectors.* Mol Ther, 2007. **15**(11): p. 1947-54.
388. Yanez-Munoz, R.J., et al., *Effective gene therapy with nonintegrating lentiviral vectors.* Nat Med, 2006. **12**(3): p. 348-53.
389. Hamra, F.K., et al., *Production of transgenic rats by lentiviral transduction of male germ-line stem cells.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14931-6.
390. Chandrashekran, A., et al., *Efficient generation of transgenic mice by lentivirus-mediated modification of spermatozoa.* Faseb j, 2014. **28**(2): p. 569-76.
391. Kim, B.G., et al., *Enrichment of testicular gonocytes and genetic modification using lentiviral transduction in pigs.* Biol Reprod, 2010. **82**(6): p. 1162-9.
392. Zeng, W., et al., *Viral transduction of male germline stem cells results in transgene transmission after germ cell transplantation in pigs.* Biol Reprod, 2013. **88**(1): p. 27.
393. Kim, K.J., et al., *Lentiviral modification of enriched populations of bovine male gonocytes.* J Anim Sci, 2014. **92**(1): p. 106-18.
394. Sehgal, L., et al., *Generation of transgenic mice by exploiting spermatogonial stem cells in vivo.* Methods Mol Biol, 2014. **1194**: p. 327-37.

395. Qin, J., et al., *An efficient strategy for generation of transgenic mice by lentiviral transduction of male germline stem cells in vivo.* J Anim Sci Biotechnol, 2015. **6**: p. 59.
396. Li, X., et al., *Rescuing infertility of Pick1 knockout mice by generating testis-specific transgenic mice via testicular infection.* Sci Rep, 2013. **3**: p. 2842.
397. Ikawa, M., et al., *Restoration of spermatogenesis by lentiviral gene transfer: offspring from infertile mice.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7524-9.
398. Zhao, K., et al., *Tissue-specific inhibition of urokinase-type plasminogen activator expression in the testes of mice by inducible lentiviral RNA interference causes male infertility.* Reprod Fertil Dev, 2017. **29**(11): p. 2149-2156.
399. Wang, F., et al., *The microtubule plus end-binding protein EB1 is involved in Sertoli cell plasticity in testicular seminiferous tubules.* Exp Cell Res, 2008. **314**(1): p. 213-26.
400. Park, S.J., et al., *hCG-induced endoplasmic reticulum stress triggers apoptosis and reduces steroidogenic enzyme expression through activating transcription factor 6 in Leydig cells of the testis.* J Mol Endocrinol, 2013. **50**(2): p. 151-66.
401. Schaffner, W., *Direct transfer of cloned genes from bacteria to mammalian cells.* Proc Natl Acad Sci U S A, 1980. **77**(4): p. 2163-7.
402. Fennelly, G.J., et al., *Mucosal DNA vaccine immunization against measles with a highly attenuated Shigella flexneri vector.* J Immunol, 1999. **162**(3): p. 1603-10.
403. Bergmann-Leitner, E.S., et al., *Self-adjuvanting bacterial vectors expressing pre-erythrocytic antigens induce sterile protection against malaria.* Front Immunol, 2013. **4**: p. 176.
404. Giacalone, M.J., et al., *Immunization with non-replicating E. coli minicells delivering both protein antigen and DNA protects mice from lethal challenge with lymphocytic choriomeningitis virus.* Vaccine, 2007. **25**(12): p. 2279-87.
405. Byrne, W.L., et al., *Bacterial-mediated DNA delivery to tumour associated phagocytic cells.* J Control Release, 2014. **196**: p. 384-93.
406. Kazmierczak, R., et al., *Direct attachment of nanoparticle cargo to Salmonella typhimurium membranes designed for combination bacteriotherapy against tumors.* Methods Mol Biol, 2015. **1225**: p. 151-63.
407. Xu, X., et al., *Effective cancer vaccine platform based on attenuated salmonella and a type III secretion system.* Cancer Res, 2014. **74**(21): p. 6260-70.
408. Patyar, S., et al., *Bacteria in cancer therapy: a novel experimental strategy.* J Biomed Sci, 2010. **17**(1): p. 21.
409. Theys, J., et al., *Repeated cycles of Clostridium-directed enzyme prodrug therapy result in sustained antitumour effects in vivo.* Br J Cancer, 2006. **95**(9): p. 1212-9.
410. Rieck, S., K. Zimmermann, and D. Wenzel, *Transduction of murine embryonic stem cells by magnetic nanoparticle-assisted lentiviral gene transfer*. Methods Mol Biol, 2013. **1058**: p. 89-96.
411. Shim, G., et al., *Therapeutic gene editing: delivery and regulatory perspectives.* Acta Pharmacol Sin, 2017.

412. Wu, Y., et al., *Correction of a genetic disease in mouse via use of CRISPR-Cas9.* Cell Stem Cell, 2013. **13**(6): p. 659-62.
413. Yin, H., et al., *Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype.* Nat Biotechnol, 2014. **32**(6): p. 551-3.
414. Xu, L., et al., *CRISPR-mediated Genome Editing Restores Dystrophin Expression and Function in mdx Mice.* Mol Ther, 2016. **24**(3): p. 564-9.
415. Wang, D., et al., *Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses.* Hum Gene Ther, 2015. **26**(7): p. 432-42.
416. Tabebordbar, M., et al., *In vivo gene editing in dystrophic mouse muscle and muscle stem cells.* Science, 2016. **351**(6271): p. 407-11.
417. Yoshimi, K., et al., *Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR-Cas platform.* Nat Commun, 2014. **5**: p. 4240.
418. Guan, Y., et al., *CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse.* EMBO Mol Med, 2016. **8**(5): p. 477-88.
419. Fanslow, D.A., et al., *Genome Editing in Mouse Spermatogonial Stem/Progenitor Cells Using Engineered Nucleases.* PLoS ONE, 2014. **9**(11): p. e112652.
420. Sato, T., et al., *Genome Editing in Mouse Spermatogonial Stem Cell Lines Using TALEN and Double-Nicking CRISPR/Cas9.* Stem Cell Reports, 2015. **5**(1): p. 75-82.
421. Wu, Y., et al., *Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells.* Cell Res, 2015. **25**(1): p. 67-79.
422. Chapman, K.M., et al., *Targeted Germline Modifications in Rats Using CRISPR/Cas9 and Spermatogonial Stem Cells.* Cell Rep, 2015. **10**(11): p. 1828-35.
423. Choi, J.G., et al., *Lentivirus pre-packed with Cas9 protein for safer gene editing.* Gene Ther, 2016. **23**(7): p. 627-33.
424. Tabebordbar, M., et al., *In vivo gene editing in dystrophic mouse muscle and muscle stem cells.* Science, 2016. **351**(6271): p. 407-411.
425. Sauer, B., *Inducible gene targeting in mice using the Cre/lox system.* Methods, 1998. **14**(4): p. 381-92.
426. Madisen, L., et al., *A robust and high-throughput Cre reporting and characterization system for the whole mouse brain.* Nat Neurosci, 2010. **13**(1): p. 133-40.
427. Srinivas, S., et al., *Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus.* BMC Dev Biol, 2001. **1**: p. 4.
428. Welsh, M., et al., *New Insights into the Role of Androgens in Wolffian Duct Stabilization in Male and Female Rodents.* Endocrinology, 2009. **150**(5): p. 2472-2480.
429. Lécureuil, C., et al., *Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice.* genesis, 2002. **33**(3): p. 114-118.
430. He, W., et al., *Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15712-7.

431. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.
432. Colaco, S. and D. Modi, *Genetics of the human Y chromosome and its association with male infertility.* Reprod Biol Endocrinol, 2018. **16**(1): p. 14.
433. Jungwirth, A., et al., *European Association of Urology guidelines on Male Infertility: the 2012 update.* Eur Urol, 2012. **62**(2): p. 324-32.
434. Vigen, R., et al., *Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels.* JAMA, 2013. **310**(17): p. 1829-1836.
435. Ohlander, S.J., B. Varghese, and A.W. Pastuszak, *Erythrocytosis Following Testosterone Therapy.* Sexual Medicine Reviews, 2018. **6**(1): p. 77-85.
436. Pan, M.M., et al., *Male Infertility Diagnosis and Treatment in the Era of In Vitro Fertilization and Intracytoplasmic Sperm Injection.* Med Clin North Am, 2018. **102**(2): p. 337-347.
437. Chen, L., et al., *Birth prevalence of congenital malformations in singleton pregnancies resulting from in vitro fertilization/intracytoplasmic sperm injection worldwide: a systematic review and meta-analysis.* Arch Gynecol Obstet, 2018. **297**(5): p. 1115-1130.
438. Xie, C., et al., *Multicenter study of genetic abnormalities associated with severe oligospermia and non-obstructive azoospermia.* J Int Med Res, 2018. **46**(1): p. 107-114.
439. Sharpe, A., P. Avery, and M. Choudhary, *Reproductive outcome following pre-implantation genetic diagnosis (PGD) in the UK.* Hum Fertil (Camb), 2018. **21**(2): p. 120-127.
440. Pradhan, B.S. and S.S. Majumdar, *An Efficient Method for Generation of Transgenic Rats Avoiding Embryo Manipulation.* Mol Ther Nucleic Acids, 2016. **5**: p. e293.
441. Blanchard, K.T. and K. Boekelheide, *Adenovirus-mediated gene transfer to rat testis in vivo.* Biol Reprod, 1997. **56**(2): p. 495-500.
442. Scobey, M., et al., *Delivery of a cyclic adenosine 3',5'-monophosphate response element-binding protein (creb) mutant to seminiferous tubules results in impaired spermatogenesis.* Endocrinology, 2001. **142**(2): p. 948-54.
443. Kanatsu-Shinohara, M., et al., *Adenovirus-mediated gene delivery and in vitro microinsemination produce offspring from infertile male mice.* Proceedings of the National Academy of Sciences, 2002. **99**(3): p. 1383-1388.
444. Sehgal, L., et al., *Lentiviral mediated transgenesis by in vivo manipulation of spermatogonial stem cells.* PLoS One, 2011. **6**(7): p. e21975.
445. Araki, M. and T. Ishii, *International regulatory landscape and integration of corrective genome editing into in vitro fertilization.* Reprod Biol Endocrinol, 2014. **12**: p. 108.
446. Renaud-Gabardos, E., et al., *Internal ribosome entry site-based vectors for combined gene therapy.* World J Exp Med, 2015. **5**(1): p. 11-20.
447. Ellis, J., *Silencing and variegation of gammaretrovirus and lentivirus vectors.* Hum Gene Ther, 2005. **16**(11): p. 1241-6.
448. Minoguchi, S. and H. Iba, *Instability of retroviral DNA methylation in embryonic stem cells.* Stem Cells, 2008. **26**(5): p. 1166-73.
449. He, J., Q. Yang, and L.-J. Chang, *Dynamic DNA Methylation and Histone Modifications Contribute to Lentiviral Transgene Silencing in Murine*

Embryonic Carcinoma Cells. Journal of Virology, 2005. **79**(21): p. 13497-13508.
450. Pfeifer, A., et al., *Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos.* Proceedings of the National Academy of Sciences, 2002. **99**(4): p. 2140-2145.
451. Herbst, F., et al., *Extensive Methylation of Promoter Sequences Silences Lentiviral Transgene Expression During Stem Cell Differentiation In Vivo*. Molecular Therapy, 2012. **20**(5): p. 1014-1021.
452. Klug, C.A., S. Cheshier, and I.L. Weissman, *Inactivation of a GFP retrovirus occurs at multiple levels in long-term repopulating stem cells and their differentiated progeny.* Blood, 2000. **96**(3): p. 894-901.
453. Kunkiel, J., et al., *The CpG-sites of the CBX3 ubiquitous chromatin opening element are critical structural determinants for the anti-silencing function.* Scientific Reports, 2017. **7**(1): p. 7919.
454. Piras, F., et al., *Lentiviral vectors escape innate sensing but trigger p53 in human hematopoietic stem and progenitor cells.* EMBO Molecular Medicine, 2017.
455. Lyon, M.F. and S.G. Hawkes, *X-linked Gene for Testicular Feminization in the Mouse.* Nature, 1970. **227**: p. 1217.
456. Yeh, S., et al., *Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13498-503.
457. Chang, C., et al., *Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells.* Proc Natl Acad Sci U S A, 2004. **101**(18): p. 6876-81.
458. Denolet, E., et al., *The Effect of a Sertoli Cell-Selective Knockout of the Androgen Receptor on Testicular Gene Expression in Prepubertal Mice.* Molecular Endocrinology, 2006. **20**(2): p. 321-334.
459. O'Shaughnessy, P.J., et al., *Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice.* Mol Cell Endocrinol, 2009. **306**(1-2): p. 2-8.
460. De Gendt, K., et al., *Expression of Tubb3, a Beta-Tubulin Isotype, Is Regulated by Androgens in Mouse and Rat Sertoli Cells1*. Biology of Reproduction, 2011. **85**(5): p. 934-945.
461. Tsai, M.Y., et al., *Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells.* Proc Natl Acad Sci U S A, 2006. **103**(50): p. 18975-80.
462. Carreau, S., et al., *The promoter(s) of the aromatase gene in male testicular cells.* Reprod Biol, 2004. **4**(1): p. 23-34.
463. Teerds, K.J. and I.T. Huhtaniemi, *Morphological and functional maturation of Leydig cells: from rodent models to primates.* Hum Reprod Update, 2015. **21**(3): p. 310-28.
464. Willems, A., et al., *Early effects of Sertoli cell-selective androgen receptor ablation on testicular gene expression.* International Journal of Andrology, 2010. **33**(3): p. 507-517.
465. Welsh, M., et al., *Deletion of androgen receptor in the smooth muscle of the seminal vesicles impairs secretory function and alters its responsiveness to*

exogenous testosterone and estradiol. Endocrinology, 2010. **151**(7): p. 3374-85.
466. Badran, H.H. and L.S. Hermo, *Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development.* J Androl, 2002. **23**(3): p. 358-73.
467. Soffientini, U., et al., *Identification of Sertoli cell-specific transcripts in the mouse testis and the role of FSH and androgen in the control of Sertoli cell activity.* BMC Genomics, 2017. **18**(1): p. 972.
468. Moller, A.P., *Ejaculate Quality, Testes Size and Sperm Production in Mammals.* Functional Ecology, 1989. **3**(1): p. 91-96.
469. Bujan, L., et al., *Testicular size in infertile men: relationship to semen characteristics and hormonal blood levels.* Br J Urol, 1989. **64**(6): p. 632-7.
470. Lenz, S., et al., *Ultrasonic testicular texture and size in 444 men from the general population: correlation to semen quality.* Eur Urol, 1993. **24**(2): p. 231-8.
471. Condorelli, R., A.E. Calogero, and S. La Vignera, *Relationship between Testicular Volume and Conventional or Nonconventional Sperm Parameters.* Int J Endocrinol, 2013. **2013**: p. 145792.
472. Wang, R.S., et al., *Androgen receptor in sertoli cell is essential for germ cell nursery and junctional complex formation in mouse testes.* Endocrinology, 2006. **147**(12): p. 5624-33.
473. Chen, M., et al., *Wt1 is involved in leydig cell steroid hormone biosynthesis by regulating paracrine factor expression in mice.* Biol Reprod, 2014. **90**(4): p. 71.
474. Wang, X.N., et al., *The Wilms tumor gene, Wt1, is critical for mouse spermatogenesis via regulation of sertoli cell polarity and is associated with non-obstructive azoospermia in humans.* PLoS Genet, 2013. **9**(8): p. e1003645.
475. Mäkelä, J.-A., et al., *Hedgehog signalling promotes germ cell survival in the rat testis.* Reproduction (Cambridge, England), 2011. **142**(5): p. 711-721.
476. Calamita, G., et al., *Possible involvement of aquaporin-7 and -8 in rat testis development and spermatogenesis.* Biochem Biophys Res Commun, 2001. **288**(3): p. 619-25.
477. Bernardino, R.L., et al., *Hepatocyte and Sertoli Cell Aquaporins, Recent Advances and Research Trends.* International Journal of Molecular Sciences, 2016. **17**(7): p. 1096.
478. I., V., et al., *Peritubular myoid cells of human and rat testis are smooth muscle cells that contain desmin-type intermediate filaments.* The Anatomical Record, 1986. **215**(1): p. 10-20.
479. Chen, L.-Y., et al., *Peritubular Myoid Cells Participate in Male Mouse Spermatogonial Stem Cell Maintenance.* Endocrinology, 2014. **155**(12): p. 4964-4974.
480. Paulin, D. and Z. Li, *Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle.* Exp Cell Res, 2004. **301**(1): p. 1-7.
481. Zwain, I.H., et al., *Regulation of Sertoli cell alpha 2-macroglobulin and clusterin (SGP-2) secretion by peritubular myoid cells.* Biol Reprod, 1993. **48**(1): p. 180-7.

482. Wang, G.M., et al., *Effects of insulin-like growth factor I on steroidogenic enzyme expression levels in mouse leydig cells.* Endocrinology, 2003. **144**(11): p. 5058-64.
483. Donnelly, M.L.L., et al., *Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'.* Journal of General Virology, 2001. **82**(5): p. 1013-1025.
484. Liu, Z., et al., *Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector.* Scientific Reports, 2017. **7**: p. 2193.
485. Tuck, R.R., et al., *The composition of fluid collected by micropuncture and catheterization from the seminiferous tubules and rete testis of rats.* Pflugers Arch, 1970. **318**(3): p. 225-43.
486. Richburg, J.H., D.M. Redenbach, and K. Boekelheide, *Seminiferous tubule fluid secretion is a Sertoli cell microtubule-dependent process inhibited by 2,5-hexanedione exposure.* Toxicol Appl Pharmacol, 1994. **128**(2): p. 302-9.
487. Watanabe, S., et al., *Adeno-associated virus-mediated delivery of genes to mouse spermatogonial stem cells.* Biol Reprod, 2017. **96**(1): p. 221-231.
488. Nagano, M., et al., *Lentiviral vector transduction of male germ line stem cells in mice.* FEBS Lett, 2002. **524**(1-3): p. 111-5.
489. Takehashi, M., et al., *Adenovirus-mediated gene delivery into mouse spermatogonial stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(8): p. 2596-601.
490. Nagano, M., et al., *Transgenic mice produced by retroviral transduction of male germ-line stem cells.* Proceedings of the National Academy of Sciences, 2001. **98**(23): p. 13090-13095.
491. Cherry, S.R., et al., *Retroviral expression in embryonic stem cells and hematopoietic stem cells.* Mol Cell Biol, 2000. **20**(20): p. 7419-26.
492. Tajik, P., et al., *Transfection of bovine spermatogonial stem cells in vitro.* Iran J Vet Res, 2017. **18**(2): p. 113-118.
493. Cooper, A., et al., *HIV-1 causes CD4 cell death through DNA-dependent protein kinase during viral integration.* Nature, 2013. **498**(7454): p. 376-9.
494. Park, S.-J., et al., *hCG-induced endoplasmic reticulum stress triggers apoptosis and reduces steroidogenic enzyme expression through activating transcription factor 6 in Leydig cells of the testis.* Journal of molecular endocrinology, 2013. **50**(2): p. 151-166.
495. Tung, P.S. and I.B. Fritz, *Morphogenetic restructuring and formation of basement membranes by Sertoli cells and testis peritubular cells in co-culture: inhibition of the morphogenetic cascade by cyclic AMP derivatives and by blocking direct cell contact.* Dev Biol, 1987. **120**(1): p. 139-53.
496. Kohler, B., et al., *Coexpression of Wilms' tumor suppressor 1 (WT1) and androgen receptor (AR) in the genital tract of human male embryos and regulation of AR promoter activity by WT1.* J Mol Endocrinol, 2007. **38**(5): p. 547-54.
497. Wang, X.N., et al., *The Wilms Tumor Gene, Wt1, Is Critical for Mouse Spermatogenesis via Regulation of Sertoli Cell Polarity and Is Associated with Non-Obstructive Azoospermia in Humans.* PLOS Genetics, 2013. **9**(8): p. e1003645.