MICROSCOPIC AND MACROSCOPIC INVESTIGATIONS OF MALE DEVELOPMENT
ANATOMY AND FERTILITY, AND THE ROLE OF Y CHROMOSOME GENES

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By

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„It’s in the genes.”
-Carole B. Ruthig-
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Abstract

There have been decades of work on the Y chromosome and how it relates to maleness and male reproduction. In light of the increasing decline of human male fertility and dependence on assisted reproduction more knowledge about the causes behind male infertility is needed to find more solutions for affected men. This dissertation details histological investigations aiming to elucidate function of Y chromosome genes in male development and fertility using a mouse model. The testis and the seminiferous tubules within were explored and spermatogenesis was quantitatively assessed. Males lacking Y chromosome and transgenic for either the key Y-derived transgenes (Sry and Eif2s3y) or transgenes of their non Y-derived homologues (Sox9 and Eif2s3x) were shown to produce haploid germ cells that could be used for assisted reproduction technologies (ART) and yield live offspring. Another Y chromosome gene, Zfy2, was identified as a gene allowing for transformation of round spermatids into sperm. While in males with only two Y chromosome genes, Sry and Eif2s3y, spermatogenesis progressed to round spermatid stage, addition of Zfy2 allowed for complete spermatogenesis and formation of sperm capable of generating offspring with ART. Investigations into the testicular abnormalities in mice with limited Y chromosome gene contribution showed relationship between the number of Y genes present and the severity and distribution of cellular abnormalities in the seminiferous epithelium and defects of the testis interstitium. Human spermatogenesis was also histologically investigated utilizing testis biopsies from normal and infertile men, some with Y chromosome azoospermic factor (AZF) deletions, validating mice with Y chromosome deficiencies as a model for human male Y-linked infertility. Gross anatomy investigations into human male urogenital anatomy were also undertaken using a novel dissection method in order to grow the repository of male urogenital
teaching tools at the medical school. Both examined specimens had common male genital pathologies (direct inguinal hernia, varicocele) and so also showcase physical abnormalities that can affect male reproductive health.
Publications Arising from this Dissertation

Journal Publications


Presentations and Abstracts

2016 – Presented

Talks
1. 2016 Victor A. Ruthig: *Y, men need it*. Talk at University of Hawa’i at Mānoa Three Minute Thesis Competition; Honolulu, HI; April 2016. (this presentation advanced to the final round in the competition)
3. 2016 Victor A. Ruthig: *Histological assessment of the genetic factors initiating and regulating spermatogenesis in mice and men*. Talk at the Developmental and Reproductive Biology Graduate Program semi-annual Graduate Student Retreat; Honolulu, HI; February 2016

Posters
6. 2016 Victor A. Ruthig, Yasuhiro Yamauchi, Jonathan M. Riel, Monika A. Ward: *Quantitative assessment of spermatogenesis in testicular biopsies from azoospermic men to validate a mouse model for human male infertility*. Poster at JABSOM Biomedical Sciences and Health Disparities Symposium; Honolulu, HI; April 2016. (this work was awarded best graduate poster)

2016 – Co-Authored

8. 2016 Torbjoern Nielsen, Victor A. Ruthig, Monika A. Ward: *Quantitative assessment of cell populations in the seminiferous epithelium of infertile male mice with minimal or no Y chromosome gene contribution*. Poster at JABSOM Biomedical Sciences and Health Disparities Symposium; Honolulu, HI; April 2016.

2015 – Presented

9. 2015 Victor A Ruthig, Yasuhiro Yamauchi, Jonathan A. Riel, Monika A Ward: *Testis structure and seminiferous epithelium characteristics in mice with a single or no Y*
chromosome genes. Poster at the Gordon Research Conference on Germinal Stem Cell Biology; Hong Kong, China; June 2015.

10. 2015 Victor A Ruthig, Yasuhiro Yamauchi, Jonathan M Riel, Michael J Mitchell, Monika A Ward: Overexpression of X-linked Eif2s3x can substitute for the loss of Y-linked Eif2s3y and allows for spermatogonial proliferation and differentiation in the mouse. Poster at the 7th International Health Symposium on Vertebrate Sex Determination (VSD); Kona, HI; April 2015.

2015 – Co-Authored


14. 2015 Egle A. Ortega, Yasuhiro Yamauchi, Victor A. Ruthig, Monika A. Ward: Sox9 overexpression can sufficiently supplement for a lack of Sry in terms of function in spermatogenesis and adult male fertility. Poster at the 7th International Health Symposium on Vertebrate Sex Determination (VSD); Kona, HI; April 2015.

2014 – Presented

Talk


Posters

16. 2014 Victor A. Ruthig, Yasuhiro Yamauchi, Jonathan M. Riel, Michael J. Mitchell, Monika A. Ward: Overexpression of X-linked Eif2s3x can substitute for the loss of Y-linked Eif2s3y and allows for spermatogonial proliferation and differentiation in the mouse. Poster at the 70th Annual Meeting of the American Society for Reproductive Medicine (ASRM); Honolulu, Hawaii; October 2014.

17. 2014 Victor A. Ruthig, Yasuhiro Yamauchi, Jonathan M. Riel, Monika A. Ward: X-linked Eif2s3x can substitute for Y-linked Eif2s3y in regulation of spermatogonial proliferation and differentiation in the mouse. Poster at JABSOM Biomedical Sciences
2014 – Co-Authored

18. 2014 Jonathan M. Riel, Yasuhiro Yamauchi, Victor A. Ruthig, Julie Cocquet, Monika A. Ward: Progress in defining whether lack of the Y chromosome long arm (NPYq) encoded gene Sly is the sole or the contributing cause of spermiogenic defects in mice with severe NPYq deletions. Poster at the 70th Annual Meeting of the American Society for Reproductive Medicine (ASRM); Honolulu, Hawaii; October 2014.


2013 – Presented

Talk

22. 2013 Victor A. Ruthig: Identification of novel protein partners of Y chromosome encoded, testis specific protein SLY. Talk at Developmental and Reproductive Biology Graduate Program semi-annual Graduate Student Retreat; Honolulu, HI; March 2013

Poster


2013 – Co-Authored

2012 – Co-Authored

2016 Fellowship Grant to Victor A. Ruthig, “Role of Lamin B Receptor in Male Germ Cell Differentiation and Epigenetic Regulation”, Lalor Foundation. Post-doctoral position with principal investigator, Dr. Blanche Capel. Total project period September 1st, 2016 to September 1st, 2017. A modified excerpt of this work can be found in Chapter 1.
Seminars (in part, utilizing the specimens described in Chapter 6)

2016 Victor A. Ruthig: Anatomy Review Seminar; gross anatomy prosection and cadaver presentation to Windward Community College undergraduate class Human Anatomy and Physiology I Zool141 Dr Allison Beale; John A. Burns School of Medicine; Honolulu, HI; July 2016

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* moved entirely or in part to Chapter 8. "Supplements to All Chapters"

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<tbody>
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<td>2/3NPYq-</td>
<td>two-thirds deletion of the NPYq region</td>
</tr>
<tr>
<td>9/10NPYq-</td>
<td>nine-tenths deletion of the NPYq region</td>
</tr>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>A7l-A7aligned</td>
<td>aligned type A spermatogonia</td>
</tr>
<tr>
<td>ABP</td>
<td>androgen binding protein</td>
</tr>
<tr>
<td>APPBP2</td>
<td>beta-amyloid precursor protein (cytoplasmic tail)-binding protein 2</td>
</tr>
<tr>
<td>A_p/A_paired</td>
<td>paired type A spermatogonia</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ART</td>
<td>assisted reproduction technology</td>
</tr>
<tr>
<td>A7s-A7single</td>
<td>single type A spermatogonia</td>
</tr>
<tr>
<td>AZFa,b,c</td>
<td>azoospermic factor region a, b or c</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>CME</td>
<td>cassette mediated exchange</td>
</tr>
<tr>
<td>COR1</td>
<td>chromosomal core protein 1</td>
</tr>
<tr>
<td>DKKL1</td>
<td>dickkopf-like 1</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dpc</td>
<td>days post coitus</td>
</tr>
<tr>
<td>Dpp</td>
<td>days post partum</td>
</tr>
<tr>
<td>DSD</td>
<td>disorders of sex development</td>
</tr>
<tr>
<td>E</td>
<td>efficiency</td>
</tr>
<tr>
<td>Eif2/s3</td>
<td>translation initiation factor 2 subunit 3</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>G1/G0</td>
<td>gap one or zero of the cell cycle</td>
</tr>
<tr>
<td>GCT</td>
<td>germ-cell tumor</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GW</td>
<td>gestational week</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>ICSI</td>
<td>intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>INM</td>
<td>inner nuclear membrane</td>
</tr>
<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
</tr>
<tr>
<td>KAT5</td>
<td>histone H4 lysine acetyl transferase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases nucleotide bases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>L5</td>
<td>lumbar vertebra 5</td>
</tr>
<tr>
<td>LAD</td>
<td>lamina associated domain</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>M7pro-spermatogonia</td>
<td>mitotic pro-spermatogonia</td>
</tr>
<tr>
<td>MI</td>
<td>meiosis one</td>
</tr>
<tr>
<td>MIIL</td>
<td>meiosis two</td>
</tr>
<tr>
<td>MSY</td>
<td>male specific region of Y chromosome</td>
</tr>
<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NOA</td>
<td>non-obstructive azoospermia</td>
</tr>
<tr>
<td>NPY</td>
<td>non-pseudoautosomal region of Y chromosome</td>
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<tr>
<td>NPYp</td>
<td>NPY short arm</td>
</tr>
<tr>
<td>NPYq</td>
<td>NPY long arm</td>
</tr>
<tr>
<td>NPYq-</td>
<td>full deletion of the NPYq region</td>
</tr>
<tr>
<td>NPYq-2</td>
<td>(XY&lt;sup&gt;Y&lt;/sup&gt;Sex&lt;sup&gt;p&lt;/sup&gt;) – inheritable full deletion of the NPYq region</td>
</tr>
<tr>
<td>ONM</td>
<td>outer nuclear membrane</td>
</tr>
<tr>
<td>P.I.</td>
<td>primary investigator</td>
</tr>
<tr>
<td>PAR</td>
<td>pseudo-autosomal region</td>
</tr>
<tr>
<td>PAS-H</td>
<td>periodic acid Schiff and hematoxylin</td>
</tr>
<tr>
<td>PGC</td>
<td>primordial germ cell</td>
</tr>
<tr>
<td>PMSC</td>
<td>post-meiotic sex chromosome</td>
</tr>
<tr>
<td>PSCR</td>
<td>post-meiotic sex chromosome repression</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAR&lt;alpha&gt;</td>
<td>retinoic acid receptor alpha</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROSI</td>
<td>round spermatid injection</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCP</td>
<td>supporting cell precursors</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>shSLY</td>
<td>SLY specific shRNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>S6X</td>
<td>Sycp3-like, X-linked</td>
</tr>
<tr>
<td>S6xl1</td>
<td>S6xl-like 1</td>
</tr>
<tr>
<td>Sly</td>
<td>(Sly&lt;sup&gt;1&lt;/sup&gt; and Sly&lt;sup&gt;2&lt;/sup&gt;) – Sycp3-like, Y-linked</td>
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<tr>
<td>Sox&lt;9&gt;</td>
<td>Sry-related HMG box gene 9</td>
</tr>
<tr>
<td>Sry</td>
<td>sex determining region, Y chromosome</td>
</tr>
<tr>
<td>SSC</td>
<td>spermatogonial stem cell</td>
</tr>
<tr>
<td>Sxr&lt;sup&gt;a&lt;/sup&gt; or Sxr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>sex reversal domain</td>
</tr>
<tr>
<td>Sycp3</td>
<td>synaptosomal complex protein 3</td>
</tr>
<tr>
<td>T1</td>
<td>pro-spermatogonia – transition one pro-spermatogonia</td>
</tr>
<tr>
<td>T2</td>
<td>pro-spermatogonia – transition two pro-spermatogonia</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>triiodothyronine</td>
</tr>
<tr>
<td>TA</td>
<td>transactivating</td>
</tr>
<tr>
<td>Tdy&lt;sup&gt;m1&lt;/sup&gt;</td>
<td>11kb deletion on the Y chromosome that removes Sry</td>
</tr>
<tr>
<td>X&lt;sup&gt;E&lt;/sup&gt;</td>
<td>X&lt;sup&gt;E&lt;/sup&gt;/X&lt;sup&gt;c&lt;/sup&gt; (X&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>X&lt;sup&gt;I&lt;/sup&gt;</td>
<td>X-chromosomal, lymphocyte regulated</td>
</tr>
<tr>
<td>X&lt;sup&gt;W&lt;/sup&gt; or X&lt;sup&gt;Paf&lt;/sup&gt;</td>
<td>X chromosome with X-linked coat marker</td>
</tr>
<tr>
<td>Patchy-fur</td>
<td></td>
</tr>
<tr>
<td>Y&lt;sup&gt;Tdy&lt;/sup&gt;&lt;sup&gt;m1&lt;/sup&gt;</td>
<td>Y chromosome with Tdy&lt;sup&gt;m1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Z&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Zfy2 transgene</td>
</tr>
<tr>
<td>Zfy&lt;sup&gt;(Zfy1 and Zfy2)&lt;/sup&gt;</td>
<td>zinc finger Y-linked</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

Section 1.1 History

„To understand a man, you must first understand his past.”

Peter Falk

Boys and men are often more curious and proud, than girls and ladies, when it comes to the inner workings and products of their male anatomy. In 1677 this innate and overabundant curiosity got the better a young man named Johan Ham, who decided to microscopically examine the ejaculate of a man suffering with gonorrhea. Ham was of one of Antonie van Leeuwenhoeks medical students at the time and is credited with the first observations of the animicules present in human semen [10]. Ham and van Leeuwenhoeks research on these „animicules” set the stage for centuries of investigation into male fertility (Fig. 1).

By the nineteenth century the testis and the cells within were under full investigation. In 1850 Franz Leydig described cells that resided in the interstitium of the testis and would eventually bear his name [11]. Around the same time Enrico Sertoli was very busy exploring the seminiferous tubules of the testis which led to his description of the supportive cells within (at the time called cellule ramificata or branched cells) that would eventually be named after him at the behest of another contemporary of the era, Victor von Ebner (Fig. 1) [12].

Extensive histological research into the nature of the seminiferous epithelium of the testis and its classification began in 1888 with Victor von Ebner (Fig. 1) [13]. The work was in the rat and stands as the inception of diagnostic investigations into spermatogenesis [8]. By the mid twentieth century research on spermatogenesis had jumped species. In 1940 Charles Charny published results, which demonstrated the value of human testis biopsy in the diagnosis of sterility
Thirteen years later, in 1953 biopsies had become an „It” in human fertility assessment and Warren Nelson proposed a classification system for their evaluation [15].

During the 1960’s research on human spermatogenesis took quantum leaps. In 1964 Carl Heller and Yves Clermont described the histological characteristic and kinetics of human spermatogenesis [16]. By 1967 Anna and Emil Steinberger along with Djin Tjioe and C. Alvin Paulsen had devised a method for quantification of spermatogenesis with testis biopsies, unfortunately it was incredibly labor intensive and so never rose to wide spread use (Fig. 1) [1, 17, 18].

In the world of rodents, murine spermatogenesis started to make real headway in the 1950’s. In 1952 Charles Leblond and Yves Clermont published an initial description of murine spermatogenesis [19]. Four years later in 1956 Eugene Oakberg proposed the 12 stage classification system of murine spermatogenesis that is still used today [20]. What Clermont was for human spermatogenesis Oakberg would be for the mouse. In the mid to late 1970’s Eugene Oakberg and Claire Huckins published in-depth studies on mouse spermatogenesis with a focus on murine spermatogonia (Fig. 1) [21, 22].

The Hilschers, Barbara and Werner were studying phenomena in the mouse testis the same time as Oakberg and Huckins (Fig. 1). However, this couple’s biggest lasting contribution was a restructuring and logical organization of the nomenclature used in prespermatogenesis. In 1974 the Hilschers identified and named the subtle cellular changes that take place during prespermatogenesis [23]. Their system can be applied to mice and men and will be used in this text.
Figure 1. Major researchers in the field of sperm, spermatogenesis and testis research. An abridged historical context for major contributors in sperm, spermatogenesis and testis research. Johann Ham first described sperm after examining semen from a gonorrhea victim. Ham’s initial findings fueled years of extensive research by Antonie van Leeuwenhoek. Franz Leydig first described the steroidogenic testosterone producing cells of the testis that bear his name. Enrico Sertoli first described the somatic supportive cells of the testis that facilitate spermatogenesis and bear his name. Victor von Ebner made the first description of spermatogenesis in any mammal. Charles Leblond and Yves Clermont gave the first description of spermatogenesis in the mouse. Eugene Oakberg meticulously devised the spermatogenesis staging system that is used in the mouse to this day. Yves Clermont and Carl Heller did the initial groundwork describing and classifying human spermatogenesis. Anna and Emil Steinberger were at the lead of much of the work in quantitative analysis of human testis biopsies. Barbara and Werner Hilscher did much of the early work in mouse spermatogenesis, but their system for organizing and renaming the events of prespermatogenesis has been of great and lasting significance. Lonnie Russell, Michael Griswold, Marvin Meistrich and Dirk de Rooij are the modern day leaders in the field of murine spermatogenesis assessment. Larry Johnson, Niels Skakkebaek, Rupert Amann and Ewa Rajpert-De Meyts are the current leading experts in human spermatogenesis and testis biopsy assessment, especially in regard to testis pathologies.
Section 1.2 The Y Chromosome

In humans and mice, the Y chromosome or lack thereof determines gender. Offspring lacking a Y, and in the case of humans bear a second X, are by default female and those with a Y are male. Genes important to male development are scattered throughout the mammalian genome, however the Y chromosome holds the genes that are the prime movers for male anatomical and primordial germ cell development [24, 25]. Testis formation is an important aspect of male development. During the lifespan of a male it is the testis that produces the male gamete, the spermatozoon and the majority of the male hormone, testosterone [26, 27]. The Y chromosome also plays a vital role in the process of spermatozoa production, spermatogenesis [28].

Spermatogenesis can be divided into three distinct phases: the proliferative phase when spermatogonia undergo mitosis, the meiotic phase when spermatocytes undergo meiosis and change to haploid germ cells, and the spermiogenic phase when haploid spermatids undergo morphological and chromatin condensation changes as they differentiate into spermatozoa (Fig. 2) [29]. The mouse Y chromosome can be divided into a pseudoautosomal region (PAR), which acts as a pairing partner with a similar region on the X chromosome during meiosis, and a non-pairing Y chromosome (NPY, also called male specific Y or MSY) region (Fig. 3) [30]. The NPY region can be further subdivided into a short arm (NPYp) and a long arm (NPYq), determined to have roles at least in spermatogonial proliferation and spermiogenesis, respectively (Fig. 3, Table 1 and Table 2) [31-33]. Contemporaries
Figure 2. Stages of murine spermatogenesis. Spermatogenesis is the process of sperm development and involves mitosis and meiosis and morphological cell changes. In mice, spermatogenesis is divided into 12 stages (I-XII) and 16 spermatid developmental steps. Spermatogenesis takes place within the seminiferous tubules of the testis with the support of somatic Sertoli cells. A, In, and B are type A, intermediate, and type B spermatogonia, respectively. Pl, L, Z, P, D, M, and 2º are preleptotene, leptotene, zygotene, pachytene, diplotene, meiotic, and secondary spermatocytes, respectively. Steps of spermatid development are numbered 1-16.
Figure 3. Selected murine chromosome gene content. Mouse Y chromosome can be divided into the pseudoautosomal region (PAR) and the non-pairing region of the Y chromosome (NPY); the latter can be subsequently divided into the NPY short arm (NPYp) and the NPY long arm (NPYq). NPYp contains predominantly single copy genes with two duplicate genes (Zfy and H2al2y) and one multicopy gene (Rbmy). NPYq contains predominantly multicopy genes arranged in amplicons. Chromosome 11 contains the autosomal gene Sox9, which is downstream of Y-linked Sry in the testis formation cascade. X chromosome contains Eif2s3x, which is homologous to Y-linked Eif2s3y. Both chromosome 11 (ch11) and X chromosome contain many other genes. NPYq multicopy genes are not arranged in any specific order on the NPYq. All chromosomes are not to scale, NPY regions are not to scale. Locations of Sox9 and Eif2s3x in figure are approximate.
Table 1. Mouse NPYp genes and protein function.

<table>
<thead>
<tr>
<th>Gene Abbreviation</th>
<th>Gene Name</th>
<th>Function</th>
<th>Accession Number (UniProt)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ddx3y (Dhy)</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked</td>
<td>ATP-dependent RNA helicase</td>
<td>Q62095</td>
<td>[34] [25]</td>
</tr>
<tr>
<td>Eif2s3y</td>
<td>Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked</td>
<td>Spermatogonial proliferation and differentiation</td>
<td>Q9Z0N2</td>
<td>[34] [35]</td>
</tr>
<tr>
<td>H2al2y (Gm6026 and Gm16501)</td>
<td>Histone 2A-like 2, Y-linked</td>
<td>Nucleosome assembly</td>
<td>A9Z055</td>
<td>[36] [37]</td>
</tr>
<tr>
<td>Prsly</td>
<td>Protease, serine-like, Y chromosome</td>
<td>Serine-type endopeptidase activity</td>
<td>KJ780361</td>
<td>[4]</td>
</tr>
<tr>
<td>Rbmy</td>
<td>RNA binding motif protein, Y chromosome</td>
<td>mRNA processing / splicing</td>
<td>O35698</td>
<td>[38] [39]</td>
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<tr>
<td>Smcy (Kdm5d or Jarid1d)</td>
<td>Jumonji, AT rich interactive domain 1D</td>
<td>Histone demethylation</td>
<td>Q62240</td>
<td>[40] [41]</td>
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<tr>
<td>Sry (Tdy)</td>
<td>Sex-determining region Y</td>
<td>Testis formation</td>
<td>Q05738</td>
<td>[24] [42]</td>
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<tr>
<td>Teyorf1</td>
<td>Testis-expressed Y open reading frame 1</td>
<td>Unknown</td>
<td>KJ780362</td>
<td>[4]</td>
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<tr>
<td>Ubely1</td>
<td>Ubiquitin-activating enzyme</td>
<td>Protein activation / modification</td>
<td>Q61341</td>
<td>[43, 44] [45]</td>
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<td>Usp9y (Dffry)</td>
<td>Ubiquitin specific peptidase 9, Y chromosome</td>
<td>Protease</td>
<td>F8VPU6</td>
<td>[34, 46] [47]</td>
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<td>Uty</td>
<td>Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome</td>
<td>Histone demethylation</td>
<td>G3X9F2</td>
<td>[48] [34]</td>
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<td>Zfy1</td>
<td>Zinc finger protein 1, Y linked</td>
<td>Transcription regulation</td>
<td>P10925</td>
<td>[49] [50]</td>
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<td>Zfy2</td>
<td>Zinc finger protein 2, Y linked</td>
<td>Transcription regulation</td>
<td>P20662</td>
<td>[49] [50]</td>
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**Table 2. Mouse NPYq genes and protein function.**

<table>
<thead>
<tr>
<th>Gene Abbreviation</th>
<th>Gene Name</th>
<th>Function</th>
<th>Accession Number (UniProt)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rbm31y</strong></td>
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<td>Nucleotide binding</td>
<td>AK017055</td>
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<td><strong>Sly</strong></td>
<td>Sycp3 like Y-linked</td>
<td>Spermiogenesis</td>
<td>Q810R0</td>
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<td><strong>Srby</strong></td>
<td>Serine-rich secreted Y-linked</td>
<td>Unknown</td>
<td>EU052291</td>
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<td><strong>Ssty1</strong></td>
<td>Spermiogenesis specific transcript on the Y 1</td>
<td>Putative role in spermatid development</td>
<td>P13675</td>
<td>[53] [54, 55]</td>
</tr>
<tr>
<td><strong>Ssty2</strong></td>
<td>Spermiogenesis specific transcript on the Y 2</td>
<td>Putative role in spermatid development</td>
<td>Q149W4</td>
<td>[56, 57] [54, 55]</td>
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</table>
Deletion of all or a part of either the NPYp or NPYq negatively impacts the phases of spermatogenesis, in which they are involved [32, 33]. The phenotype of mice with these deletions can be more specifically traced back to a lack of specific genes [25, 58, 59]. Opposing the activity of the Y chromosome during spermatogenesis is the X chromosome, the two are seemingly locked in an age old conflict between the competing interests of X and Y bearing male gametes [52, 60].

Section 1.3 The Mouse Y Chromosome

The short arm of the mouse Y chromosome (NPYp) encodes *Sry*, the gene responsible for initiating testis formation during development (Fig. 3, Table 1) [24]. The second most important NPYp encoded gene is *Eif2s3y*, a spermatogonal proliferation factor and the master initiator of spermatogenesis (Fig. 3, Table 1) [25, 61]. Most of the genes on NPYp are single copy [34]. However there are two duplicated genes, *Zfy* and *H2al2y* as well as one multicopy gene, *Rbmy* (Fig. 3, Table 1) [38, 49, 62].

Unlike NPYp, the long arm of the mouse Y chromosome (NPYq) is composed primarily of multi-copy genes (Fig. 3, Table 2). To date most studies have focused on *Ssty* and *Sly*, both of which have been classified as having high copy number [36, 51, 55, 56, 63]. *Ssty*, and *Sly* have approximately 386 and 126 copies respectively in the NPYq region, a more recent addition *Srsy* has a putative 197 copies [4, 64]. There is also one duplicated gene on the long arm, *Rbm31y* [4]. Due to the multi-copy nature of these genes, there is a significant difference in size between NPYp and NPYq [53]. The change in gene composition between NPYp and NPYq thus results in the latter encompassing approximately 96.09% of the NPY region [4, 30, 65, 66].
As previously stated NPYq is involved in spermiogenesis [33]. In fact Ssty and Sly are expressed in round spermatids, while Srsy is at least expressed in the male germline [4, 36, 51, 53, 67]. Ssty and Sly are transcribed and also translated in round spermatids [54, 59]. Out of the three genes, Sly is the most highly expressed in round and elongating spermatids and SLY can be found in both the nucleus and cytoplasm of round spermatids, just prior to the morphological changes and chromatin condensation that are the two hallmarks of spermiogenesis (Fig. 4) [58, 59]. This suggests that Sly covers the breadth of spermiogenesis (Fig. 4) [51, 55, 58, 59].

Part 1.3.1 Mutations of the Y Chromosome Short Arm (NPYp)

The most well studied mutation of the NPYp is sex reversal factor (Sxr, now referred to as Sxra). Sxra is a portion of the NPYp region, which encodes Sry [24, 31, 35]. Sxra can produce sex-reversed female mice because the NPYp fragment can become translocated first distal to the Y PAR, and then from Y chromosome to X chromosome during homologous recombination between the X PAR and Y PAR in male meiosis (Fig. 5) [24, 68-70]. Breeding between XO and XY Sxra mice can result in a XSxraO genotype in which the X chromosome dosage is similar to that in XY mouse but the Y genes are limited to just what is on the transferred NPYp fragment [68].

XSxraO mice produce grossly abnormal sperm and have a significant decrease in the number of spermatids due to apoptosis taking place during meiosis [32, 68]. The addition of a pairing partner, Y*X, which results in XSxraY*X genotype, overcomes the XSxraO meiotic decrease of spermatids but fails to correct the sperm morphology issues [33].
Figure 4. Sly1/SLY1 expression patterns in secondary spermatocytes and spermatids. Spermatogenesis steps relevant to Sly1/SLY1 expression corresponding to stages XII-IX of spermatogenesis. Blue arrow representing Sly1 expression with shading intensity correlating to expression level. Green color represents SLY1 presence (nuclear/cytoplasmic). Red color represents the acrosome granule/acrosome.
Figure 5. Formation of XSxr$^a$ chromosome variant. Translocation of Sxr$^a$ to the distal PAR of the Y chromosome is followed by transfer of Sxr$^a$ from Y to X chromosome during homologous recombination.
Figure 6. Formation of the Y∗X chromosome variant. Inversion of the Y PAR followed by addition of a small portion of the X chromosome and its centromere forms Y∗. During meiosis homologous recombination between the inverted Y∗ PAR and the X PAR results in the Y∗X chromosome, which acts as a pairing partner for the X or Y chromosome but entirely lacks Y-linked genes.
Figure 7. Formation of Sxrb. Homologous recombination between YSxr^a and XSxr^a during meiosis leads to a further reduction in retained NPYP genes (Sxrb) due to a Zfy1/Zfy2 fusion removing a large portion of the Sxr^a.
The name for $X_{Sxr}^aY^{*X}$ mice gives the false impression that these mice have a Y chromosome. The $Y^{*X}$ of $X_{Sxr}^aY^{*X}$ mice is actually an X chromosome derived, chromosome variant containing a centromere attached to a PAR region. $Y^{*X}$ acts as a pairing partner while contributing no X or Y specific genes, leaving the $Sxr^a$ on the X chromosome as the only Y gene contributor (Fig. 6) [33]. $X_{Sxr}^aY^{*X}$ mice have germ cells that successfully pass through meiosis and produce spermatozoa, however the number of spermatozoa is low and all have abnormal morphology [33].

The $Sxr^b$ mutation is a further reduction in the gene composition of the NPYp variant $Sxr^a$. Occurring in an $X_{Sxr}^aY_{Sxr}^a$ mouse as part of a crossing over event many of the NPYp genes are removed from $Sxr^a$, but $Sry$ as well as a few other genes remain (Fig. 7) [71, 72]. $X_{Sxr}^bO$ mice form small testes lacking in germ cells due to misregulated spermatogonial differentiation and proliferation [32]. Transgenic addition of $Eif2s3y$ to an $X_{Sxr}^bO$ mouse ($X_{Sxr}^bOEif2s3y$) allows spermatogonial differentiation and proliferation to be restored, implicating $Eif2s3y$ as the gene necessary for regulating spermatogonial proliferation and differentiation for initiation of spermatogenesis [25].

Part 1.3.2 Deletions of the Y Chromosome Long Arm (NPYq)

The $Sxr^a$ mutation also led to studies of NPYq deletions (NPYq-). The self-synapsed cross over of a $Y^{RIII}_{Sxr}^a$ chromosome resulted in a two-thirds (2/3) deletion of NPYq ($XY^{RIII}qdel$ or 2/3NPYq-) (Fig. 8 and Fig. 9) [53]. 2/3NPYq- mice show a marked increase in the incidence of sperm with slight headshape defects and have an upregulation of some X-linked and Y-linked genes when compared to wild-type males [36, 53, 73]. Offspring of 2/3NPYq- mice exhibit a sex ratio distortion that favors females [53].
Figure 8. Formation of '2/3 deletion'. Self-synapsed crossing over of a $Y^{RIII}Sxa$ chromosome, which is initiated by homologous recombination between the $Sxa$ and NPYp regions, removes approximately two-thirds of the NPYq region.
Figure 9. Sex chromosome genotypes of the four NPYq deletion models. Note that in 9/10 NPYq- mice Sry is transgenically added autosomally to counteract loss of endogenous Sry due to the Tdy\textsuperscript{m1} deletion on the Y chromosome. For NPYq- it is the X chromosome that carries Y-linked genes encoded within Sxr\textsuperscript{a} region, but in NPYq-2 it is the Y\textsuperscript{*X} chromosome.
Intracytoplasmic sperm injection (ICSI) negates advantages a sperm might have over a dysfunctional member of its cohort by directly injecting the sperm into the oocyte. When ICSI was used with the 2/3NPYq- mouse sperm to produce offspring the sex ratio distortion was overcome [74]. ICSI success indicates that X and Y bearing sperm from 2/3NPYq- mice have equally good abilities to produce a zygote when injected but Y bearing sperm must be at a disadvantage when approaching or penetrating the oocyte in mating or in conventional in vitro fertilization (IVF) [74].

The Tdy<sup>m1</sup> mutation first described by Lovell-Badge and Robertson in 1990 is a 11kb deletion on the Y chromosome that removes Sry, thus XY<sup>Tdy<sub>m1</sub></sup> mice are phenotypically female (Fig. 10) [75, 76]. In 2004 Touré et al. reported finding an XXY<sup>Tdy<sub>m1</sub></sup> Sry mouse with no Ssty [55]. Tracing back through F1 and parent generations showed an extensive NPYq deletion removing about nine-tenths (9/10) of NPYq in the germ cells of the XXY<sup>Tdy<sub>m1</sub></sup> parent (Fig. 9 and Fig. 10) [55]. Male mice lacking 9/10 of NPYq (XY<sup>Tdy<sub>m1</sub></sup> qdelSry or 9/10NPYq-) have all severely abnormal sperm, spermiation delay, reduced sperm count in the epididymides, decreased sperm motility and are infertile, failing both natural mating and (IVF) [55, 74]. 9/10NPYq- mice also exhibit upregulation of some X-linked and Y-linked genes [36]. Much like in the case of the 2/3NPYq- mice, ICSI overcomes 9/10NPYq- infertility and offspring equally represent the four genotypes of possible male gametes [74].
Figure 10. Formation of '9/10 deletion'. Spontaneous partial deletion of NPYq in XXY^{Tdym1} mouse removes approximately nine-tenths of the NPYq.
XSxr\(^A\)Y\(^*\)X (NPYq-) mice can also be used to study NPYq deletions because they completely lack NPYq as well as some of the NPYP Rbmy copies ([Fig. 9 and Fig. 11]) [33, 36]. As mentioned earlier, these mice are infertile and produce sperm with abnormal head morphology [33]. NPYq-mice have an upregulation of X-linked and Y-linked genes [36]. As with the previous NPYq deletion mice, ICSI can overcome NPYq- infertility [77]. However, NPYq- mice are the product of laborious breeding. In 2009 Yamauchi et al. reported the generation of a more easily produced male mouse lacking the entire NPYq [77].

Since progeny from XSxr\(^A\)Y\(^*\)X males, that retain the XSxr\(^A\) mutation, will be XXXSxr\(^A\) males when Sxr\(^A\) is passed on by the father, the NPYq- genotype cannot be reproduced in offspring [77]. To circumvent the NPYq- mouse inability to produce offspring of the same genotype, XY\(^*\)XSxr\(^A\) (NPYq-2) mice can be used. NPYq-2 mice are produced from the gametes of an XX female and an XY\(^*\)XSxr\(^A\) male. XY\(^*\)XSxr\(^A\) is the product of a crossing over event between the X PAR and Y\(^*\)X PAR that results in the Sxr\(^A\) being translocated onto the Y\(^*\)X ([Fig. 9 and Fig. 12]) [77]. NPYq-2 mice produce sperm with grossly abnormal morphology, have a reduction in spermatid populations and are infertile, failing both natural mating and IVF [77]. NPYq-2 infertility can be overcome using ICSI with offspring representing the six genotypes of possible male gametes [77].
Figure 11. The sex chromosomes in NPYq- males. Y*X chromosome is paired with a XSr^a chromosome to produce a mouse with no NPYq genes, only NPYp genes.
Figure 12. Formation of the NPYq-2 deletion. Translocation of Sxr\textsuperscript{a} from X to Y\textsuperscript{*X} chromosome leads to a Y\textsuperscript{Y,X}Sxr\textsuperscript{a}. The translocation allows for a genotype that completely lacks the NPYq and can be passed on to offspring, unlike NPYq\textsuperscript{-}.
There is a common theme of abnormal sperm head morphology in all males with the NPYq deletions. This indicates that there should be some factor(s) on the NPYq that regulate sperm morphology. Changes in morphology during spermatogenesis occur during spermiation as round spermatids transition to elongating spermatids and eventually spermatozoa [29]. Of the multicopy genes on the NPYq it has been shown conclusively that Ssty and Sly are expressed in round spermatids [36, 51, 53]. While Srsy is only putatively expressed exclusively in the male germline [4]. Of these genes, Sly is the best candidate gene to regulate spermiogenesis. It is expressed the strongest during a crucial point in spermatid development (Fig. 4) [36, 51]. There is also a direct relationship between Sly reduction and the severity of the NPYq deletion phenotype [36, 51, 78, 79].

In 2010 Yamauchi et al. showed that partial to full deletion of the NPYq led to DNA damage, abnormal chromatin condensation, and impaired chromatin protamination in sperm. The severity of the phenotype was directly proportional to the severity of the deletion [80]. Chromatin condensation and protamination are a vital part of the nuclear changes that occur as spermatids differentiate into spermatozoa [29, 81].

Part 1.3.3 Early NPYq Project – Sycep3-like Y-linked (Sly) and Sycep3-like X-linked (Slx)

Early on in my degree pathway I participated in investigations of mice with NPYq deletions and with Sly knockdown. However, this work did not rise to the level of a chapter in my dissertation and so is included as a part of the introduction section focused on the mouse Y chromosome.

Of the NPYq multicopy genes Sly is strongly expressed in spermatids, especially round spermatids where it appears in both the cytoplasm and nucleus (Fig. 4) [36, 51, 58]. Decrease of
Sly copy number and lack of transcription is directly proportional to the extent of NPYq deletion [51].

The acquired multi-copy NPYq genes are randomly scattered throughout the NPYq region in a large series of ampliconic tandem repeats [4, 56]. Therefore, deletions of broad swaths of this region, such as the NPYq deletions mentioned previously (Fig. 9), remove NPYq genes nonspecifically, in that any and all of the multi-copy NPYq genes experience varying degrees of reduction in a random manner. An alternative approach that can specifically reduce the expression of a multi-copy gene is silencing using small interfering RNA (siRNA). In 2009 Cocquet et al. used a Sly specific short hairpin RNA (shRNA) to silence Sly expression via siRNA in transgenic mice. These shSLY transgenic mice produced sperm with grossly abnormal head morphologies, a spermiation (shedding) delay, impaired motility and had reduced fertility when mated and with IVF, with a sex ratio distortion in favor of females [58]. In the nucleus of spermatids X-link and Y-linked genes were upregulated along with a few autosomal genes. Postmeiotic sex chromosomes also lacked the hypermethylation marks that are part of heterochromatic transcriptional repression [58]. The sperm head morphology and ratio of male to female progeny for shSLY mice mirror the observations of NPYq deletion mice. The finding that the postmeiotic sex chromosomes of shSLY mice did not undergo post meiotic sex chromosome repression (PSCR) implicate SLY as a vital part of the mechanism that continues repression of sex-linked genes postmeiotically.

Sly has two transcriptional variants Sly1 and Sly2 [59]. Sly2 is a splice variant that lacks two exons (Fig. 13) [59]. A further study by Riel et al. 2013 was done with shSLY mice that were either deficient only for the Sly1 variant, or both the Sly1 and Sly2 variants [79]. Sly1/2-deficient mice produced sperm with morphology defects, impaired IVF function, chromatin condensation and protamination defects, and poor DNA integrity; these mice also had a high level of X and Y
gene upregulation in the testis [79]. Mice that were Sly1-only-deficient had no phenotype [79]. If knockdown of Sly1 only is not enough to result in the phenotype, this suggests that SLY2 plays an important role in spermiogenesis. But it is also possible that the combined expression for SLY1 and SLY2 might be necessary for maintaining normal spermiogenesis.

The protein, SLY1, is testis specific and present from 21.5 days post partum (dpp) onward [59]. It is expressed in the cytoplasm of round to early elongating spermatids from stages II to XI of spermatogenesis (Fig. 4) [59]. SLY1 is also expressed in the nuclei of spermatids from stages II to IX, where it co-localizes with the X and Y postmeiotic sex chromatin (PMSC) (Fig. 4) [58].

Sly encodes two protein variants SLY1 and SLY2. SLY1 is a 128 amino acid (AA) of approximately 40kDa. SLY2 (approximately 27kDa) is a splice variant of SLY1 that lacks exons 5-6 translating to a 34 AA omission from the SLY1 isoform (Fig. 13 and Fig. 14) [59, 67]. However both SLY1 and SLY2 contain the chromatin binding COR1 domain (Fig. 14) [51, 59]. SLY1 has been shown to interact with an acrosome protein DKKL1 [59, 82]. SLY1 has also been shown to interact with a histone acetyltransferase involved with DNA repair and apoptosis KAT5 [59, 83]. Finally, an interaction between SLY1 and APPBP2, a microtubule binding protein involved with intracellular trafficking, has also been observed [59, 84]. Originally these protein-protein interactions pointed to a possible mechanism for SLY1 action. However in Sly knockdown mice changes in KAT5 histone acetylation and DKKL1 localization and expression level were not observed [58].
Figure 13. Sly transcript variants. Exons are represented by boxes, introns are represented by lines, UTRs are represented by open boxes and ORFs are represented by filled boxes (blue). Sly2 differs from Sly1 due to a splicing event that removes two exons to form Sly2.
Figure 14. Amino acid sequence comparison of SLY1 and SLY2. Blue highlight represents SLY1 and SLY2 unique region when compared to SLX and SLXL1. Magenta highlight represent SLY1 specific region. Green highlight shows COR1 domain.

SLY1
MALKKLVIP KEGYLLLDVF DDEDDIKVS EEALESVKSP AFKDNENISP QAEADEDMGD EVDSMLDKSE VNNPAIGKDE NISPQVKGDE DMSGHEVGSMEL DKSQDDIYKT LHIKRWMET YVKESEFKGSN QKLERFCKTN ERERKNINNK FCEQYITTFQ KSDMDVQKFN EEKEKSVNSQ QKEQQALKLS KCSQNTLEA VKEMHEKSME VLMNLGTKN

SLY2
MALKKLVIP KEGYLLLDVF DDEDDIKVS EEALESVKSP AFKDNENISP QAEADEDMGD EVDSMLDKSE ****** ****** ****** ****** ****** ****** LHIKRWMET YVKESEFKGSN QKLERFCKTN ERERKNINNK FCEQYITTFQ KSDMDVQKFN EEKEKSVNSQ QKEQQALKLS KCSQNTLEA VKEMHEKSME VLMNLGTKN

SLY1 128 AA 40kDa
SLY2 94 AA 27kDa

Figure 14. Amino acid sequence comparison of SLY1 and SLY2. Blue highlight represents SLY1 and SLY2 unique region when compared to SLX and SLXL1. Magenta highlight represent SLY1 specific region. Green highlight shows COR1 domain.
The COR1 domain is proposed to be involved with chromatin binding [52]. SLY1/2 has the COR1 domain in common with a host of proteins expressed in spermatids (XLR, SLX, SLXL1 and SYCP3) [51]. XLR co-localizes with the base of chromatin loops and has a 46% AA similarity to SLY1 (Fig. 15) [51, 85]. SLX (formerly XMR) is a cytoplasmic protein highly expressed in spermatids with 48% AA similarity to SLY1 [36, 51, 86]. SLXL1 (formerly AK012913) is a testis specific protein that localizes to the acrosome, interacts with DKKL1 and has been shown to be involved in fertilization, and specifically zona pellucida binding [87]. Both Slx and Slxl1 are X chromosome genes that are implicated in an intragenomic conflict with Sly [36, 52]. SYCP3 is part of the synaptonemal complex formed during meiotic recombination and has 30% AA similarity to SLY1 (Fig. 15) [51, 88].

A silencing approach utilizing small interfering RNA (siRNA) was also used to investigate the functions of the X-linked Slx and Slxl1 [78]. The transgenic shSLX mice showed a reduction in fertility proportional to the severity of the knockdown, had impaired IVF success, a sex ratio distortion in favor of males, a spermatid elongation and spermiation delay with apoptotic elongating and condensing spermatids, reduced epididymal sperm counts and sperm motility, and abnormal sperm morphology and head tail junctions [52, 78]. In the nucleus of spermatids, autosomal genes involved with metabolism, cytoskeletal and extracellular matrix organization, and apoptosis were upregulated [78]. Importantly sex chromosome linked genes were not upregulated in spermatids and knockdown of Slx/Slxl1 in these mice led to a preference for male offspring, the opposite of shSLY mice. The shSLX mice had a spermatid phenotype that was similar to shSLY mice, however its origin was likely different. While in shSLY mice the phenotype might have
resulted from an upregulation of X-linked and Y-linked genes, in shSLX mice it could be due to an upregulation of metabolic, cellular component organization and apoptotic genes [58, 78].

Figure 15. Clustal alignment of SLY, XLR, SLX, SLX1 and SYCP3. The alignment demonstrates the sequence similarities between these five proteins. Coloring scheme is Zappo. COR1 domain boxed in orange.
Sly and Slx competition can be further elucidated by considering the effects of Sly knockdown on SLX/SLXL1 expression and localization. In wild-type mice SLX/SLXL1 are found only in the cytoplasm of spermatids [78, 86]. However, in spermatids of shSLY mice SLX/SLXL1 enter nuclei and occupy areas on the PMSC and autosomal Speer genes on chromosome 5, sites normally occupied by SLY1 [52]. This indicates that SLX/SLXL1 replaces SLY1, with regards to post meiotic sex chromosome inactivation in the nucleus. Thus SLX/SLXL1 can be thought of as a transcription enhancer in a Sly knockdown, which explains why X-linked and Y-linked genes are upregulated in shSLY mice.

Lastly, the effects of a reduction in both Sly and Slx was examined by breeding shSLX female mice with shSLY males. Slx/y deficient males exhibited a partial correction of the gene upregulation and partial restoration of the decrease of PMSC methylation marks observed in shSLY mice. Slx/y deficient males also showed partial correction of autosomal gene deregulation and lacked the delayed and apoptotic elongating spermatids observed in shSLX mice [52]. The dual knock down mice had improved sperm morphology, fertility and restored sex ratio equality when compared to shSLX and shSLY mice [52]. If knockdown of both Sly and Slx partially restores the mouse phenotype to resemble that of wild type mice, then when their products are present they must compete in such a way that they balance one another other out.

Genes on both the mouse and human Y chromosome can be divided up into ancestral genes that date back to when the Y chromosome was still an autosome and have endured the decay of the Y chromosome over the past 300 million years; or acquired genes that were not present on the autosome precursor [4, 89, 90]. There are a limited amount of ancestral genes remaining on the Y chromosome (17 for men and 9 for mice), but those that are still around are vital for testis
formation, spermatogenesis and putatively male viability, they are often also broadly expressed [89, 90]. In conjunction with all of this decay, the Y chromosome was also acquiring testis specific genes that were then massively amplified. This process of acquisition and amplification is abundantly obvious on the huge mouse NPYq and is the X and Y arms race in carnate [4]. As Sly and Slx demonstrate the acquired and amplified sex linked genes are opposing meiotic drivers that distort sex ratio. The X-linked homologue is often a meiotic driver such as Slx and the Y-linked homologue is often a meiotic suppressor such as Sly [4, 52, 79].

Section 1.4 The Human Y Chromosome

As with the mouse Y chromosome, the human Y chromosome is also acrocentric. However, unlike the mouse the human NPY is flanked by two PAR regions, one at each end (Fig. 16). In total the human Y is about 23.9 Mb and contains 78 genes. The NPY region comprises about 95% of the Y chromosome and contains a NPYp (8 Mb) and a NPYq (14.5 Mb) [4, 91]. Human SRY and ZFY are also located on the NPYp and the human NPYq also contains amplification of acquired genes, though the amplification is not as extensive as in the mouse [4, 89-91]. The human orthologue to mouse Eif2s3y, EIF2S3, has been autosomally retrotransposed but the human NPYq contains another eukaryotic translation initiation factor, EIF1A, in the AZFb region of the NPYq [90, 92]. There are actually three azoospermic factor (AZF) regions on the human NPYq: AZFa, AZFb and AZFc (Fig. 16). Deletions in these regions are a common cause of non-obstructive azoospermia (NOA), which is a lack of sperm in ejaculate not due to a physical block somewhere along the male reproductive tract. Specific genes, their functions and homologues elsewhere in the human genome can be found in Table 3, Table 4 and Table 5. In general AZFa deletions lead to a complete/type I Sertoli cell only (SCO) syndrome, AZFb
deletions lead to meiotic disruption resulting in maturation arrest or hypospermatogenesis, and AZFc deletions lead to incomplete/type II SCO syndrome with maturation arrest or hypospermatogenesis in the limited tubules with germ cells [93-99]. The AZFa, AZFb and AZFc occupy 0.8, 6.2 and 3.5 Mb of the NPYq respectively and there is slight overlap between the distal AZFb and proximal AZFc regions (Fig. 16) [95, 100-104]. The AZFc region is almost entirely amplicons (95%) and AZFb is only about 50% amplicons, the AZFa region is all single copy genes. Part of the non-ampliconic AZFb contains 5 widely expressed single copy genes [94, 104, 105]. Despite the AZFb and AZFc region overlap there is no AZFd region. This is because deletions in the overlap represent a distinct loss that is AZFb with some AZFc as well, as oppose to deletions of unaffiliated sequence [104].
Figure 16. The human Y chromosome and genetic Y-linked male factor infertility. The human Y chromosome has 3 AZF (azoospermic factor regions) regions (a, b, and c); deletions in these regions can lead to azoospermia, a lack of sperm in ejaculate. Deletions in AZFa are associated with a Sertoli cell only phenotype, in AZFb are associated with premeiotic disruption leading to maturation arrest or hypospermatogenesis, and in AZFc are associated with postmeiotic disruption leading to maturation arrest or hypospermatogenesis. There is overlap between the AZFb and AZFc regions. Human NPY is in blue, PAR is in orange.
### Table 3. Human Y chromosome AZFa region genes.

<table>
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<th>Gene (0.8Mb Total)</th>
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<th>Mouse Analogue/ Orthologue</th>
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<th>Deletion</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBY/ DDX3y</td>
<td>DEAD box Y</td>
<td>X-linked</td>
<td></td>
<td>Spermatogenic failure</td>
<td>ATP-dependent RNA helicase, putative roles RNA secondary structure alteration, splicing, spliceosome assembly and translation initiation</td>
<td>[95, 99, 105]</td>
<td></td>
</tr>
<tr>
<td>DFFRY (USP9Y)</td>
<td>Ubiquitin specific protease 9 Y</td>
<td>X-linked</td>
<td></td>
<td>Spermatogenic failure</td>
<td>promotes intracellular cleavage of ubiquitin molecules from ubiquitinated proteins</td>
<td>[46, 92, 95, 105]</td>
<td></td>
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<td>EXLM1Y</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>[100]</td>
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<tr>
<td>UTY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Excluded from involvement in infertility</td>
<td>[100]</td>
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**Table 4. Human Y chromosome AZFb region genes.**

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<th>Homologue</th>
<th>Deletion</th>
<th>Function</th>
<th>References</th>
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<td>CYorf15A</td>
<td>Chromosome Y open reading frame 15A</td>
<td></td>
<td>X-linked</td>
<td></td>
<td></td>
<td></td>
<td>[105]</td>
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<tr>
<td>CYorf15B</td>
<td>Chromosome Y open reading frame 15B</td>
<td></td>
<td>X-linked</td>
<td></td>
<td></td>
<td></td>
<td>[105]</td>
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<tr>
<td>EIF1AY</td>
<td>Eukaryotic translation initiation factor 1A, Y chromosome</td>
<td></td>
<td>X-linked</td>
<td></td>
<td></td>
<td>Essential translation initiation factor, 8 splices variant cytoplasmic proteins involved in protein biosynthesis, enhancing ribosome dissociation and stabilizing Met-tRNA binding</td>
<td>[92, 95, 105]</td>
</tr>
<tr>
<td>KDM5D</td>
<td>Lysine (K)-specific demethylase 5D</td>
<td></td>
<td>X-linked</td>
<td></td>
<td></td>
<td>Histone H3 lysine 4 (H3K4) demethylase, which forms a complex with MSH5 DNA repair factor during spermatogenesis</td>
<td>[105]</td>
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<tr>
<td>GAPD-similar</td>
<td>Similar to glyceraldehyde 3-phosphate dehydrogenase</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>[92]</td>
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<td>Gene (6.2Mb Total)</td>
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<td>Deletion</td>
<td>Function</td>
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<td>SMCY</td>
<td>Selected mouse C DNA Y chromosome</td>
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<td></td>
<td></td>
<td></td>
<td>Ubiquitously expressed, histocompatibility antigen, X-linked homologue escapes X inactivation</td>
<td>[92, 95]</td>
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<tr>
<td>TSPYq1-similar</td>
<td>Testis specific protein, Y encoded, q1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>[92]</td>
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<td>[92]</td>
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<td>USP9Y-similar</td>
<td>Similar to ubiquitin specific protease 9, Y chromosome</td>
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<td></td>
<td></td>
<td></td>
<td>Ubiquitous transcript, X-linked homologue escapes X inactivation</td>
<td>[92]</td>
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<td>RPS4Y2</td>
<td>Ribosomal protein S4, Y linked</td>
<td></td>
<td>X-link, Y-linked</td>
<td></td>
<td></td>
<td>Ribosomal subunit S4</td>
<td>[92, 95, 105]</td>
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<td><strong>Multicopy</strong></td>
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<tr>
<td>BPY2</td>
<td>Basic protein Y 2</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
<td>Testis specific highly charged, putative role in cytoskeletal regulation during spermatogenesis</td>
<td>[94, 95, 105, 106]</td>
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Table 4. (Continued) Human Y chromosome AZFb region genes.
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<tr>
<td>CDY2</td>
<td>Chromodomain protein, Y chromosome, 2</td>
<td>Yellow</td>
<td>yes</td>
<td>yes</td>
<td>Germ cell development, possibly histone to protamine transition</td>
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<tr>
<td>HSFY</td>
<td>Heat shock transcription factor 2, Y chromosome</td>
<td>Blue</td>
<td>X-linked</td>
<td>Heat shock factor DNA binding domain</td>
<td>[92, 95, 105]</td>
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Table 4. (Continued) Human Y chromosome AZFb region genes.

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<th>PRY</th>
<th>PTPN13-like Y-linked</th>
<th>Blue</th>
<th>Rbm</th>
<th>Putative role in apoptosis of germ cells</th>
<th>[94, 95, 105]</th>
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<td>RBMY1A1/YRRM</td>
<td>RNA binding motif Y chromosome 1, subtype A1</td>
<td>Turquoise</td>
<td>yes</td>
<td>RNA binding domain, putative role in storage and transport of mRNA from nucleus during spermatogenesis</td>
<td>[92, 95, 99, 105]</td>
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<td>XKRY</td>
<td>X Kell blood group precursor related protein, Y chromosome</td>
<td>Yellow</td>
<td></td>
<td>Predicted similar to XK, putative membrane transport protein</td>
<td>[92, 95, 105]</td>
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Table 5. Human Y chromosome AZFc region genes.

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Table 5. (Continued) Human Y chromosome AZFc region genes.
Section 1.5 Gonadal Development

Part 1.5.1 Testis Formation and Spermatogonial Proliferation

In 1990 Gubbay et al. reported that the gene responsible for initiating the testis formation cascade in the mouse is the NPYp encoded gene \textit{Sry} (sex determining region, Y chromosome) (Fig. 3) [24, 107, 108]. SRY is expressed between E10.5-E12.0, while its downstream target \textit{Sox9} is expressed from E11.5 onward [109-111].

Once SRY reaches a critical threshold, it initiates the testis formation cascade causing downstream repression of Müllerian duct formation, and stimulation of Wolffian duct formation as well as seminiferous tubule organization in the embryonic gonad (Fig. 17) [27, 112]. Without \textit{Sry}, even if the rest of the Y chromosome is present, the embryonic gonad develops down the female pathway forming Müllerian ducts and ovaries [75].

Simply having a testis and spermatic chord is not enough to produce sperm. Once primordial germ cells reach the gonad and settle on the basal lamina as pro-spermatogonia these germ cells must be signaled to become spermatogonia that will proliferate and differentiate via spermatogenesis eventually forming sperm [23, 32]. The gene responsible for initiating this process was elusive. In the 1980’s and early 1990’s mice with deletions in the NPYp region of the Y chromosome failed to initiate spermatogenesis implying that the important factor, then termed Spy, was on the NPYp. In 2001 Mazeyrat et al. identified this factor as the gene encoding the $\gamma$ portion of eukaryotic translation initiation factor 2 (eIF-2$\gamma$, \textit{Eif2s3y}) [25].
Figure 17. Molecular sex determination cascade in the mouse. For males this cascade is initiated by \( Sry \) and leads to testis development and repression of the female developmental pathway. Assertion to maintains male and female structures continues in the adult. Inspired by the work of David Zarkower.
Mazerat et al. 2001 used Sxr and Sry bearing transgenic mice to determine the role of 
Eif2s3y in spermatogonial proliferation and differentiation. The Sxr\textsuperscript{a} is a portion of the NPY\textsubscript{p} region, which can end up attached to the X chromosome. (Fig. 5) [24, 31, 35]. X\textsubscript{Sxr}\textsuperscript{a}O mice are sex reversed females [68]. Sxr\textsuperscript{b} is a reduction in the gene content of Sxr\textsuperscript{a}, which results in a loss of all Y-linked genes except H2al2y, Sry, Rbmy and a Zfy1/2 fusion gene [25]. X\textsubscript{Sxr}\textsuperscript{b}O mice (Fig. 7) are phenotypically male but have a block in spermatogonial proliferation and differentiation. When Eif2s3y was transgenically added to X\textsubscript{Sxr}\textsuperscript{b}O mice spermatogonia numbers returned to normal and the spermatogonia began to differentiate [25]. X\textsubscript{O}Sry transgenic mice have a phenotype similar to that of X\textsubscript{Sxr}\textsuperscript{b}O males. The addition of an Eif2s3y transgene to X\textsubscript{O}Sry mice rescued the phenotype in the same way as Eif2s3y rescued the X\textsubscript{Sxr}\textsuperscript{b}O phenotype [25].

Part 1.5.2 Non-Y-linked Genes Related to Testis Formation and Spermatogenesis

The Sox9 gene is the downstream target of Sry (Fig. 17) and is expressed from 11.5 dpc onward and therefore has a prolonged signaling time when compared to Sry [113, 114]. Sox9 is also the most prominent downstream gene of the testis formation cascade [114-116]. In the mouse Sox9 is encoded on chromosome 11 and is therefore autosomal (Fig. 2) [117]. It has been shown previously that in XX female mice transgenic for Sox9 (i.e. with Sox9 overexpression) the primitive gonad finishes development with the structure of a testis [118, 119]. Once Sox9 is upregulated, SOX9 continues to promote Sox9 expression as well as upregulate other downstream genes in the cascade (Fig. 17) [114-116, 120-122]. In humans SOX9 is encoded on chromosome 17. In 1999 Huang et al. reported a SOX9 duplication on a single chromosome 17 in a patient [123]. This duplication increased SOX9 expression enough for the child to develop as a phenotypic male despite having an XX genotype [123]. These findings show that if Sox9 is overexpressed during
the sex determination timeframe then the gonad can develop into a testis even when Sry is not present and there are two X chromosomes.

A Sox9 null mutation results in an embryonic lethal phenotype because Sox9 is involved in other important developmental cascades such as chondrogenesis and neural development [124, 125]. In 2011 Lavery et al. reported on an XY Sox9 embryonic loss-of-function mutant specifically losing Sox9 expression in the developing gonad, which avoids the embryonic lethal phenotype [113]. Gonads of these loss-of-function mutants followed the expression patterns similar to XX gonads. Histologically the developing gonads contained meiotic cells and follicular cell signaling, with a distinct lack of Sertoli cells and pro-spermatogonia. Gross anatomy observations of adult loss-of-function mutants revealed external and internal genitalia of a female [113]. These results show that while Sry is responsible for initiating Sox9 expression for testis development, Sry alone is not enough to produce a functional testis.

Eif2s3y is ubiquitously expressed in the mouse [126]. In the testis Eif2s3y expression is necessary for the initiation of spermatogenesis because it is part of spermatogonial proliferation and differentiation regulation [25]. Eif2s3y has 97% amino acid and 86% nucleic acid sequence homology with its X-linked homologue, Eif2s3x (Fig. 2) [126]. Considering the expression level of Eif2s3x in XSexrO and XOSry mice provides another way to interpret the phenotype observed by Mazeyrat et al. 2001 and their observed rescue when Eif2s3y is transgenically added. In the mouse brain Eif2s3x expression in females is higher than in males, because males only carry one copy of Eif2s3x on their one X chromosome [127, 128]. In spite of this EIF2S3 expression is the same in both sexes due to Eif2s3y expression in males, which avoids EIF2S3 haplo-insufficiency [127, 128]. Eif2s3x is also one of a handful of genes to escape X inactivation, providing further evidence that copy number is the cause for an expression differences of Eif2s3x in males and
females [126]. When considering the two homologues together there is a double dose expressed in XX females (two copies of Eif2s3x) and in XY males (one copy of Eif2s3x and one copy of Eif2s3y). Therefore, another cause for the phenotype of XSexrO and XOSry could also be haplo-insufficiency of EIF2S3.

Prevention of haplo-insufficiency or haplo-lethality is a role sometimes played by X-Y gene pairs. Instead of being locked in a battle for dominance the way acquired sex linked genes such as Sly and Slx are, these ancestral X-Y gene pairs work together to ensure sufficient expression dosage regardless of sex chromosome content [4, 52, 89, 90]. X-Y gene pairs have a tendency to be ubiquitously or at least widely expressed and be involved with basic pathways such as transcription, translation and protein stability [89, 90]. The gene pairs date back to the autosomes that diverged into the modern day sex chromosomes in mammals. As the X chromosome was growing and retaining, the Y chromosome was shrinking and decaying, in terms of these autosomal ancestral genes [89]. However, the Y chromosome did keep some ancestral genes, case in point, the EIF pairs (EIF1A in humans and Eif2s3 in mice) [90].

Section 1.6 Prespermatogenesis

This section is a modified excerpt from a grant that was written by the author and funded for a post-doctoral position.

Epigenetic regulation is a vital part of directing a cell’s commitment to a specific fate, especially in pluripotent cells like germ cells. During differentiation, genes that maintain pluripotency must be repressed/methylated and those that direct differentiation toward a specific fate must be activated/acetylated [129]. Although this process is critical throughout the developing embryo, in the germ cell lineage, failure to repress the genes that promote pluripotency and activate
those that lead to spermatogonial stem cell differentiation can lead to tumor formation and/or a failure to establish the spermatogonial stem cell (SSC) population with resulting infertility. Primordial germ cells (PGCs) are first identifiable as they cluster around the future allantois at 7.25-7.5 dpc [130]. PGCs will migrate from the embryonic ectoderm to the genital ridge [130, 131]. In the developing murine embryo, primordial germ cells (PGCs) have migrated into the gonadal tissue by 11.5 dpc. In the fetal testis, PGCs enter prespermatogenesis as mitotic (M) pro-spermatogonia, which proliferate until about 14.5 dpc (Fig. 18). At 14.5 dpc M pro-spermatogonia synchronously enter a cell cycle arrest (G1/G0) as transition 1 (T1) pro-spermatogonia [130, 132, 133]. Between 14.5 and 19.5 dpc extensive epigenetic modifications occur before the pro-spermatogonia return to a mitotically active state as transition 2 (T2) pro-spermatogonia [132, 133]. During this time of epigenetic modification T1 pro-spermatogonia aggregate in the center of the testis cord [131, 134].

The quiescent period of T1 pro-spermatogonia represents an extended interphase. Failure to enter G1/G0 arrest is associated with teratoma formation [135-137], suggesting that this period of epigenetic remodeling may be critical to stabilize the germ cell genome. During this period the DNA in the nucleus is arranged in a non-random manner. In general, heterochromatin aggregates at the periphery of the nucleus, in close apposition to the nuclear envelope (NE), which acts as a physical barrier between cytoplasm and nucleoplasm, and regulates what will leave and enter the nucleus [129, 138, 139]. The NE has a number of sub-layers/structures: the outer nuclear membrane (ONM), inner nuclear membrane (INM), and, internal to the INM, a nuclear lamina (NL) made up of intermediate filament lamins (A/C, B1, B2) [140, 141]. In other cell types, chromatin that is co-localized with nuclear lamins (called lamina associated domains (LADs)) is enriched in methylated chromatin [129, 138, 139, 142]. In general, LADs are spatially close to inactive genes, present on all chromosomes and most likely play a role in epigenetic regulation
and transcriptional repression [129, 142]. Although the localization of heterochromatin with lamins is a basic nuclear pattern, associations between specific LADs and the NL are very dynamic in nature and change with each cell division [129, 138].

**Figure 18. Time line of prespermatogenesis in the mouse.** Pre-Sertoli cell activity is marked in orange along the orange arrow. Germ cell activity is plotted under the general gonocyte arrow (black). M pro-spermatogonia (red arrow) are mitotically active, T1 pro-spermatogonia (green arrow) are quiescent in a G1/G0 cell cycle arrest to undergo epigenetic modification (example green box), T2 pro-spermatogonia (blue arrow) resume mitosis (orange arrow, example orange box) and have a nucleus distinct from that of T1 pro-spermatogonia when not mitotically active (example blue box). T2 pro-spermatogonia will transition into differentiating and undifferentiated type A spermatogonia (purple arrow) for the first wave and spermatogonial stem cell population respectively (example purple box). Scale = 10 µm, applies to all cell images.
Around 1-3 dpp T2 pro-spermatogonia migrate from the center to the periphery of the seminiferous tubule [131, 134, 143]. At the same time T2 pro-spermatogonia also resume mitosis [131]. In mice the first wave of spermatogenesis begins around 3-6 dpp with the T2-prospermatogonia to A₁ spermatogonia transition [134, 144]. T2 pro-spermatogonia will also transition into a primitive type A spermatogonia, the spermatogonia stem cell (SSC). SSCs are about 0.02% of the total testicular germ cell population in the adult mouse [131]. For boys the early stages of the first wave occur during the first 2-3 months with a gradual transition of T2-prospermatogonia to type A_dark and A_pale spermatogonia. Type A_pale spermatogonia will begin a limited transition to type B spermatogonia around age 5 [144].

The PGC to pro-spermatogonia transition is a critical process in development that lays the foundation for fertility in the adult and protects against tumorigenesis [145]. Events that occur during the T1 to T2 pro-spermatogonia transition establish the spermatogonial stem cell population responsible for lifetime fertility in males. Failures in this process lead to early demise of fertility and are believed to be the cause of teratomas and other adult germ cell tumors, presumably due to the inability to repress pluripotency and/or genes associated with differentiation pathways [146-148]. Pure teratomas, which contain cells of all three germ cell layers, make up about 3% of the germ-cell tumor (GCT) cases afflicting adult males and 38% of cases in infants and children [149]. Seminomas, a testicular cancer subcategory identified by its mixed cell population, constitute about half of the GCT population. Of this half a further 50% contain elements of teratomas [148, 150, 151]. Fully 95% of testicular cancers originate from germ cells and GCT is the leading cancer for young men in their reproductive prime, with 7% of cases found among young boys [135, 148, 150-152]. Although testicular cancer has a high survival rate, a patient typically survives at the
expense of one or both testes, along with his fertility and primary natural testosterone source [152, 153].

Section 1.7 Mouse Spermatogenesis

Part 1.7.1 Anatomical Organization

In the mouse testis the seminiferous tubules are generally arranged in a superior to inferior orientation as they loop from pole to pole (Fig. 19 A & C) [29, 154-157]. This gross anatomy structure is ideal for spermatogenesis assessment. A transverse cut along the equator of the mouse testis yields sectioning that contains seminiferous tubules that are predominantly in cross section (Fig. 20). Seminiferous tubules in cross section are the established method for spermatogenesis staging and germ cell counts in mice [19, 20, 29].

Part 1.7.2 First Wave

The very first round of spermatogenesis starts with the first wave around 3-6 dpp [134, 144]. By 10 dpp primary spermatocytes of the first wave are present and by 18 dpp pachytene/secondary spermatocytes can be observed [131]. The first wave is unique as this is the only time that a T2 pro-spermatogonia will directly transition to a differentiated type A₁ spermatogonia (Fig. 18) [143, 158].
Figure 19. **Mouse and human testis anatomy.** A-B: Testis, epididymis and vas deferens from mouse (A) and human (B); fresh tissue is shown in A and plastinated tissue in B. C-D: Diagrammatic representation of human and mouse testis interior. The seminiferous tubules of the mouse have a pole to pole looping swooding structure (C). The human testis is divided into a series of radiating septa that each contain a highly coiled loop of seminiferous tubule (D). Scale = 5 mm in A and 5 cm in B. Image in D was inspired by work of Netter [5].
Figure 20. Mouse testis section panoramic. Section demonstrates how an equatorial cross section of a mouse testis results in seminiferous tubules that are predominantly in cross section.
Part 1.7.3 Germ Cells and the Spermatogenic Cycle

Unlike men, male mice have highly organized and efficient spermatogenesis. Murine spermatogenesis is a cycle that takes ~8.6 days [20, 143, 159]. The time necessary for a germ cell to go from a type $A_{\text{paired}}$ spermatogonia to a spermatozoa (the complete process or duration of spermatogenesis) is about 35 days [20, 159, 160]. The concentric rings of spermatogonia; spermatocytes; and spermatids, both round and elongating/ed, (from periphery to lumen respectively) are arranged in a very dependable manner (Fig. 21) [20, 29]. These cellular associations are consistent as germ cells develop and form the basis of spermatogenesis staging of seminiferous tubule cross sections (Fig. 2). Furthermore, in the mouse, there is typically only one stage per cross section of seminiferous tubule (Fig. 22 A & B) [19, 20, 29, 159].

In general, the progression of germ cell development in the mouse proceeds as follows: type A spermatogonia, intermediate spermatogonia, type B spermatogonia, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, diplotene spermatocytes, secondary spermatocytes, round spermatids, elongating spermatids, elongated spermatids and spermatozoa (Fig. 2) [20, 29, 159]. There are two major subcategories for type A spermatogonia: differentiating ($A_1$, $A_2$, $A_3$, $A_4$) and undifferentiated ($A_{\text{single}}$ ($A_s$), $A_{\text{paired}}$ ($A_p$), $A_{\text{aligned}}$ ($A_{al}$)) (Fig. 2) [130]. Gain of Kit expression marks the transition of undifferentiated to differentiated type A spermatogonia, this expression will persist until meiosis begins [143]. Spermatogonia undergo mitosis to proliferate and differentiate, spermatocytes undergo meiosis to give rise to haploid germ cells and spermatids undergo spermiogenesis to form the spermatozoa which will be spermiated from the seminiferous epithelium to be stored in the epididymis until ejaculation occurs (Fig. 2 and Fig. 19 A & C) [29].
Figure 21. Cartoon diagram of a seminiferous tubule. Demonstrating the concentric rings of developing germ cells supported by the somatic Sertoli cells. Directionality of development is from periphery towards the lumen. Mitotically active spermatogonia (blue cell bodies) proliferate and differentiate into early spermatocytes (green cell bodies) which enter meiosis. Late spermatocytes (red cell bodies) will finish meiosis and transition to spermatids (diploid to haploid respectively). Spermatids undergo spermiogenesis when they will morphologically transition from round spermatids (purple cell bodies) to elongating/ed spermatids (black and white nucleus with red wedge for acrosome) and eventually be spermiated into the lumen as spermatozoa. Sertoli cells (pink nuclei, grey cell bodies engulfing germ cells) are somatic cells that support this entire process.
Figure 22. Diagrammatic representation of mouse and human testis cross-section organization. The mouse seminiferous tubule in cross section typically has only one stage per cross section (A and B) whereas the human has multiple stages per section (C and D). Inspired by work of Amann et al. [9].
Part 1.7.4 Staining Method, Identification Basics, Quantitative Methods

Murine spermatids possess a very hardy acrosome that survives the fixation process intact and is very receptive to Periodic Acid-Schiff (PAS) staining [161, 162]. In general PAS staining is used for demonstrating carbohydrates and glycoproteins, as luck would have it the acrosome of developing spermatids is a carbohydrate rich and morphologically dynamic structure [161]. For decades murine spermatogenesis staging has relied on acrosome morphology with PAS staining and germ cell identification via chromatin structure with hematoxylin staining [19, 20, 29, 163]. Once a seminiferous tubule has been staged and the germ cells within identified and quantified spermatogenesis assessment relies on the gold standard: Sertoli cell ratios, the value of which is discussed in Section 1.9.

Section 1.8 Human Spermatogenesis

Part 1.8.1 Anatomical Organization

The human testis is divided up into a series of 200-300 radiating septa or lobules with the rete testis at the focus. Each septa contains 1-3 highly coiled loops of seminiferous tubule 70-80 cm in length (Fig. 19 B & D) [164, 165]. When human testes are sectioned equatorially, or in most any other orientation, there is a lack of organization to seminiferous tubule appearance (Fig. 22 C & D). Due to the radiating septa structure seminiferous tubules can be found in cross section but also longitudinal section and oblique section (Fig. 23) [9, 162].
Figure 23. Human testis section panoramic. Section demonstrates how an equatorial cross section of a human testis results in seminiferous tubules that are in many orientations (cross, longitudinal and oblique section).
Part 1.8.2 First Wave

In humans, the prepubertal period lasts for about 12 years and begins during the second to third month after birth when spermatogonial stem cells transition to type $A_{\text{dark}}$ and type $A_{\text{pale}}$ spermatogonia. At five years of age some of the type $A_{\text{pale}}$ spermatogonia with undergo mitosis and differentiate to type B spermatogonia [144]. A GnRH (gonadotropin releasing hormone) surge in the hypothalamus initiates puberty in boys around age twelve to fourteen [166]. Full productive spermatogenesis begins at puberty in conjunction with a major rise in testicular testosterone levels [167, 168]. Puberty is usually achieved by age fourteen and is marked by the growth of the testis to greater than or equal to 4 mL and nighttime LH (luteinizing hormone) secretion [169]. The pervading theory is that once the first wave starts the cycle at a specific site of the seminiferous tubule, that site is committed to that particular cycle regardless of the timing at neighboring sites [9, 170].

Part 1.8.3 Germ Cells and the Spermatogenic Cycle

Spermatogenesis is a 16 day cycle for men with a duration that was classically determined to be 64 days but modern methods show it to be closer to 74 days [16, 171-175]. Differentiated diploid germ cell ratios in man are 1:2:4 (type $A_{\text{pale}}$ spermatogonia : type B spermatogonia : primary spermatocytes) in mice this ratio is 1:2:4:8:16:32:64 (type $A_1$ spermatogonia : type $A_2$ spermatogonia : type $A_3$ spermatogonia : type $A_4$ spermatogonia : intermediate spermatogonia : type B spermatogonia : primary spermatocytes) (Fig. 24 A & B) [29, 158, 173, 176-178]. Spermatogonial mitosis only occurs twice per cycle (type $A_{\text{pale}}$ spermatogonia to type B spermatogonia and type B spermatogonia to primary spermatocytes) and is most prevalent during stages II (type B spermatogonia) and V (type $A_{\text{pale}}$ spermatogonia) [172, 173]. Due to the limited rounds of mitosis, daughter cells of a progenitor type $A_{\text{pale}}$ spermatogonia occupy a small site of
the seminiferous epithelium [158, 178]. The limited size of clonal expansion by any one progenitor cell in conjunction with the fact that there is a lack of pressure from neighboring sites to have a synchronous progression of the spermatogenesis along the length of the seminiferous tubule leads to a typical seminiferous tubule cross section containing about two to four stages that may or may not be temporally related (Fig. 22 C & D) [162, 179]. This is divergent from the mouse which has a synchronous progression of the spermatogenesis along the length of the tubule with a site of reversal and the middle of the seminiferous loop farthest from the rete testis (Fig. 19 A & C and Fig. 22 A & B) [29, 180]. For reasons explained in the next part, the human spermatogenesis cycle is classically divided into six stages (Fig. 24 A) [16, 162, 174].

In general spermatogenesis in men is much less efficient than in the mouse [181]. In mice the meiotic index (spermatids per primary spermatocyte) is 2.3-3.1 in humans the meiotic index is 1.3. Another way to look at efficiency is the spermatogenic rate (spermatids per differentiated type A_pale/A_1 spermatogonia) men have a rate of 3.2 while in mice the rate is 44-84 [175]. Again, the limited rounds of mitosis for clonal expansion is part of the cause for lower human efficiency. However, there is also a loss of specific generations of spermatocytes and spermatids that can occur during human spermatogenesis, which also affects efficiency [182]. Some of this loss is due to degeneration of low quality germ cells, but there is also a pervading theory that the carrying capacity of Sertoli cells is also responsible for some of the generational loss. In short spermatogonia over-proliferate to ensure Sertoli cells are supporting spermatogenesis at maximum capacity. Germ cell over proliferation would overburden Sertoli cells except that they allow excessive germ cells to degenerate which in turn lowers overall efficiency [183].
Figure 24. Seminiferous epithelial stages of human and murine spermatogenesis. A: In human, spermatogenesis is divided into 6 stages (I-VI) and 6 spermatid developmental steps. A\textsubscript{dark}, A\textsubscript{pale} and B are type A dark, type A pale and type B spermatogonia, respectively. Pl, L, Z, P, D, M, A and 2\textsuperscript{º} are preleptotene, leptotene, zygotene, pachytene, diplotene, meiotic metaphase, meiotic anaphase and secondary spermatocytes, respectively. Steps of spermatid development are labeled Sa, Sb1, Sb2, Sc, Sd1 and Sd2. Sections were stained with H&E, which is a conventional staining for human testis histology assessment. Scale = 20 µm. B: In mice, spermatogenesis is divided into 12 stages (I-XII) and 16 spermatid developmental steps. A, In, and B are type A, intermediate, and type B spermatogonia, respectively. Pl, L, Z, P, D, M, and 2\textsuperscript{º} are preleptotene, leptotene, zygotene, pachytene, diplotene, meiotic, and secondary spermatocytes, respectively. Steps of spermatid development are numbered 1-16. Sections were stained with PAS-H, which is a conventional staining for staging of mouse testis sections. Scale = 20 µm.
Part 1.8.4 Staining Method, Identification Basics, Quantitative Methods

PAS-H staining is not useful for human spermatogenesis staging due to the delicacy of the human acrosome. Fixation for histological studies is too rough on the human acrosome, which loses integrity and does not resolve well with PAS-H [162, 174]. Thus spermatogenesis staging with human testis sections is typically dependent on germ cell identification using nuclear morphology. The limitations on identification of very specific spermatid steps is what led to the commonly used six stage spermatogenesis system (Fig. 24 A) [1, 8, 162]. Over the decades a number of methods for quantitative analysis of human spermatogenesis have been devised. The most common methods are: cells per unit length circumference, germ cells per Sertoli cells per grams testis homogenate, cells within cellular association per Sertoli cell, and germ cells per unit length (Fig. 25, Fig. 26 and Fig. 27) [1, 2, 8, 17, 181, 183]. Typically testis biopsies are taken for spermatogenesis assessment and not the entire testis via orchidectomy, a biopsy is also very fragile and this delicacy leaves it highly susceptible to irrevocable damage which can further complicate the ability of the histologist to assess spermatogenesis [17, 173].

Section 1.9 Sertoli Cells

Part 1.9.1 Sertoli Cell Development in Rodents

From a molecular perspective it is now a well-established fact that the gene Sry is responsible for initiating testis formation in the undifferentiated gonad of the developing male [24]. However, from a cellular perspective it is the immature or pre-Sertoli cell (pre-Sertoli cell will be used in this text), expressing Sry that is necessary for testis formation [184, 185]. Post-partum Sertoli cells are to be a critically important somatic cell in the germ cell filled seminiferous
tubules. In the adult, Sertoli cells support spermatogenesis thus being instrumental in the production of spermatozoa [186].
Figure 25. Testis biopsy assessment method described by Tjioe and Steinberger. Tjioe and Steinberger modeled their method after the general method described by Chalkley [1, 2]. A light aperture was used to magnify, trace and measure the circumference of seminiferous tubules of interest. Germ cell counts were then within these measure tubules with analysis done using cell counts per unit length of circumference.
Figure 26. Testis biopsy assessment method described by Johnson. A testis biopsy is homogenized and the loose cells spread on a slide. Different germ cell types and Sertoli cells are identified based on morphology. Cell counts are analyzed per gram of tissue homogenized or per Sertoli cell [6].
Figure 26. Testis biopsy assessment method described by Rowley and Heller. Cell counts were either done within a specific region of a cross section of a seminiferous tubule all in the same stage of spermatogenesis; or along a measured length/circumference of seminiferous tubule longitudinal/cross section respectively. In the case of the latter method parameters were established to determine suitability of a seminiferous tubule for quantitative analysis. For both methods germ cells were analyzed per Sertoli cell to give a Sertoli cell ratio (SCR).
Enrico Sertoli first described Sertoli cells in 1865, nearly a century later a debate waged about their embryonic origins. Specifically, whether Sertoli cells originated in the mesonephric tubules, the coelomic epithelium or a mix of both [187]. In 1998 Karl and Capel provided strong evidence for the second theory. A method employing coelomic epithelium cell specific labeling, and time course plotting and immunohistochemistry of labeled cell migration, proliferation and fate showed that coelomic epithelium cells migrate into the developing gonad [188].

The coelomic epithelium is a single cell layer the covers the entire coelomic cavity. The tissue is labile concurrent with the beginning of gonadogenesis (11.5-12.5 dpc), which allows cells to leave the epithelium. Not only do the epithelial cells of the coelomic epithelium migrate into the gonad, a subpopulation ends up specifically in the testis cords. This testis cord subpopulation shows a distinct morphology from coelomic epithelium cells in the gonadal interstitium, which is outside of the testis cord. The morphology of this testis cord subpopulation observed is a cell with elongated shape (cuboidal or columnar) located on the basement membrane [188].

Pre-Sertoli cells first appear at 11.5 dpc (Fig. 18) [189]. The earliest observed time of coelomic epithelium cell migration is 9.5 dpc. Migration continues until 10.5 dpc and by 11 dpc coelomic epithelium cells do not contribute to the pre-Sertoli cell population. The formation of the tunica albuginea is the gross anatomy indicator of cessation of the coelomic epithelium cell migration into the developing gonad (Fig. 19 C) [188]. Murine pre-Sertoli cells are often described as having only one proliferative peak during the fetal to neonatal period. However, they do have the peripubertal proliferative peak that is observed in primates, but in rodents this second peak blends with the first due to the short time period between the peaks (Fig. 18) [186].
During development of the male gonad it is pre-Sertoli cells that differentiate first, thus enabling seminiferous cord formation by pressuring other cell lineages to choose the testis fate [112, 186]. Shortly after 11.5 dpc pre-Sertoli cells will surround germ cells, these aggregates will then be encapsulated by peritubular myoid cells, ultimately forming testis cords [112]. When pre-Sertoli cells first appear at 11.5 dpc they begin their proliferative phase, which peaks at 16 dpc and stops sometime between 12-14 dpp with a significant decrease in proliferation from early post-partum to 9 dpp (Fig. 18) [134, 189, 190]. During this time period pre-Sertoli cells prevent germ cells from beginning meiosis while also inhibiting the differentiation and function of Leydig cells [186]. Vergouwen et al. 1991 described the morphological change of pre-Sertoli cell nuclei moving towards the center of the tubule and becoming droplet shaped from 20 dpc to early post partum. Vergouwen et al.’s observations should follow the morphology described by Nistal et al. 2002 of pre-Sertoli cells as having small, spindle-shaped nuclei arranged in a palisade that have yet to form a lumen[134, 191].

Between 12-14 dpp pre-Sertoli cells begin to mature into Sertoli cells (Fig. 18). During this transition pre-Sertoli cells lose their proliferative ability establishing a static cell population, form inter-Sertoli tight junctions and change their expression patterns [186]. Pre-Sertoli cells secrete anti-Müllerian hormone to block development of the Müllerian duct [26, 192, 193]. Secretion of FSH (follicle stimulating hormone) from the pars distalis increases the pre-Sertoli cell proliferative rates but prompts Sertoli cell secretion of androgen binding protein (ABP) in the adult [26, 194, 195]. Thyroid hormones regulate maturation of pre-Sertoli cells [196, 197]. Sertoli cells secrete inhibin B, which is a Sertoli cell specific product that inhibits FSH secretion [26, 198]. In rat pre-Sertoli cells FSH and T3 induce androgen receptor (AR) expression [199].
Sertoli cells are described as polarized, elongated and attached to the basement membrane with occluding junctions between Sertoli cells and a tripartite nucleolus [26, 29, 200, 201]. They function to physically and metabolically support germ cells during the cycle of spermatogenesis. The number of Sertoli cells has a direct impact on the number of spermatozoa produced because of the germ cell dependence on Sertoli cells [186]. Due to the germ cell morphology changes during spermatogenesis the overall morphology of Sertoli cells follows a related cycle of minor morphological changes [29]. This germ cell dependency is present before spermatogenesis begins as fetal pre-Sertoli cells support primordial germ cells (PGCs) and pro-spermatogonia, and neonatal pre-Sertoli cells support pro-spermatogonia and spermatogonia [186].

Part 1.9.2 The Sertoli Cell in the Adult

Once pre-Sertoli cells have completed formation of the testis it is the non-proliferating Sertoli cells that will support spermatogenesis [8, 186]. Cessation of proliferation in pre-Sertoli cells begins shortly before the onset of the first wave of spermatogenesis, which is supported by Sertoli cells (Fig. 18) [186, 202]. T2-prospermatogonia transition to A1 spermatogonia around 3-6 dpp initiates of the first spermatogenic wave (Fig. 18) [134, 144]. The first wave is dependent on indirect retinoic acid (RA) signaling via the retinoic acid receptor α (RARα) expressed by Sertoli cells [203]. Sertoli cells have a cyclic expression of AR that is tied to integrity of the blood-testis barrier, morphological transition of spermatids during spermiogenesis and spermiation [204]. Sertoli cell secretion of GDNF (glial cell line-derived neurotrophic factor), affects spermatogonial stem cell proliferation [205-207]. Germ cells are supported by Sertoli cells through antioxidant secretion [208].

In humans there is a linear relationship between testis size and sperm count [186, 209]. This is due to the link between testis size and Sertoli cell number along with the Sertoli cells role
in supporting spermatogenesis, thus the more Sertoli cells, the more supported germ cells, the more sperm, the more cellular mass, the larger the testis. As men age, not only germ cell populations decline, so do Sertoli cell populations. A young adult male in his reproductive prime (<30 years old) should have about 500-1350 million Sertoli cells per testis, this number drops to around 300 million after age 50, this number reduces even further to 23-30 million in the case of Sertoli cell only syndrome [165, 181, 210]. This relationship has led to the theory that part of the inefficiency of human spermatogenesis is due to degeneration of germ cells to keep germ cell populations at levels the Sertoli cells can support [9, 181, 183].

In 1971 Mavis Rowley and Carl Heller established the Sertoli cell as the reliable constant for germ cell ratio comparisons in healthy men [8]. Sertoli cell ratios (SCRs) became a gold standard with the caveat that they were not suitable for germ cell assessments on infertile men due to the high level of Sertoli cell number variability in this group [18, 165, 211-213]. Sertoli cells serve as the baseline to germ cell ratios in murine spermatogenesis assessment as well [20, 159, 214].
CHAPTER 2. TWO GENES SUBSTITUTE FOR THE MOUSE Y CHROMOSOME FOR SPERMATOGENESIS AND REPRODUCTION


Section 2.1 Abstract

The Y chromosome is often considered a symbol of maleness as it encodes a gene driving male sex determination, Sry, as well as a battery of other genes important for male reproduction. We previously demonstrated that in the mouse successful assisted reproduction can be achieved when the Y gene contribution is limited to only two genes, Sry and spermatogonial proliferation factor Eif2s3y. Here we show that the function of these two genes can be replaced, and that live offspring can be obtained from male mice without any Y chromosome DNA. Our findings are relevant to discussions on Y chromosome gene function and evolution.

Section 2.2 Introduction

Sexual characteristics depend on sex chromosomes, with females carrying XX and males XY. The presence of the Y chromosome is a prerequisite for male development as it encodes the testis determining gene Sry [107]. The mammalian Y encodes a battery of other genes thought to benefit maleness including broadly expressed genes encoding regulators of transcription, translation and protein stability [4, 89]. Numerous studies have shown that deficiencies in Y chromosome gene content are associated with spermatogenic dysfunction and reduced fertility. In the era of assisted reproduction technologies (ART) most of these defects can be overcome. As long as a haploid gamete is available, fertilization and subsequent embryo development can be achieved. We recently reported that in the mouse only two Y chromosome genes, testis
determinant \textit{Sry} and spermatogonial proliferation factor \textit{Eif2s3y}, are needed for successful assisted reproduction [61]. When the \textit{Sry} and the \textit{Eif2s3y} transgenes were placed in the context of a single X chromosome (X\textsuperscript{E}O\textit{Sry}, \textbf{Table 6}), the resulting mice developed as phenotypic males whose gametes could fertilize oocytes when injected.
Table 6. Summary characteristics of mice used in the study.

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Y gene contribution</th>
<th>Sex determination driver</th>
<th>Spermatogenesis driver</th>
<th>Spermatogenesis</th>
<th>Haploid round spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY</td>
<td>Intact Y</td>
<td>endogenous Sry</td>
<td>endogenous Eif2s3y</td>
<td>normal</td>
<td>frequent</td>
</tr>
<tr>
<td>XOSry</td>
<td>Sry</td>
<td>Sry transgene</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>XOSry</td>
<td>Sry</td>
<td>Sry transgene</td>
<td>Eif2s3y transgene</td>
<td>impaired</td>
<td>rare</td>
</tr>
<tr>
<td>XOSry</td>
<td>Eif2s3y &amp; Sry</td>
<td>Sox9 transgene</td>
<td>Eif2s3y transgene</td>
<td>impaired</td>
<td>rare</td>
</tr>
<tr>
<td>XOSox9, Eif2s3x</td>
<td>Sry</td>
<td>Sox9 transgene</td>
<td>Eif2s3x transgene</td>
<td>impaired</td>
<td>extremely rare</td>
</tr>
<tr>
<td>XOSox9, Eif2s3x</td>
<td>none</td>
<td>Sox9 transgene</td>
<td>Eif2s3x transgene</td>
<td>impaired</td>
<td>extremely rare</td>
</tr>
</tbody>
</table>
Here, we substituted the function of \( Sry \) and \( Eif2s3y \) by transgenic activation of their relatives encoded on other chromosomes and demonstrated that male mice without any Y chromosome DNA generate haploid germ cells functional in assisted fertilization, and sire live healthy offspring.

Section 2.3 Materials and Methods

Part 2.3.1 Chemicals and Media

Pregnant mares’ serum gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Round spermatid and oocyte collection and subsequent manipulation, including microinjections were done in HEPES-buffered CZB medium (HEPES-CZB) [215]. Culture of round spermatid-injected oocytes and embryos was done in CZB medium [216].

Part 2.3.2 Animals

Six-to-twelve week-old B6D2F1 (C57BL/6J x DBA/2) females (NCI, Raleigh, NC) were used as oocyte donors for injections and CD-1 (Charles River, Wilmington, MA) or Swiss Webster (NCI, Raleigh, NC) mice were used as vasectomized males and surrogate/foster females for embryo transfer.

The mice of interest in this study were mice with a single X chromosome (XO) and limited Y gene complement:

1. \( X^{Eif2s3yOSox9} \) (abbreviated as \( X^{EOSox9} \)) are males carrying an autosomally-encoded Sox9 transgene driven by Wt1 (Wills tumor 1) promoter [118] and the X chromosome-
located transgene encoding spermatogonial proliferation factor \textit{Eif2s3y} [217]; the X chromosome carrying the \textit{Eif2s3y} transgene is designated as X\textsubscript{E}. We obtained cryopreserved sperm samples from XY\textit{Sox9} transgenic males from Andreas Schedl (Inserm, France). These samples were used for intracytoplasmic sperm injection (ICSI) to generate live XY\textit{Sox9} males. The X\textsubscript{E}O\textit{Sox9} males were then produced in 3 steps (Fig. 28 A). In step 1, XY\textit{Sox9} males were bred to sex reversed XY\textit{Tdym1} females, which carry an X chromosome and a Y chromosome with an 11-kb deletion removing the testis determinant \textit{Sry} (dl1Rlb) [24, 76]. This resulted in obtaining XY\textit{Tdym1}\textit{Sox9} males, in which sex determination was driven by \textit{Sox9} in the absence of \textit{Sry} but with the remaining Y chromosome genes present. In step 2, the XY\textit{Tdym1}\textit{Sox9} males were crossed to X\textsubscript{E}X\textsubscript{E} females, in which both X chromosomes carried the \textit{Eif2s3y} transgene, in order to produce X\textsubscript{E}\textit{Y}\textit{Tdym1}\textit{Sox9} males. In step 3, the X\textsubscript{E}\textit{Y}\textit{Tdym1}\textit{Sox9} males were bred to X\textsubscript{PafO} (abbreviated as X\textsubscript{P}\textsubscript{O}) [218] carrying the X-linked coat marker Patchy-fur [219].

2. \textbf{XO\textit{Sry}, \textit{Eif2s3x}} are males carrying an autosomally-encoded \textit{Sry} transgene [38] and an autosomally encoded \textit{Eif2s3x} transgene. We first generated mice transgenic for \textit{Eif2s3x}. A bacterial artificial chromosome (BAC) clone containing \textit{Eif2s3x} and 21 kb upstream of its transcription site was placed in pBeloBAC11 vector, resulting in a \~118 kb construct that was used for pronuclear microinjections. Out of 7 founders that were obtained, 6 had germline transmission, and those were further propagated. The males from lines with significant \textit{Eif2s3x} expression in testes and other tissues were used to obtain XX\textit{Eif2s3x} transgenic females. The XO\textit{Sry}, \textit{Eif2s3x} males were then produced in two steps (Fig. 28 B). In step 1, the XX\textit{Eif2s3x} females were bred to XY\textit{Tdym1}\textit{Sry} males that have the X chromosome carrying an \textit{Eif2s3y} transgene [217] and a Y
chromosome with an 11 kb deletion removing the testis determinant Sry (dl1Rlb) [24, 76], complemented by an autosomally-located Sry transgene [Tg(Sry)2Ei] [38]. In step 2, the XY\textsuperscript{Tdym1 Sry,Eif2s3x} males (XY\textsuperscript{Tdym1 Sry} transgenic for autosomally encoded Eif2s3x) obtained in step 1 were bred with X\textsuperscript{P}O females.

3. **XO\textit{Sox9},\textit{Eif2s3x}** are males carrying an autosomally encoded Sox9 transgene driven by Wt1 (Wills tumor 1) promoter [118] and an autosomally encoded Eif2s3x transgene. The XO\textit{Sox9},\textit{Eif2s3x} males were produced by crossing XX\textit{Eif2s3x} females with XY\textsuperscript{Tdym1 Sox9} males and then the resulting XY\textsuperscript{Tdym1 Sox9,Eif2s3x} to X\textsuperscript{P}O females (Fig. 28 C).

In addition to these three primary genotypes of interest, we also examined mice with two Y chromosome genes, X\textsuperscript{Eif2s3yOSry} (abbreviated as X\textsuperscript{E}OSry). These males carry an autosomally-encoded Sry [38] and X chromosome encoded Eif2s3y [217] transgenes, in the context of a single X chromosome. The X\textsuperscript{E}OSry males were produced ‘in house’ by breeding X\textsuperscript{Paf}O or X\textsuperscript{Paf}\textsuperscript{Y*}\textsuperscript{X} females [218] carrying the X-linked coat marker Patchy-fur [219] and X\textsuperscript{Eif2s3y}Y\textsuperscript{Tdym1 Sry} males that have the X chromosome carrying an Eif2s3y transgene [217] and a Y chromosome with an 11 kb deletion removing the testis determinant Sry (dl1Rlb) [24, 76], complemented by an autosomally-located Sry transgene [Tg(Sry)2Ei] [38].

The breeding crosses yielded a variety of progeny genotypes; the males of interest were identified among the progeny by genotyping for Y chromosome markers, scoring fur appearance, and evaluation of testes size. All mice with limited Y gene complement were on partial MF1 genetic background. The XY\textsuperscript{RIII} on MF1 background and XY B6D2F1 males were used as wild-type controls; Y\textsuperscript{RIII} chromosome is the strain of Y chromosome from which Sxr\textsuperscript{a} and Sxr\textsuperscript{b} derive.
Figure 28. Breeding themes. The breeding themes applied to produce males of interest, shown in yellow filled boxes: X^E^O_Sox9 (A), XOSry,Eif2s3x (B) and XOSox9,Eif2s3x (C) are shown. Females are depicted in pink and males in blue font. X and Y represent normal sex chromosomes. X^E^ is an X chromosome carrying the Eif2s3y transgene. X^P^ is an X chromosome carrying the coat marker Patchy-fur. Y^Tdyn1^ is a Y chromosome with the Sry deleted. Eif2s3x, Sry, Sox9 represent autosomally encoded transgenes. The offspring from the crosses were recognized by a combination of genotyping methods involving PCR, qPCR, testis size and fur appearance. Crossed genotypes do not survive to birth.
The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22°C, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawai‘i and guidelines presented in National Research Council’s (NCR) “Guide for Care and Use of Laboratory Animals” published by Institute for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawai‘i.

Part 2.3.3 Testicular Material Collection and Preparation

Testes were collected twice from each male following initial semi-castration. Each dissected testis was weighed, photographed if needed, and divided into 4 parts: half for Bouin fixation and subsequent sectioning, one-fourth for preparation of testicular cell suspension for injections, and one-fourth for storage at -80°C and subsequent molecular or cytogenetic analyses.

Part 2.3.4 Histology Analysis

For histology analysis, part of the testes was fixed in Bouin overnight and then stored in 70% ethanol prior to embedding in paraffin wax, sectioning at 5 µm, and staining with hematoxylin-eosin (H&E) and Periodic acid Schiff and hematoxylin (PAS-H). The stages of seminiferous tubules were identified based on the composition of cells near the basal membrane according to the method described by Ahmed [220]. This was necessary because of meiotic and post-meiotic arrests present in males with limited Y gene complement, which prevented staging based on the changes of acrosome and nuclear morphology of spermatids.

Part 2.3.5 Round Spermatid Injection (ROSI) *

This part can be found in Chapter 8, Section 1 Part 1.
Part 2.3.6 ROSI Progeny Analyses *

This part can be found in Chapter 8, Section 1 Part 2.

Part 2.3.7 Transgene Copy Number *

This part can be found in Chapter 8, Section 1 Part 3.

Part 2.3.8 Real-Time RT-PCR *

This part can be found in Chapter 8, Section 1 Part 4.

Part 2.3.9 Statistical Analyses

Fisher's Exact Test was used to assess the differences between the genotypes for ROSI data and to analyze the progeny genotype frequencies. One-way ANOVA with post-hoc test was used to analyze body weight data. Student's t-test was used for all other analyses.

Section 2.4 Results

Part 2.4.1 Replacement of Sry Function by Transgenic Activation of Sox9 *

In most mammals, including humans, Sry is the leading gene in the sex determination pathway, which acts in developing gonads and induces the development of testes [24, 107]. Sox9 (Sry-related HMG box gene 9), encoded on chromosome 11, is a direct target of SRY [115] and plays a pivotal role in male sexual development. In both mice and humans, ablation of Sox9 causes male-to-female phenotypic sex reversal [221-223] while Sox9 gain-of-function results in male gonad differentiation [118, 123]. Transgenic overexpression of Sox9 driven by Wt1 (Wilms Tumor 1) promoter leads to sex reversal, with XX mice developing as males; these XXSox9 males have testes devoid of germ cells due to a toxic dosage of X chromosome genes and the lack of the Y chromosome genes critical for spermatogonial proliferation [118]. We placed the Wt1-Sox9 transgene in the context of a single X chromosome carrying the Eif2s3y transgene (Fig. 28 A).
The resulting $X^E OS_{ox9}$ males (Table 6) had testis determination driven by transgenic overexpression of $Sox9$ and the Y chromosome gene contribution limited to $Eif2s3y$. These mice had testes smaller than wild-type XY and $X^E OS_{ry}$ males (Fig. 29). The analysis of testicular sections confirmed that spermatogenesis was ongoing allowing the development of germ cells with round spermatid-like morphology in all examined males (10/10). The numbers of spermatids were low and they arrested at step 7 of development (Fig. 30 D). The quantitative analysis of spermatogenesis progression showed that spermatogonia/Sertoli cell and round spermatid/Sertoli cell ratios in $X^E OS_{ox9}$ males were similar to those observed in $X^E OS_{ry}$, but 2- and 8-fold lower, respectively, than in XY (Fig. 30 G & H, Table 7). We also observed a variety of defects in seminiferous tubules (Fig. 31 A), resembling those reported earlier for $X^E OS_{ry}$ males [61].

ART and fertility analysis from this part can be found in Chapter 8, Section 1, part 5.

We conclude that for the production of spermatids competent in assisted reproduction transgenic $Sox9$ activation can substitute for the lack of $Sry$ in mice without the Y chromosome but transgenic for $Eif2s3y$.

Part 2.4.2 Replacement of $Eif2s3y$ Function by Transgenic Overexpression of $Eif2s3x$

Mice with one X chromosome and transgenic for $Sry$ (XOSry, Table 6) develop testes populated with spermatogonia, first-line germ cells from the mitotic phase of spermatogenesis. In the absence of other Y chromosome genes these spermatogonia undergo proliferation arrest, and meiotic and post-meiotic stages of spermatogenesis are absent (Fig. 30 B). This spermatogonial proliferation block can be overcome by transgenic $Eif2s3y$ addition [61, 217] (Fig. 30 C).
Figure 29. Testis weight in males with limited or no Y gene contribution. Four types of males with limited or no Y gene complement (tested; X^{OSry}, X^{O Sox9}, X^{Sry,Eif2s3x} and X^{Sox9,Eif2s3x}) were compared to wild-type XY males (control). Graph bars represent the average testis weight, with n=8, 6, 20, 80, 106 testes for XY, X^{OSry}, X^{O Sox9}, X^{Sry,Eif2s3x} and X^{Sox9,Eif2s3x}, respectively. Statistical significance (t-test): a different than all others at P<0.05. Error = SEM.
Figure 30. Testis histology analysis. (A-F) Exemplary tubules of PAS-H stained sections of testis from wild-type XY (A, positive control), XOSry (B, negative control), and males with limited Y gene complement X^{E}{Sry}, X^{E}{O}{Soya}, XOSry,Eif2s3x and XOSox9,Eif2s3x (tested, B-F). XY males have normal spermatogenesis with expected germ cell types present, including step 16 spermatids with developed tails (inset, A). XOSry males have spermatogonial proliferation arrest resulting in tubules devoid of germ cells except for occasional normal (inset) and abnormal (*) spermatogonia (B). The remaining genotypes have meiotic and postmeiotic arrests that occasionally allow formation of round spermatids (insets), arresting at step 7. All tubules are of stage VII-VIII except for XOSry which could not be staged due to lack of germ cells. Scale bar = 50 µm; insets, x3 magnification. (G & H). Quantitative analysis of spermatogenesis progression. For each male 10 tubules were examined per stage and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data are expressed as germ cell/Sertoli cell ratios. Bars represent the average ± SEM, with n shown under the X axis. Statistical significance (t-test): (G): a different than all other (P<0.05), ** P<0.01; (H) bars with different letters are significantly different (P<0.05).
Figure 31. Testis histology - abnormalities. Exemplary tubules from $X^E OSox9$ (A), $XOSry,Eif2s3x$ (B) and $XO Sox9,Eif2s3x$ (C) showing various spermatogenic defects, such as residual body (RB), sloughed Sertoli cell (SS), vacuole (V), hypercondensed nuclei (HN), apoptotic cell (A), and apoptotic metaphase (AM). All tubules are stage XII-I. Scale = 50 µm.
*Eif2s3y* belongs to the eukaryotic translation initiation factor 2 subunit 3 protein family. It has an X chromosome encoded homologue, *Eif2s3x*, with which it shares high sequence identity, 86% at nucleic acid and 98% at protein level [126]. Both genes are ubiquitously expressed with strong expression in germ cells and represent a typical ancestral, single copy X-Y homologous gene pair, in which the X gene escapes X chromosome inactivation, and both X and Y copies are widely expressed in males [4, 89]. We therefore hypothesized that if expression of *Eif2s3x* is high enough, it could substitute for *Eif2s3y* and replace its function during the earliest phase of spermatogenesis. We produced mice transgenic for *Eif2s3x* using pronuclear microinjection and a bacterial artificial chromosome (BAC) clone encoding *Eif2s3x* and 21 kb upstream of its transcription site. We obtained 6 transmitting lines (ESX1,2,4,5,6,7), all with the transgene incorporated on an autosome. The analysis of *Eif2s3x* expression in testes from F1 and F2 transgenic XY males revealed that males of the ESX1,2,6&7 lines had elevated Eif2s3x levels (Fig. 32). The *Eif2s3x* transgene was incorporated as a single copy in the ESX2,6,7 lines, and as 3 copies in the ESX1 (Fig. 33). The *Eif2s3x* transgene from the ESX1 line, which resulted in the strongest *Eif2s3x* overexpression, was placed in the context of XOSry through selective breeding (Fig. 28 B). The resulting XOSry,*Eif2s3x* males (Table 6) had the Y chromosome contribution limited to one gene, Sry.

Testis size in XOSry,*Eif2s3x* males was similar to that of X^E^OSox9 males but smaller than in XY or X^E^OSry (Fig. 29). In all males examined (13/13) the spermatogonial proliferation block was overcome and spermatogenesis progressed through meiosis up to step 7 of spermatid development (Fig. 30 E). The spermatogonia/Sertoli cell ratio was similar to that observed in X^E^OSry (Fig. 30 G). Round spermatids, however, were dramatically depleted, with
spermatid/Sertoli cell ratio 10- and 88-fold lower than in $X^E O S r y$ and XY, respectively (Fig. 30 H, Table 7). The defects in seminiferous tubules were observed as for $X^E O S o x 9$ (Fig. 31 B).

ART and fertility analysis from this part can be found in Chapter 8, Section 1, part 5.

We conclude that overexpression of $E i f 2 s 3 x$ can substitute for the lack of $E i f 2 s 3 y$ and initiate spermatogenesis in the $X O S r y$ context. Moreover, our data suggest that the role of $E i f 2 s 3 x / y$ during spermatogenesis is not limited to spermatogonial proliferation and differentiation, but extends to meiotic progression.

**Figure 32.** $E i f 2 s 3 x$ transcript levels in XY males. Pronuclear microinjection with $E i f 2 s 3 x$ transgene resulted in 7 founders (F0, ESX1-7). Six of them transmitted the transgene to next generations (F1 & F2). XY transgenic males provided tissues (testis, spleen or kidney) for $E i f 2 s 3 x$ transcript level quantification by real-time PCR, with actin as a loading control, and primers recognizing both the endogenous and transgenic $E i f 2 s 3 x$. The controls were non-transgenic XY siblings. Values are mean ± SEM. The number of males examined is shown under each bar. $E i f 2 s 3 x$ transcript levels remained at the level comparable to controls in lines ESX4&5, were slightly elevated in lines ESX2,6&7, and were strongly elevated in line ESX1.
Figure 33. Transgene copy number in *Eif2s3x* transgenic lines. *Eif2s3x* transgene copy number was assessed by quantitative PCR after amplification of three single copy X chromosome genes *Eif2s3x*, *Amelx* and *Prdx4*, with *Atr* used for normalization. The primers for detection of *Eif3s3x* were designed to recognize both endogenous and transgenic copies. Control are non-transgenic XO females, in which only one copy of all genes is expected. ESX1, 2, 6 & 7 are XOSry and XO mice transgenic for *Eif2s3x*. All transgenic mice had a single copy of *Prdx* and *Amelx*, as expected. Transgenic lines ESX2, 6, 7 were scored as having 2 copies of *Eif2s3x* (1 endogenous and 1 transgenic). Transgenic ESX1 showed 4 copies of *Eif2s3x* (1 endogenous and 3 transgenic). The final gene copy number was established using a typical mathematical rounding, i.e. readout <1.5 was scored as 1 and readout >/=1.5 as 2. Values are mean ± SEM, with n= 4, 10, 5, 3, 4 for Control, ESX1, ESX2, ESX6, and ESX7, respectively.
Part 2.4.3 Spermatogenesis and Germ Cell Function in Males with No Y Chromosome DNA *

Having demonstrated that functional substitutions of Sry-to-Sox9 and Eif2s3y-to-Eif2s3x are possible, and males with single Y chromosome genes (either Sry or Eif2s3y) can produce haploid gametes and yield live offspring, we tested whether spermatogenesis can take place in males with a complete absence of Y chromosome genes. We generated males transgenic for Sox9 and Eif2s3x in the XO context (XOSox9,Eif2s3x, Fig. 28 C) using the same transgenes that were successful in single Y gene substitutions (Wt1-Sox9 and Eif2s3x ESX1). The testes from XOSox9,Eif2s3x males were smaller than those from the other genotypes (Fig. 29) and were variable in morphology, with some males having relatively normal testes, except for their reduced size and roundish rather than oval shape (Fig. 34 B & D), and other males (XOSox9,Eif2s3x-alt) having severely disfigured testes with patchy discoloration throughout, an abundance of interstitial tissue, and a low number of seminiferous tubules essentially devoid of germ cells, except for a few spermatogonia (Fig. 34 C, E & G). These latter testes likely developed from ovotestes as they showed an elevated expression of ovarian markers, FoxL2, Rspo1, and Wnt1 (Fig. 35).

In testes from all examined XOSox9,Eif2s3x males (10/10) spermatogonial proliferation arrest was overcome (Fig. 30 F) and spermatogenesis progression was comparable to XOSry,Eif2s3x males, with a spermatogonia/Sertoli cell ratio similar to that of XESry, a very significant depletion of round spermatids (Fig. 30 F, G & H, Table 7), and the presence of defects in the seminiferous epithelium (Fig. 31 C).
Figure 34. Testis morphology in XOSox9,Eif2s3x males. Morphology of the testis from a wild-type male with an intact Y chromosome (XY; A) and males with no Y chromosome genes (XOSox9,Eif2s3x; B&C). D&E are panoramics of cross sections of testes shown in B and C, respectively. The male whose testis is shown in B&D represents "good" XOSox9,Eif2s3x males, in which the seminiferous tubules contain germ cells indicative of ongoing spermatogenesis (F). The male whose testis is shown in C&E represents XOSox9,Eif2s3x-alt males, in which the seminiferous tubules resemble those of XOSry, with few spermatogonia present and no spermatogenesis (G). Scale is 1 mm (A-C, shown in C) and 500 μm (D&E, shown in E), and 50 μm (F&G,
Figure 35. Transcript levels of ovary-specific genes in testes from males with no Y chromosome genes. Testes were obtained from two groups of males with no Y gene complement, XOSox9,Eif2s3x representing males with ongoing spermatogenesis and XOSox9,Eif2s3x-alt with abnormal testes and no spermatogenesis (see Fig. 34). Ovaries from wild-type females (XX) and testes from wild-type males (XY) served as controls. Transcript levels of FoxL2 (A), Rspo1 (B) and Wnt1 (C) were quantified by real-time PCR, with Actin or Sdha as loading controls. Values are mean ± SEM, with n=3-4 males per group, Statistical significance (t-test, P<0.05): * different than XX, † different than XY, * P<0.05.
Table 7. Quantitative analysis of spermatogenesis progression.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spermatogonia/Sertoli cell ratio (mean±SEM)</th>
<th>XII-I</th>
<th>II-IV</th>
<th>V-VI</th>
<th>VII-VIII</th>
<th>IX-X</th>
<th>XI</th>
<th>All stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY</td>
<td></td>
<td>0.41±0.11</td>
<td>1.42±0.13a</td>
<td>2.38±0.61</td>
<td>0.11±0.02</td>
<td>0.20±0.01</td>
<td>0.27±0.02</td>
<td>0.79±0.08a</td>
</tr>
<tr>
<td>X&lt;sup&gt;E&lt;/sup&gt;Sry</td>
<td></td>
<td>0.30±0.07</td>
<td>0.79±0.08</td>
<td>1.17±0.44</td>
<td>0.07±0.01</td>
<td>0.08±0.00</td>
<td>0.10±0.01</td>
<td>0.41±0.09</td>
</tr>
<tr>
<td>X&lt;sup&gt;E&lt;/sup&gt;Sox9</td>
<td></td>
<td>0.15±0.02a</td>
<td>0.70±0.06a</td>
<td>1.15±0.12a</td>
<td>0.02±0.01</td>
<td>0.07±0.02</td>
<td>0.20±0.02</td>
<td>0.39±0.03a</td>
</tr>
<tr>
<td>XO Sry, Eif2s3x</td>
<td></td>
<td>0.34±0.03</td>
<td>0.92±0.08a</td>
<td>1.45±0.07a</td>
<td>0.05±0.01a</td>
<td>0.12±0.03</td>
<td>0.21±0.03</td>
<td>0.52±0.03a</td>
</tr>
<tr>
<td>XO Sox9, Eif2s3x</td>
<td></td>
<td>0.28±0.03</td>
<td>0.94±0.08</td>
<td>1.52±0.13a</td>
<td>0.03±0.00a</td>
<td>0.07±0.01</td>
<td>0.17±0.04</td>
<td>0.51±0.03a</td>
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</table>

<table>
<thead>
<tr>
<th>Round spermatid/Sertoli cell ratio (mean±SEM)</th>
<th>XII-I</th>
<th>II-IV</th>
<th>V-VI</th>
<th>VII-VIII</th>
<th>IX-X</th>
<th>XI</th>
<th>All stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY</td>
<td>8.03±0.45a</td>
<td>14.72±2.04a</td>
<td>12.48±1.08a</td>
<td>13.21±0.95a</td>
<td>0</td>
<td>0</td>
<td>7.88±0.50a</td>
</tr>
<tr>
<td>X&lt;sup&gt;E&lt;/sup&gt;Sry</td>
<td>0.40±0.02a</td>
<td>1.39±0.45a</td>
<td>1.08±0.10a</td>
<td>1.43±0.36a</td>
<td>0.85±0.13**</td>
<td>0.34±0.07**</td>
<td>0.92±0.15a</td>
</tr>
<tr>
<td>X&lt;sup&gt;E&lt;/sup&gt;Sox9</td>
<td>0.27±0.03a</td>
<td>0.68±0.13a</td>
<td>1.01±0.16a</td>
<td>0.83±0.16a</td>
<td>0.81±0.25a</td>
<td>0.50±0.15a</td>
<td>0.67±0.12a</td>
</tr>
<tr>
<td>XO Sry, Eif2s3x</td>
<td>0.04±0.02</td>
<td>0.08±0.04a</td>
<td>0.11±0.04a</td>
<td>0.16±0.10b</td>
<td>0.06±0.02b</td>
<td>0.05±0.02b</td>
<td>0.09±0.04a</td>
</tr>
<tr>
<td>XO Sox9, Eif2s3x</td>
<td>0.06±0.02</td>
<td>0.13±0.04a</td>
<td>0.24±0.06b</td>
<td>0.28±0.06a</td>
<td>0.18±0.04c</td>
<td>0.13±0.03a</td>
<td>0.16±0.04a</td>
</tr>
</tbody>
</table>

For each male 10 tubules were examined per stage and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data are expressed as germ cell/Sertoli cell ratios. In wild-type males no round spermatids are present in stages IX-XI so those observed in the remaining genotypes represent ‘delayed spermatids’. Statistical significance (t-test): * different than XY; & different than all other, a,b,c columns with different superscript letters are significantly different. The number of males per genotype was n=3, 3, 8, 13, 10 for XY, X<sup>E</sup>Sry, X<sup>E</sup>Sox9, XO Sry, Eif2s3x, XO Sox9, Eif2s3x.
ART and fertility analysis from this part can be found in Chapter 8, Section 1, part 5.

The data support that males entirely devoid of the Y chromosome genes are able to produce haploid gametes and yield progeny when these cells are used for assisted fertilization. However, simultaneous Sry-to-Sox9 and Eif2s3y-to-Eif2s3x substitutions leads to problems with both testis development and spermatogenesis initiation so that only a subset of XOSry,Eif2s3x males were able to sire offspring with the help of ART.

Part 2.4.4 Healthy and Normal Progeny from Males with a Single or with No Y Chromosome Genes *

This part can be found in Chapter 8, Section 1 Part 5.

Part 2.4.5 Relationship Between Spermatogenesis and Eif2s3x/y Expression

Males carrying the Eif2s3x transgene had significantly depleted round spermatid number when compared to Eif2s3y transgensics (Fig. 30 H, Table 7). This suggests that Eif2s3y and Eif2s3x genes may differ functionally and/or the observed variety of spermiogenic phenotypes are due to the differences in the global level of Eif2s3 genes/transgenes. In order to relate spermatogenesis progression to Eif2s3x/y expression, we independently quantified Eif2s3x, Eif2s3y, and both (Eif2s3x/y), in mice of interest (Fig. 36 H-J). Mice transgenic for Eif2s3y and for Eif2s3x had their respective transgenic transcript levels significantly elevated when compared to XY (Fig. 36 H & I). For global Eif2s3x/y quantification males transgenic for Eif2s3y had higher Eif2s3x/y levels than males transgenic for Eif2s3x (Fig. 36 J). The drop in fold overexpression in males transgenic for Eif2s3x (compare Fig. 36 I & J) was due to Eif2s3y transcripts being ~7-times more abundant than Eif2s3x in wild-type testes [224] and was not present when the data were normalized to XOSry (Fig. 37). The higher global Eif2s3x/y expression in X^{E}OSry and X^{E}OSox9 is consistent with the
higher incidence of round spermatids in these males (relate Fig. 36 J to Fig. 30 H). However, such elevated \textit{Eif2s3x/y} expression is clearly not essential for normal spermatogenesis since the levels of \textit{Eif2s3x/y} in XY males were lower than in either transgenic model, raising the question of how spermatogenesis would progress in the XO context with fewer transgenic copies of either \textit{Eif2s3y} or \textit{Eif2s3x}. For the \textit{Eif2s3y} transgenics, we had only one line available, with 10 copies of the transgene \cite{ref}. However, among the \textit{Eif2s3x} transgenics, mice from lines ESX2 and ESX7 carried a single copy of the transgene (Fig. 33) and had lower \textit{Eif2s3x} expression in the XY context (Fig. 32). We placed these \textit{Eif2s3x} transgenes in the XO\textit{Sry} context, and examined testes from the resulting XO\textit{Sry},\textit{Eif2s3x} males (ESX2 and ESX7). The \textit{Eif2s3x} transcript levels in the ESX2 and ESX7 groups were respectively 2.4- and 2.9-fold lower than in the ESX1 group of mice that produced round spermatids (Fig. 36 E). The spermatogonial proliferation arrest was overcome in ESX2 and ESX7 mice and no differences in spermatogonia/Sertoli cell ratio were observed compared to the ESX1 mice (Fig. 36 B, D & F). However, no round spermatids were observed in ESX2 or ESX7 males (Fig. 36 B, D & G).

Altogether, our data support that \textit{Eif2s3x} can functionally replace \textit{Eif2s3y} role during spermatogenesis but only if sufficiently overexpressed. We also conclude that in males lacking the Y chromosome spermatogonial proliferation and spermatogenesis initiation can efficiently take place within a broad window of \textit{Eif2s3x/y} expression. However, progression through meiosis is more sensitive and requires higher \textit{Eif2s3x/y} transcript levels.
Figure 36. Relationship between Eif2s3 expression and spermatogenesis progression. (A-D) Exemplary tubules of PAS-H stained sections of testis from XOSry,Eif2s3x males lines ESX2 (A&B) and ESX7 (C&D). Spermatogonial proliferation arrest was overcome and spermatogenesis was initiated (A&C) but contradictory to line ESX1 (Fig. 30 E & H, Table 7), no round spermatids could be found (B&D). Scale bar, 50 µm; (E) Eif2s3x (endogenous and transgenic) transcript levels in testes from XOSry,Eif2s3x males (ESX1-A, ESX1-B, ESX2 and ESX7). (F-G) Spermatogenesis progression in males shown in E. For each male 10 tubules were examined per stage and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data are expressed as germ cell/Sertoli cell ratios. (H-J) Transcript levels of endogenous and transgenic (H) Eif2s3y; (I) Eif2s3x; and (J) Eif2s3x/y. The same males were used in H-J except that in (H) XY males were included as a reference control and XOSry,Eif2s3x and XOSox9,Eif2s3x were omitted. Transcripts were quantified by real-time PCR with Actin as a loading control. XOSry males were a reference control in E,I&J and XY in H. Statistical significance (t-test, P<0.05): bars with different letters are different. Bars are mean ± SEM, with n shown under the X axis.
Figure 37. Relationship between Eif2s3 expression and spermatogenesis progression. Transcript levels of endogenous and transgenic (A) Eif2s3y; (B) Eif2s3x; and (C) Eif2s3x/y. Transcripts were quantified by real-time PCR with Actin as a loading control, and XY (A) and XOSry (B&C) males serving as reference controls. The data in (A) are the same as in Fig. 36 H and is shown here for comparison. The data in (B) and (C) are the same as in Fig. 36 I & J but the normalization here is done with XOSry instead of XY which was used in Fig. 36 I & J. Statistical significance (t-test, P<0.05): bars with different letters are different. Bars are mean ± SEM, with n shown under the X axis.
Section 2.5 Discussion

We have shown that a male mouse without any Y chromosome genes can generate haploid gametes and father offspring with the help of assisted fertilization. This indicates that pathways guiding development and fertility of a male are not exclusively directed by the Y chromosome genes, and that alternative mechanisms regulated by genes encoded elsewhere in the genome exist and can take over under certain circumstances. Here, we revealed two of such strategies by genome manipulation.

Transgenic activation of Sox9 is one of many examples of an alternative pathway driving sex determination. Manipulation of expression of other genes from the SOX family (Sox3, Sox8, and Sox10) as well as other genes (e.g. Dax1, Dmrt1/2, Rspo1, Fgfr2, FoxL2, Cbx2, Map3k1/4, Igfr1, and others) have both been shown to result in sex fate change (reviewed in [112, 225, 226]). A surrogate sex determination mechanism can also be activated without human manipulation. A few rodent species, mole voles [227] and spiny rats [228], have lost the Y chromosome and Sry. Although the exact mechanism of sex determination in these animals is not clear, additional copies of the Cbx2 gene detected in spiny rat species might be responsible [229]. Disorders of sex development (DSD) in humans involve mutations of many of the aforementioned genes (reviewed in [230]) implying that sex determination pathways and players are for the most part conserved. The mutations in humans, however, are almost exclusively associated with sterility.

The second strategy that we revealed was achieved by manipulation of the Eif2s3x expression in order to substitute for the lack of Eif2s3y. Eif2s3y and Eif2s3x represent a typical ancestral, single copy X-Y homologous gene pair, and it has been previously proposed [61, 217] that their function may be interchangeable, as has already been shown for two human,
RPS4Y1/RPS4X and DDX3X/Y [231, 232] and one mouse, Uty/Utx [233-235] ancestral X-Y gene pairs. Our data support that this is true to some extent for the Eif2s3x/y gene pair: the X gene can take over the function of the Y gene in spermatogenesis initiation. For progression through meiosis, in the context of no Y chromosome, at least 3 copies of the Eif2s3x transgene are necessary and the global level of the Eif2s3x/y transcripts must reach a certain threshold. With the currently available mouse models we cannot explicitly distinguish whether Eif2s3y and Eif2s3x genes are functionally equivalent (at 1 copy per genome). This would require producing XOSry mice with fewer copies of the Eif2s3y transgene that would be more comparable to existing Eif2s3x transgenics (carrying 1 or 3 copies). The final clarification of the roles of both homologues in spermatogenesis could be acquired through transgenic rescue experiments in mice engineered to have Eif2s3y or Eif2s3x independently inactivated in male germ cells.

Our data also suggest that the Eif2s3x/y may play a role in gonad formation. The Wt1:Sox9 transgene is not as efficient as Sry in driving male pathway and in the XX fetuses often induces development of ovotestes [119]. Here, when sex determination was driven by Wt1-Sox9, severe abnormalities of mature testes, indicative of impaired gonadal development, were observed in XO males transgenic for Eif2s3x but not in XO males transgenic for Eif2s3y. Since the global Eif2s3x/y expression is lower in the former males, this suggests that a certain threshold of Eif2s3x/y may be required for proper testis differentiation. Investigations of the processes taking place during the sex determination window in the genotypes used in this study would clarify the interplay between the sex determinants (Sry and Sox9) and the Eif2s3x/y genes, although the distinct transcript levels from the Eif2s3y and Eif2s3x transgenes might be a limitation. Altogether our analyses of Eif2s3x/y gene pair expression viewed in the context of gonadal and germ cell development indicate that the dosage of these genes is of tremendous importance for spermatogenesis. It will
now be a prerogative to explore the mechanism whereby \textit{Eif2s3x/y} exert their functions, and whether they play a general role in protein translation in the cells of the developing and mature testis, or are involved in some gonad-specific pathways.

\textit{Eif2s3y} is conserved on the Y chromosome in eutherians but is not present in simian primates, including humans [126]. Considering its important role in spermatogenesis: how do species lacking \textit{Eif2s3y} handle the loss? Comparative gene mapping studies have provided evidence that Y gene loss can be compensated by gene transposition to an autosome, and that this mechanism is widespread among mammals [90]. A \textit{EIF2S3} copy with testis specific expression was identified in human [126] and in several primates, with the phylogenic analysis indicating that the \textit{EIF2S3} retrogenes originate from the X chromosome and arose independently at least 3 times during evolution indicating strong purifying selection [90]. In the Japanese spiny rat, which has lost its Y chromosome, four ancestral Y chromosome genes, including \textit{EIF2S3Y}, have been identified in the genome, transposed either to X or to an autosome [236, 237].

It is generally believed that widely expressed genes on the human Y chromosome, with X-homologues that escapes X-inactivation, are dosage sensitive. It has recently been proposed that these X and Y gene pairs are haplo-lethal, and that the human Y chromosome is essential to male viability [89]. We did not observe developmental defects interfering with viability in mice with no Y chromosome genes. Indeed, when haploid gametes were formed, the success of embryo development to term was similar to that of mice with an intact Y chromosome. Mice and humans differ in respect to ancestral gene retention and behavior, with mice having fewer ancestral genes on the Y (9 vs. 17) and fewer that escape sex chromosome inactivation (4/9 vs. 12/17) [89]. Moreover, while X monosomy in humans (XO, Turner syndrome) is predominantly embryonic lethal with surviving individuals often having detectable sex chromosome mosaicism [238, 239],
XO mice are viable and fertile, although less efficient in breeding than XX females. These differences likely explain why lack of the Y chromosome in mice did not affect embryo viability.

Our demonstration that offspring can be obtained from males with no Y chromosome genes shows that for assisted reproduction in the mouse, the Y chromosome is no longer necessary. However, there is extensive evidence from both phenotype characterization [7, 58, 240] and genomic analyses [4, 89, 90] that unequivocally supports the importance of Y chromosome genes for normal, unassisted fertilization. So, while our data suggest that in some cases it is possible to bypass the requirement for the Y chromosome in male reproduction, the Y clearly remains the genetic determinant of full natural masculinity.

Section 2.6 Contributions

Dr. Monika A. Ward – P.I., lead author
Dr. Jonathan M. Riel – all expression data*
Dr. Yasuhiro Yamauchi – all ART related work
Eglė Ortega - *exception, expression on FoxL2, Rspo1 and Wnt1
Chapter 3. Mouse Y-encoded transcription factor Zfy2 is essential for sperm formation and function in assisted fertilization


Section 3.1 Abstract

Spermatogenesis is a key developmental process allowing for a formation of a mature male gamete. During its final phase, spermiogenesis, haploid round spermatids undergo cellular differentiation into spermatozoa, which involves extensive restructuring of cell morphology, DNA, and the epigenome. Using mouse models with abrogated Y chromosome gene complements and Y-derived transgenes we identified Y chromosome encoded Zfy2 as the gene responsible for sperm formation and function. In the presence of Zfy2 transgene, mice lacking the Y chromosome and transgenic for two other Y-derived genes, Sry driving sex determination and Eif2s3y initiating spermatogenesis, are capable of producing sperm which when injected into the oocytes yield live offspring. Therefore, only three Y chromosome genes, Sry, Eif2s3y and Zfy2, constitute the minimum Y chromosome complement compatible with successful assisted fertilization in the mouse.

Section 3.2 Introduction

We recently investigated spermatogenesis progression and germ cell function in males with significantly abrogated Y chromosome complement [61]. We have shown that males with the Y chromosome contribution provided by two transgenes, the testis determinant Sry and the spermatogonial proliferation factor Eif2s3y (Fig. 38 B, X^E0Sry and X^EY*X*Sry) have meiotic and postmeiotic arrest, the rare spermatids present in the testes do not elongate, and sperm are not
formed. When round spermatids from these males were injected into the oocytes, live mouse progeny were obtained. The success of round spermatid injection (ROSI) was low, with less than 10% of transplanted embryos developing to live offspring. When the Sry transgene was replaced with the Y chromosome derived sex reversal factor Sxr^b, encoding for Sry, H2al2y, Rbmy gene cluster, and Zfy2/1 fusion gene (Fig. 38 A, Sxr^b) the resulting males (Fig. 38 B, X^E Sxr^b O and X^E Sxr^b Y*X*) had more advanced spermatid development with clear elongation of these cells, occasional appearance of sperm, and increased ROSI efficiency.

These findings indicated that a gene/s encoded within Sxr^b plays a role in spermiogenesis progression and germ cell function. Here, we identify Zfy2 as the gene responsible. We present evidence that the Y chromosome gene Zfy2 promotes sperm morphogenesis, and use assisted reproduction (round spermatid injection, ROSI and intracytoplasmic sperm injection, ICSI) for identifying Zfy2-dependent functional changes in the spermiogenic cells.
Figure 38. Diagrammatic representation of the mouse X and Y chromosomes, variant sex chromosomes, and mouse genotypes relevant to this study. (A) The mouse Y chromosome contains ~90 Mb of male specific DNA and ~0.7 Mb constituting the pseudoautosomal region (PAR) situated at the end of the long arm. The PAR is the region of homology with the X that mediates pairing and recombination between the X and Y in normal males. The remaining non-pairing male specific part of Y (NPY) contains several genes and gene families. On the short arm (NPYp), there are single-copy genes: Prsly, Teyorf, Uba1y, Smcy/Kdm5d, Eif2s3y, Uty, Dby/Ddx3y, Usp9y, Sry, duplicated gene Zfy (Zfy1 and 2), duplicated gene H2al2y, and a multi-copy gene Rbmy. The non-pairing region of the long arm (NPYq), representing ~90% of all NPY, contains mostly repetitive sequences, and encodes multiple copies of 5 distinct genes that are expressed in spermatids: Ssty1 and Ssty2, Sly, Srsy, Rbm31y [4]. Y* is an X chromosome derivative encoding PAR, X centromere and near centromeric region. Sxr is a sex reversal variant Tp(Y)1Ct encoding almost intact NPY complement but with Rbmy gene family reduced. Sxr is a Sxr derivative with a 1.3 Mb deletion that has removed the majority of the NPY complement and created a Zfy2/1 fusion gene. (B) The mice used in this study and their Y chromosome contribution. The X chromosome located Eif2s3y and autosomally located Sry transgenes are shown in light blue frames. The Zfy2 transgene, shown in brown frame, is located on the X chromosome in the Hrpt locus in close proximity to the Eif2s3y transgene. The genotype designations without the Zfy2 transgene are shown above the diagrammatic representation of sex chromosomes and with the Zfy2 transgene below them (brown font). Sxr and Sxr gene content is shown in A. n/a = these genotypes with transgenic Zfy2 addition were either not produced or not examined in this study.
Section 3.3 Materials and Methods

Part 3.3.1 Chemicals and Media

See Chapter 2, Section 3, Part 1 for more information.

Part 3.3.2 Animals

The mice of interest in this study were mice with limited Y gene complement (Fig. 38 B):

1. $X^{Eif2s3y}OSry$ (abbreviated as $X^E OSry$) are males carrying an autosomally-encoded transgene of testis determinant $Sry$ [38] and the X chromosome-located transgene encoding spermatogonial proliferation factor $Eif2s3y$ [217]. These mice have only one sex chromosome (hence the designation XO).

2. $X^{Eif2s3y}Y^*X^{Sry}$ (abbreviated as $X^E Y^*X^{Sry}$) males have the same Y gene complement as $X^E OSry$ but carry a minute X chromosome derivative ($Y^*X$) with a complete pseudoautosomal region (PAR) but lacking most of the other X genes [241].

3. $X^{Eif2s3y}Sxr^bO$ (abbreviated as $X^E Sxr^bO$) males have the X chromosome carrying an $Eif2s3y$ transgene [217] together with $Tp(Y)1Ct^{Sxr^b}$, a $Sxr^a$ derivative with a 1.3 Mb deletion that has removed the majority of the Yp gene complement and created a $Zfy2/1$ fusion gene [32, 242].

4. $X^{Eif2s3y}Sxr^bY^*X$ (abbreviated as $X^E Sxr^bY^*X$) have the same Y gene complement as $X^E Sxr^bO$ but carry also $Y^*X$.

5. $XY^*XSxr^a$ have a single X chromosome and $Tp(Y)1Ct^{Sxr^a}$ [68] attached distal to the $Y^*X$ PAR.
6. Mice with Zfy2 transgene. The Zfy2 transgene (abbreviated as $Z^2$) was added to the genotypes described in 1, 2 & 4. It was provided as a single copy Zfy2 BAC inserted by cassette mediated exchange (CME) into the Hprt locus on the X chromosome [64, 243].

The $X^EoSry$ and $X^EY^XSry$ males were produced ‘in house’ by breeding $X^{Paf}O$ or $X^{Paf}Y^X$ females [218] carrying the X-linked coat marker Patchy-fur [219] and $X^{Eif2s3y}Y^{Tdy1}Sry$ males that have the X chromosome carrying an Eif2s3y transgene [217] and a Y chromosome with an 11 kb deletion removing the testis determinant Sry (dl1Rlb) [24, 76], complemented by an autosomally-located Sry transgene [Tg(Sry)2Ei] [38]. The $X^ESxr^bO$ and $X^ESxr^bY^X$ males were produced ‘in house’ by breeding $X^{Paf}O$ or $X^{Paf}Y^X$ females described above and $X^{Eif2s3y}YSxr^b$ males that have the X chromosome carrying an Eif2s3y transgene [217] and a Y-chromosome that has Tp(Y)1Ct$Sxr^b$ sex reversal factor [34, 71] attached distal to its PAR region. The $XY^XSxr^a$ males were produced by ICSI with sperm from males of the same genotype and oocytes from wild-type females. Males transgenic for Zfy2 ($X^{E,Z^2}O\text{Sry}, X^{E,Z^2}Y^XSry$ and $X^{E,Z^2}Sxr^bY^X$) were produced as described above but with the father carrying $X^{E,Z^2}$ rather than $X^E$.

See Chapter 2, Section 3, Part 2 for general information

Part 3.3.3 Histology Analysis

See Chapter 2, Section 3, Part 2 for more information

Part 3.3.4 Round Spermatid Injection (ROSI) and Intracytoplasmic Sperm Injection (ICSI) *

This part can be found in Chapter 8, Section 2, Part 1.

Part 3.3.5 Zygote Chromosome Analysis *

This part can be found in Chapter 8, Section 2, Part 2.
Part 3.3.6 Progeny Genotyping

Offspring produced with ICSI and ROSI were genotyped by PCR to identify presence of \textit{Eif2s3y}, \textit{Zfy2}, and \textit{Sry} transgenes. Presence of \textit{Y}^{*X} was recognized by copy number assessment. Genomic DNA was isolated from mouse tails using phenol chloroform extraction and ethanol precipitation. DNA was used to amplify single copies of X-linked \textit{Prdx4} (absent in \textit{Y}^{*X}) and \textit{Amelx} (present in \textit{Y}^{*X}), and \textit{Atr} (on chromosome 9) for normalization using \textit{Power SYBR Green} PCR Master Mix on a Quant Studio 12K Flex machine (Applied Biosystems). The following conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Two PCR reactions were used to detect the presence of \textit{Y}^{*X} and the number of X chromosomes. An 82-bp \textit{Prdx4} fragment were amplified using primers \textit{Prdx4}-F and \textit{Prdx4}-R and a 162-bp \textit{Amelx} fragment with primers \textit{Amelx}-F and \textit{Amelx}-R. All samples were tested in quadruplicate per assay using XO samples as a reference control. Copy number estimation for each gene was calculated with the \textit{ΔΔCt} method. Briefly, \textit{ΔCt} values were calculated as difference between tested gene and \textit{Atr}. \textit{ΔΔCt} values were calculated by subtracting \textit{ΔCt} of tested genes from the reference samples. The copy numbers were calculated by raising 2 to the power of \textit{ΔΔCt} (2^{ΔΔCt}). The genotypes were inferred from the copies of each target gene: XO, \textit{1 Prdx4} + \textit{1 Amelx}; \textit{XY}^{*X}, \textit{1 Prdx4} + \textit{2 Amelx}; \textit{XX}, \textit{2 Prdx4} + \textit{2 Amelx}; \textit{XXY}^{*X}, \textit{2 Prdx4} + \textit{3 Amelx}. Primer sequences are shown in Table 8.

Part 3.3.7 Real-Time RT-PCR *

This part can be found in Chapter 8, Section 2, Part 3.
Part 3.3.8 Statistical Analyses

Fisher's Exact Test was used to assess the differences between the genotypes for ROSI and ICSI and zygotic chromosome analysis data. Student t-test was used for expression and histology analyses.
Table 8. Primers for genotyping and expression analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ID</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
<th>Reference</th>
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<tr>
<td></td>
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<tr>
<td><strong>qPCR primers for sex chromosome copy number estimation</strong></td>
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<tr>
<td>Prdx</td>
<td>Prdx4-F</td>
<td>CATGATATCCACTGAAAGCTAC</td>
<td>82 bp</td>
<td>[244]</td>
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<tr>
<td></td>
<td>Prdx4-R</td>
<td>GAGCAGTGTTATCTATCCCTG</td>
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<tr>
<td>Amelx</td>
<td>Amelx-F</td>
<td>GTTGGGTGGAGTCATGGAG</td>
<td>162 bp</td>
<td>[244]</td>
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<td></td>
<td>Amelx-R</td>
<td>GGCTGCACCACACAAATCATC</td>
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</tr>
<tr>
<td>Atr</td>
<td>Atr-WT L1</td>
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<td>143 bp</td>
<td>[64]</td>
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<tr>
<td></td>
<td>Atr-WT R1</td>
<td>AGCCGATTTGCCACAGTAAC</td>
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</tr>
<tr>
<td><strong>Standard genotyping PCR primers</strong></td>
<td></td>
<td></td>
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<tr>
<td>Myog</td>
<td>Om1a</td>
<td>TTACGTCCATCGTGACGACAT</td>
<td>246 bp</td>
<td>[245]</td>
</tr>
<tr>
<td></td>
<td>Om1b</td>
<td>TGGGCTGGTGGTTAGCCTAT</td>
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<tr>
<td>Zfy</td>
<td>Zfyp1</td>
<td>AAGATAAGGCTTACATAATCATGGGA</td>
<td>600 bp</td>
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<td>Zfyp2</td>
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<tr>
<td>Sxrb</td>
<td>o3452</td>
<td>GTTAATGAAAAAGGGATGGG</td>
<td>852 bp</td>
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<td></td>
<td>o3072</td>
<td>GTATTAAGTCTTAAGACATGG</td>
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<td>Ssty</td>
<td>Ymtfp1</td>
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<td>Muty3</td>
<td>GTGCTCAAAGGCTGCTCTTC</td>
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<td>Eif2s3y tg</td>
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<td>600 bp</td>
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<td>Zfy2-R</td>
<td>ggtatttctagagctcgc</td>
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<td><strong>Real-time PCR primers for Zfy expression quantification</strong></td>
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<td>Zfy Global</td>
<td>ZfyGlobalF</td>
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<td>95 bp</td>
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<td>ZfyGlobalR</td>
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<td>Zfy1-spF</td>
<td>TGGGATTTTGTTGACTCA</td>
<td>117 bp</td>
<td>[248]</td>
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<td>ZfyGlobalR</td>
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<tr>
<td>Acrv</td>
<td>Acrv-IF</td>
<td>TGGACTACACCTCTCAAGA</td>
<td>60 bp</td>
<td>[249]</td>
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<td></td>
<td>Acrv-IR</td>
<td>AAGCACAATGTGTTGGAAATTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Act</td>
<td>Act-F</td>
<td>CAGCTCGACCAAAACCA</td>
<td>117 bp</td>
<td>[250]</td>
</tr>
<tr>
<td></td>
<td>Act-R</td>
<td>CACCCACCAAGGAGCCGCA</td>
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<tr>
<td>Actin</td>
<td>Actin-F</td>
<td>GGCAACACACCCCTCTCAAGT</td>
<td>352 bp</td>
<td>[251]</td>
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<tr>
<td></td>
<td>Actin-R</td>
<td>GTGTTGTTGAGCTGCTAGCC</td>
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<td>Sadha</td>
<td>Sadha-F</td>
<td>TCTCCACACACCCCTCTGT</td>
<td>66 bp</td>
<td>[252]</td>
</tr>
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<td></td>
<td>Sadha-R</td>
<td>TAMGATTTTACAGCCACAGA</td>
<td></td>
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</tbody>
</table>

*Amplified product was digested with 10U of Rsal for at least 1h at 37ºC; a 224 bp band confirms the presence of Sxr*. 

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Section 3.4 Results

Part 3.4.1 Functional of Sperm from $X^E Sxr^b O$ and $X^E Sxr^b Y^{*X}$ Males in Assisted Fertilization *

This part can be found in Chapter 8, Section 2, Part 4.

Part 3.4.2 Addition of Zfy2 to $X^E Y^{*X} Sry$ Males Enables Spermatid Elongation

We next investigated which of the $Sxr^b$ genes is responsible for spermatid elongation. The gene content of $Sxr^b$ is represented by few copies of $Rbmy$, two copies of $H2al2y$, one copy of $Sry$, and a $Zfy2/1$ fusion gene spanning the $Sxr^b$ deletion breakpoint (Fig. 38 A). $Rbmy$ appears early during spermatogenesis and is not expressed, and certainly not translated, after the zygotene stage [253]. $H2al2y$ has been shown to be expressed late during spermiogenesis [37] and $Sry$ transcripts in adult gonads are thought to be aberrant and not translatable [110, 254]. Based on the expression pattern, we excluded $Rbmy$, $H2al2y$, and $Sry$ as the candidates for ensuring sperm head morphogenesis in males with $Sxr^b$, and focused our attention on the $Zfy2/1$ fusion gene.

Postnatal expression of $Zfy1$ and $Zfy2$ is restricted to spermatogenic cells [255-257]. Both genes are first expressed in the testis around the time when germ cells enter meiosis. They then undergo transcriptional silencing as the early spermatocytes transition to pachytene spermatocytes. The expression is reactivated in secondary spermatocytes and continues postmeiotically [50, 244]. In round spermatids there is a clear predominance of $Zfy2$ transcripts in $Y$-bearing round spermatids; this strong expression appears because of the activity of a strong Cypt-derived spermatid-specific promoter driving $Zfy2$ expression. At this stage expression of $Zfy1$, which lacks the Cypt promoter, is limited. The $Zfy2/1$ fusion gene is driven by the Cypt promoter and is strongly
expressed in spermatids [50, 255, 258]. Our expectation therefore was that Zfy2, and not Zfy1, would mimic the effect of Sxr b.

To assess Zfy2 role in spermatogenesis progression we investigated testis histology in XEY*XSry males transgenic for Zfy2. These males are subsequently called XE,Z2Y*Xsry (Fig. 38 B). While in XEY*Xsry males, spermatid development did not progress beyond the round spermatid stage, in XEY*Xsry males spermatids elongated (Fig. 39 A). The elongated, condensed spermatids were more frequently observed in XEY*Xsry than in XE Sxr b Y*X males; in the latter genotype elongation often ceased earlier (at step 12-13) and the spermatid nuclei were less compacted, with lighter staining pattern. Quantitative analysis of spermatogenesis progression (Fig. 39 B) demonstrated that XE,Z2Y*Xsry had more round spermatids than XEY*Xsry (~2.8-fold increase), reaching a level similar to that observed with XE Sxr b Y*X males, but less than in wild-type controls. The number of elongating/elongated spermatids in XE,Z2Y*Xsry and XE Sxr b Y*X was not significantly different, and ~10-fold lower than in wild-type controls. Testicular sperm from XE,Z2Y*Xsry males were also observed in live cell suspension (Fig. 40 A) and on silver stained testicular cell spreads (Fig. 40 B). The head shape of these sperm was abnormal, as expected from males lacking Y chromosome long arm [7].

Part 3.4.3 Increases of Success with Round Spermatids Injection (ROSI) and ICSI Yielding Live Offspring with Addition of Zfy2 to XO Sry and EY*Xsry Males *

This part can be found in Chapter 8, Section 2, Part 5.

Part 3.4.4 Determination of Zfy2, and Zfy1 Role in Sperm Function with ZfyExpression Analysis *

This part can be found in Chapter 8, Section 2, Part 6.
Figure 39. Histology analysis. (A) Exemplary tubules of stage VII-VIII testis sections. \(X^{E,Y \times X}Sry\) males have meiotic and post-meiotic arrest that only occasionally allow formation of round spermatids that do not develop beyond step 7 of spermatid development. In \(X^{E,Sxr}Y^{X}Xsry\) spermatid elongation is observed but usually ceases at step 11-12, with few occurrences of more advanced stages. In \(X^{E,Z2}Y^{X}SXry\) males spermatogenesis is progressing with good spermatid elongation and many spermatids developing to step 15-16; these elongated spermatids are morphologically abnormal, which is expected from males lacking NPYq genes [7]. Tubule stages are shown in Roman and steps of spermatid development (St) in Arabic numerals. Bar = 50 µm; insets = x3 magnification.

(B) Quantitative analysis of spermatogenesis progression. For each male 10 tubules were examined per indicated stages and the numbers of round spermatid (steps 1-8), elongating/ed spermatid (steps 9-16), and Sertoli cells were counted. The data are expressed as spermatid/Sertoli cell ratios. In wild-type males no round spermatids are present in stages IX-XI so those observed in males with limited Y gene complement represent 'delayed spermatids'. Statistical significance (t-test): \(^a\) different than \(X^{E,Y \times X}Sry\); \(^b\) different than \(X^{E,Sxr}Y^{X}Xsry\); \(^c\) different than all other. Three males per genotypes were included in the analysis.
Figure 40. Sperm from $X^{E,Y\times Sry}$ males. (A) Examples of sperm found in live epididymal cell suspension from $X^{E,Y\times Sry}$ males. Top and bottom panels represent examples of sperm from two different males. Round cells likely represent shed testicular germ cells. (B) Examples of testicular sperm from $X^{E,Y\times Sry}$ males identified on silver stained spreads of testicular cells. Scale = 10 µm.
Y chromosome encoded zinc finger protein genes, Zfy, had once been the center of attention as potential candidates for the testis-determining factors [49, 259, 260]. When the fame went to another Y gene, Sry [24, 107, 108], Zfy genes were quickly forgotten and it has taken more than two decades for these genes to re-emerge with newly ascribed spermiogenic roles. Zfy1 and Zfy2 were shown to play spermatogenic quality functions during the pachytene stage of meiosis and during MI by triggering the apoptotic elimination of spermatocytes [64, 243] and to facilitate the second meiotic division [244]. It has also been shown that a gene/s from Sxr\(^a\), partially retained in Sxr\(^b\), is necessary for the initiation of sperm morphogenesis [35] and increases the efficiency of round spermatid injection [61]; Zfy genes were proposed as the most likely candidates. Here we tested this assumption by investigating the effects of transgenic Zfy2 addition into Y chromosome deficient males, which have a postmeiotic arrest at the round spermatid stage. We demonstrated that Zfy2 is responsible for formation of sperm functional in assisted fertilization.

In our previous study we reported that only two Y chromosome genes, the testis determinant factor Sry and the spermatogonial proliferation factor Eif2s3y, are sufficient to make a male mouse whose germ cells are functional in assisted fertilization and yield live progeny [61]. When Y chromosome contribution was expanded by substituting Sry for Sxr\(^b\), ROSI efficiency improved, and we speculated that this was due to the presence of the Zfy2/1 fusion gene, which facilitated the second meiotic division in the testis in the presence of Y*\(^X\), or in the oocytes after fertilization when the meiotic pairing partner was missing [61]. Spermatid elongation and occasional formation of mature testicular sperm were previously observed in males with Sxr\(^b\) [35,
but their function in fertilization has not been tested. Here we have shown that these sperm are not successful in assisted fertilization. In \(X^E Sxr^bO\) males the great majority of elongating spermatids are diploid [35] and so are the testicular sperm as shown in this study. The fact that live offspring were obtained with ROSI, and not with ICSI, could therefore be due to the highly condensed nature of the sperm chromatin. In diploid round spermatids from \(X^E Sxr^bO\) males the chromatin is still histone-bound and the homologous chromosomes are presumably still paired as in meiosis II (MII). Secondary spermatocytes, with the same chromosomal state prematurely condense upon injection into oocytes and complete MII along with the maternal chromatin, expelling a polar body-like structure with the haploid complement of paternal DNA [261]. We have proposed that a similar process occurs with the round spermatids from \(X^E Sxr^bO\) males, resulting in normal, diploid zygotes [61]. The diploid spermatozoa, however, cannot have the normal histone component because in order to complete spermiogenesis most of the histones would have had to be replaced by protamines. This condensed sperm chromatin must be completely reorganized, which normally takes one to two hours after ICSI [262]. By this time, the maternal chromatin has already completed MII, and the zygote can no longer support the completion of MII for the paternal DNA. Congruent with this explanation, ICSI should be successful with sperm from \(X^E Sxr^b Y^a\) males, which yielded predominantly diploid zygotes. However, only one offspring was obtained, suggesting that sperm ability to support embryonic and fetal development was highly impaired. \(X Y^a Sxr^a\) males can be reproduced by ICSI [7]. Thus, one or more Y genes that are present and active in \(Sxr^a\), and not in \(Sxr^b\), are likely to be responsible for rendering sperm functional. We now show that this gene is \(Zfy2\), and that sperm dysfunction \(X^E Sxr^b Y^a\) males can be overcome with the transgenic addition of \(Zfy2\).
Why is it Zfy2 and not the Zfy2/1 fusion gene that renders sperm functional in assisted fertilization? The Zfy2/1 fusion gene, present within Sxr\textsuperscript{b}, encodes a protein that is almost identical to that encoded by Zfy1 but the transcription is driven by Zfy2 specific promoter [50]. Both Zfy2 and Zfy2/1 are therefore strongly expressed postmeiotically because both have additionally acquired (Zfy2-specific) Cypt-derived promoter, which drives strong expression in spermatids [255]. However, in case of Zfy2/1 fusion gene, it is predominantly Zfy1 transcript that is present. The alternative splicing results in the majority of Zfy1 transcripts lacking exon 6, which encodes a protein transactivating domain (TA), while most of the Zfy2 transcripts retain exon 6 and functional TA domain [50]. The protein encoded by Zfy1 lacking exon 6 is expected to bind but not transactivate target genes and consequently can serve as a competitive inhibitor of full length ZFY proteins. Moreover, the TA domain in ZFY1 protein, when present, is ~5.5-fold less active than that of ZFY2 protein [244]. In Zfy2/1 fusion gene the produced transcripts are spliced like those of Zfy1 so that a substantial proportion of them lack the exon 6 encoding the transcription factor function [50] and when a TA domain is present, it is equivalent to that encoded by ZFY1 and therefore less potent. These transcript and protein specific differences explain why Zfy2/1 in X\textsuperscript{ESxr}\textsuperscript{b}Y\textsuperscript{X}\textsuperscript{*}X males is not sufficient for promoting sperm function, and why addition of Zfy2 to this genotype rescues this defect.

The fact that we obtained ICSI offspring from X\textsuperscript{ESr}Y\textsuperscript{X}\textsuperscript{*}Sry males represents a significant advancement in establishing a minimum Y complement compatible with successful assisted fertilization. Although we have shown earlier that only two Y genes are sufficient to generate progeny [61], this was achieved with round spermatid injection (ROSI), a method which is considered experimental in human ART due to concerns regarding the safety of injecting immature
germ cells and technical difficulties [263]. Intracytoplasmic sperm injection (ICSI), however, is a common procedure in human ART, rendering our mouse data more directly translational.

With the reemergence of Zfy genes from the backstage and their recently acknowledged roles during spermatogenesis [35, 50, 61, 64, 243, 244] (and this study), it will now be important to characterize the mechanism and identify the target genes that these transcription factors regulate. In humans, there is a single ZFY gene on the Y chromosome, which is ubiquitously expressed [90]. No mutations of ZFY have been described and there is therefore no information concerning its possible contribution to human germ cell development or male fertility. The newly acquired mouse data regarding the role of Zfy gene in spermatogenesis may therefore trigger re-evaluation of ZFY in humans.

Section 3.6 Contributions

Dr. Monika A. Ward – P.I., lead author

Dr. Jonathan M. Riel – all expression data

Dr. Yasuhiro Yamauchi – all ART related work
CHAPTER 4. SEMINIFEROUS EPITHELIUM CELL POPULATIONS DURING PRESPERMATOGENESIS AND THE FIRST WAVE IN SRY TRANSGENIC MICE WITH VARIABLE SEX CHROMOSOMES

Section 4.1 Abstract

Here we report on neonatal prespermatogenesis in male mice lacking a Y chromosome (XO and XX) with an autosomal Sry transgene driving testis formation. These mice have testes but previous work has shown they do not initiate spermatogenesis. Testicular cell populations in XO\textit{Sry} and XX\textit{Sry} males, and XY\textit{Tdy}\textit{Sry} as controls with normal spermatogenesis, at 3 dpp and 11 dpp were quantitatively assessed. Results showed differences in germ cell populations arising as early as 3 dpp in these mice. By 11 dpp there were significant differences in pre-Sertoli cell and germ cell populations.

Section 4.2 Introduction

It is an established dogma that the Y-linked gene \textit{Sry} is the initiator of male sex determination through testis formation [24]. \textit{Sry} is so powerful that even without any other Y chromosome genes (XX and XO context) a testis will still form (Fig. 41) [107]. But \textit{Sry} is just the initiator, the real workhorse is autosomal \textit{Sox9}, which acts downstream of \textit{Sry} in the testis formation cascade [114-116]. From a cellular perspective the pre-Sertoli cells are the prime movers of testis formation. \textit{Sry} signaling targets pre-Sertoli cells to switch on \textit{Sox9} expression. With the molecular cascade started pre-Sertoli cells can then begin to pressure neighboring cells to commit to the male lineage fate [184, 185]. Once matured, pre-Sertoli cells will change names (Sertoli cell) as well as jobs, by managing spermatogenesis in the testis of which they influenced the formation [186].
Work with \textit{Sry} has focused more on whether a testis is formed, how it is formed molecularly and the appearance of spermatogenesis in the resulting adult male [3, 27, 61, 107, 109-112]. We recently became very interested in X chromosome dosage effects (XX and XO context) on spermatogenesis progression in neonatal mice transgenic for \textit{Sry} (XX\textit{Sry} and XO\textit{Sry}). While pursuing investigations of adult mice with limited Y genes, we observed that an XX\textit{Sry} male at 21 dpp showed more spermatogonia when compared to XX\textit{Sry} males that were all 54+ dpp (Fig. 42 B & C) (unpublished data) [3, 61]. We wondered if males with only one gene present for testis formation and no genes present for spermatogenesis initiation always exhibited temporal differences in spermatogonia populations or if this was an isolated event.

We then hypothesized, if there were temporal differences in adult XX\textit{Sry} males then the same would probably be true of other males with limited Y genes, with more pronounced differences in neonates than adults. The landscape of the testis in neonatal mice from birth to about 14 dpp is different than in the adult. Temporally pro-spermatogonia are transitioning out of a quiescent state and re-entering mitosis [130, 132, 133]. Pro-spermatogonial proliferation will establish the soon-to-be spermatogonial stem cell (SSC) population and the differentiated type A spermatogonia that will enter the first wave of spermatogenesis [131, 134, 144]. At the same time pre-Sertoli cells are approaching a proliferative nadir as they end mitosis permanently and mature into non-proliferating Sertoli cells (Fig. 18 in Chapter 1) [134, 190]. Our results show that prespermatogenesis related differences between XY\textit{Tdy}m\textsuperscript{1}Sry, XO\textit{Sry} and XX\textit{Sry} mice arise as early as 3 dpp and, in males lacking a Y chromosome, continue to be divergent throughout prespermatogenesis.
Figure 41. Mice demonstrating the influence of transgenic *Sry* on gender. Wild type XY male mouse displaying normal male genitalia. XO (one X chromosome) female mouse displaying normal female genitalia. XO mouse transgenic for *Sry* (XOSry) is male despite the lack of Y chromosome or Y chromosome genes other than *Sry* and displays normal male genitalia.
Figure 42. Sertoli cell and spermatogonia population comparisons in adult male mice with testis formation driven by Sry or Sox9. (A) Sertoli cell number in XY, XOSry, XXSry and XOSox9 males with a distinction between XXSry males at 21 and 54+ dpp. XXSry and XOSox9 males exhibit a more than two-fold increase in Sertoli cell number over XY males. (B) Spermatogonia number in XY, XOSry, XXSry and XOSox9 males with a distinction between XXSry males at 21 and 54+ dpp. In all males except XY, spermatogonia are present but are a non-proliferating and non-differentiating population. There is a greater than six-fold drop in spermatogonia numbers between XXSry males at 21 and 54+ dpp. (C) Spermatogonia per Sertoli cell ratios in XY, XOSry, XXSry and XOSox9 males with a distinction between XXSry males at 21 and 54+ dpp. The drop in spermatogonia number between 21 and 54+ dpp XXSry males is apparent with ratios as it was with spermatogonia counts in B. The n for each genotype is shown in A and is the same for all graphs.
Section 4.3 Materials and Methods

Part 4.3.1 Chemicals and Media

See Chapter 2, Section 3, Part 1 for more information.

Part 4.3.1 Animals

The mice of interest in this study were mice with a single X chromosome (XO) or double X chromosomes (XX) transgenic for Sry and XY<sup>Tdym1</sup>Sry males serving as control:

1. **XY<sup>Tdym1</sup>Sry** males carry a Y chromosome with an 11-kb deletion removing the testis determinant gene Sry (<i>dl1Rlb</i>) [24, 76], designated as Y<sup>Tdym1</sup>, which is complemented by an autosomally-located Sry transgene [Tg(Sry)2Ei] [38].

2. **XO</i>Sry** and **XX</i>Sry** are both males carrying an autosomally-encoded Sry transgene [38]. These males were produced by breeding XY<sup>Tdym1</sup>Sry males that have a Y chromosome with an 11-kb deletion removing the testis determinant Sry (<i>dl1Rlb</i>) [24, 76], complemented by an autosomally-located Sry transgene [Tg(Sry)2Ei] [38], with XO females.

The breeding crosses yielded 3 male and 3 female progeny genotypes; the males were identified among the progeny by genotyping for Y chromosome markers and scoring fur appearance. Mice were on mixed (50% C57BL/6 and 50% MF1) genetic background.

The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22ºC, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawai‘i and guidelines presented in National Research Council’s (NCR) “Guide for Care and Use of Laboratory Animals” published by Institute
for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawai’i.

Part 4.3.3 Testicular Material Collection and Preparation

Testes were collected and dissected. Each dissected testis was weighed and photographed if needed. One testis was used for Bouin fixation and subsequent sectioning, and one testis for storage at -80°C and subsequent molecular or cytogenetic analyses.

Part 4.3.4 Histology Analysis

For histology analysis, the testes were fixed in Bouin overnight and then stored in 70% ethanol prior to embedding in paraffin wax, sectioning at 5 µm, and staining with hematoxylin-eosin (H&E). Testicular cells of the seminiferous tubule were identified using histological descriptions originally by the Hilschers and others more recently [23, 134, 264-266].

Part 4.3.9 Statistical Analyses

Student's t-test was used for all analyses.

Section 4.4 Results

Part 4.4.1 Sertoli Cell Populations

The first cell population affected by the masculinizing presence of Sry are supporting cell precursors (SCP). SCP differentiation to pre-Sertoli cells is initiated by Sry at 10.5 dpc [24, 112]. Therefore, we first investigated the potential effects of transgenic Sry expression on pre-Sertoli cell populations in the context of XO and XX, and in comparison to XY$^{Tdym1}$Sry. Late in prespermatogenesis, at 3 dpp, pre-Sertoli cell numbers in XY$^{Tdym1}$Sry, XOSry and XXSry mice were not significantly different (Fig. 43 A). However, by 11 dpp pre-Sertoli cell populations were
significantly different between \(XY^{Tdym1Sry}\) and \(XO^{Sry}\), or \(XY^{Tdym1Sry}\) and \(XX^{Sry}\) males (\(P < 0.001\)), and between \(XO^{Sry}\) and \(XX^{Sry}\) males (\(P < 0.05\)). The overall trend was an increase in pre-Sertoli cell population from \(XY^{Tdym1Sry}\) to \(XO^{Sry}\) to \(XX^{Sry}\) (Fig. 43 B).

Part 4.4.2 Pro-spermatogonia and Spermatogonia Populations at 3 dpp

In normal XY males, by 3 dpp almost all T1 pro-spermatogonia have entered mitosis and transitioned to T2 pro-spermatogonia. At this same temporal point there is the expectation of a very limited population of differentiated and undifferentiated spermatogonia [131, 134]. Similar to XY males, \(XY^{Tdym1Sry}\) males had a predominantly pro-spermatogonia germ cell population with some spermatogonia present as well (Fig. 44 A). \(XO^{Sry}\) males followed a similar pattern with populations that were not statistically different from that of \(XY^{Tdym1Sry}\) males (Fig. 44 B & D). \(XX^{Sry}\) males, on the other hand, had spermatogonia and pro-spermatogonia populations that were significantly less than \(XY^{Tdym1Sry}\) males (\(P < 0.05\)) (Fig. 44 C & D).
Figure 43. Pre-Sertoli cell populations at 3 dpp and 11 dpp. Comparisons of pre-Sertoli cells at 3 dpp in XY<sup>Tdym</sup>Sry, XO<sup>Sry</sup> and XX<sup>Sry</sup> mice. No statistical significance in average population between 3 dpp males. Comparisons of pre-Sertoli cells at 11 dpp in XY<sup>Tdym</sup>Sry, XO<sup>Sry</sup> and XX<sup>Sry</sup> mice. There was statistical significance in average population between 11 dpp males. For A and B n = 3 all genotypes. Statistical significance (t-test, P<0.05): ** significantly different from XY<sup>Tdym</sup>Sry and each other. Bars are mean ± SEM.
Figure 44. Cell distributions at 3 dpp. In general, there was a decline in pro-spermatogonia (marked with P) from XY<sup>Tdym<sup>1</sup>Sry</sup> (A) and XOSry (B) to XXSry (C), also seen in D. Spermatogonia (marked with G) were only minimally present in XY<sup>Tdym<sup>1</sup>Sry</sup> (A), XOSry (B) and XXSry (C), also seen in D. Pre-Sertoli cells are marked with S (A, B and C). For all genotypes n = 3. Statistical significance (t-test, P<0.05): * Significantly different from XXSry. Bars are mean ± SEM. Scale = 20 µm.
Part 4.4.3 Pro-spermatogonia and Spermatogonia Populations at 11 dpp

The seminiferous tubule is highly dynamic at 11 dpp. The first wave has been initiated and germs cells should have at least reached early meiosis I. Pre-Sertoli cells should be entering mitotic cessation to mature into Sertoli cells and most T2 pro-spermatogonia should have differentiated into type A\textsubscript{single} (A\textsubscript{s}) spermatogonia (SSCs) or type A\textsubscript{1} spermatogonia [131, 134]. XY\textsuperscript{Tdym1}Sry males exhibited the expected appearance with concentric rings of developing germ cells (spermatogonia and early spermatocytes) being supported by pre-Sertoli cells (Fig. 45 A & D). Occasionally pro-spermatogonia were found still lingering in seminiferous tubules. Without a spermatogonia proliferation and differentiation factor present at the onset of the first wave, the spermatogonia population in XO\textsubscript{Sry} males was significantly less than that of XY\textsuperscript{Tdym1}Sry males (P < 0.05) while at the same time significantly greater than that of XX\textsubscript{Sry} males (P < 0.05) (Fig. 45 B & D) [3, 61]. XO\textsubscript{Sry} males had an extremely limited population of pro-spermatogonia (Fig. 46). XX\textsubscript{Sry} males, had a spermatogonia population that was significantly less than XY\textsuperscript{Tdym1}Sry and XO\textsubscript{Sry} males (P < 0.05), and lesser quality pre-Sertoli cells that had begun to acquire vacuolations (Fig. 4F C & D).

Knowing that there had been a Sertoli cell population shift between 3 dpp and 11 dpp we also compared the average pro-spermatogonia and spermatogonia number for each genotype. This analysis yielded similar results with XY\textsuperscript{Tdym1}Sry significantly higher than XO\textsubscript{Sry} (P < 0.05) and XX\textsubscript{Sry} (P < 0.01), and XO\textsubscript{Sry} significantly higher than XX\textsubscript{Sry} (P < 0.05) (Fig. 46).
Figure 45 Cell distributions at 11 dpp. In general, there was a decline in spermatogonia (marked with G) from $XY^{Tdy\mu 1}Sry$ (A), to $XOSry$ (B) and $XXSry$ (C), also seen in D. Spermatocytes (marked with Sp) were only present in $XY^{Tdy\mu 1}Sry$ (A) and not $XOSry$ (B) and $XXSry$ (C), also seen in D. Pro-spermatogonia were a small population only in $XY^{Tdy\mu 1}Sry$ and $XOSry$ (D, magnified with inset). Pre-Sertoli cells are marked with S (A, B and C). For all genotypes n = 3. Statistical significance (t-test, P<0.05): * significantly different from $XOSry$ and $XXSry$. ** significantly different from $XXSry$. Bars are mean ± SEM. Scale = 20 µm.
Figure 46. Germ cell populations at 11 dpp. Knowing that pre-Sertoli cell populations were significantly different between $XY^{Tdy^{m1}Sry}$, $XOSry$ and $XXSry$ mice at 11 dpp (see Fig. 43 B), average germ cell number of pro-spermatogonia, spermatogonia and total (pro-spermatogonia and spermatogonia) were also compared between $XY^{Tdy^{m1}Sry}$, $XOSry$ and $XXSry$. Analysis had the same findings as analysis with germ cell ratios (see Fig. 45 D). For all genotypes $n = 3$. Statistical significance (t-test, $P<0.05$): * significantly different from $XOSry$ and $XXSry$ ** significantly different from $XXSry$. Bars are mean ± SEM.
Section 4.5 Discussion

Cessation of pre-Sertoli cell proliferation is marked by their maturation into non-proliferating Sertoli cells [8, 186, 202]. Work in rats has supported the general theory for a sliding model of non-static Sertoli cell maturity such that limited mitosis can occur after puberty [267, 268]. Recently it has been shown that, at least in vivo, glial cell line-derived neurotrophic factor (GDNF) can be used to stimulate pre-Sertoli cell proliferation [269]. GNDF is also a well-established spermatogonial stem cell growth factor secreted by Sertoli cells and is putatively part of the spermatogonial stem cell niche [132, 144, 270-272]. It is possible that the limited/declining spermatogonia population in XOSry and XXSry mice causes a misregulation of this pathway with a link to the over proliferation of Sertoli cells we observed.

In healthy fertile men this cessation of Sertoli cell mitosis is dogma [8]:

„Over a period of years we have studied biopsies obtained from approximately 2000 adult men either normal or having a variety of clinical problems without having identified a single mitotic division of a Sertoli cell.” Mavis Rowley and Carl Heller

However, the seminiferous tubules of SCO patients can sometimes contain both pre-Sertoli cells and Sertoli cells [273]. Pointing to some molecular mismanagement of their maturation.

The general trends observed with pro-spermatogonia and spermatogonia at 3 dpp and 11 dpp in XY^{Tdy^{m1}Sry} mice followed closely with what occurs in XY males at this time point and correlates to our previous findings demonstrating the similarities between adult XY and
Results with XOSry and XXSry mice matched expectations based on our unpublished findings during a previous project that included adult XOSry and XXSry mice (Fig. 42) [3, 61]. The lack of significant difference between XY^Tdym1Sry^ and XOSry spermatogonia populations at 3 dpp suggests that absence of Eif2s3y, the initiator of spermatogonial proliferation and differentiation, in the latter males did not impair processes taking place prior to this time point indicating that Eif2s3y may not play that pivotal a role in fetal and early neonatal germline development. Our previous work has shown that substitution of Eif2s3y with its X-linked homologue Eif2s3x yields ongoing spermatogenesis with the caveat of significant efficiency differences in spermatogenesis driven by Eif2s3y and Eif2s3x. Eif2s3x could play an important role in neonatal germline development, while Eif2s3y is dormant, and the dosage difference in XXSry (two copies) to XY^Tdym1Sry^ and XOSry (one copy in each) could be part of the cause behind the differences described here in these mice.

There is still a large knowledge gap in regards to pre-Sertoli cells and their role in disease, such as tumorigenesis [12]. However, a recent theory is gaining traction. New findings are showing, changes in pre-Sertoli cell differentiation and proliferation during development may have profound effects on the state of the testis in the adult [186, 275]. Work with humans indicates Sertoli cell numbers may increase with SCO syndrome [276-278].

We have observed this Sertoli cell population boost with our adult XOSry, XXSry and XOSox9 mice, which also have a SCO phenotype (Fig. 42 A). In some cases, not only do these mice have more Sertoli cells than adult XY males but many of the Sertoli cells are sloughed and in some cases will aggregate into bundles (Fig. 47 A-D). It follows that XOSry, XXSry and XOSox9 may serve as a model for some form of testicular dysgenesis syndrome (TDS) [275].
However, much more work with these mice is required before any assertions to their value as a TDS model can be made.

The next direction for this project will be a three pronged approach. It will be equally important to expand temporally in prespermatogenesis and do similar work on other mice with limited Y gene contribution. A major priority will be to investigate earlier in prespermatogenesis around 16 dpc. At this time point T1 pro-spermatogonia are present and in a quiescent state of G0/G1 arrest while germline specific epigenetic regulation occurs. This is a crucial junction for ensuring germline competency. Misregulation during the quiescent period can lead to tumorigenesis in both mice and men [135-137]. The addition of males transgenic for *Eif2s3y* or *Eif2s3x* will provide information of the role, if any, *Eif2s3* genes play in germline establishment and the effects of *Eif2s3y* and *Eif2s3x* dosage on this process. Expression data for all males at all time points will be critical for understanding the role of endogenous and transgenic *Eif2s3y* and *Eif2s3x*. These future directions will also help elucidate the link between pre-Sertoli cell proliferation and differentiation changes in fetal and neonatal males of these genotypes, and the resulting testis malformations in their adult forms.
Figure 47. Sertoli cell sloughing in adult XOSry, XXSry and XOSox9 males. Sertoli cells not only slough off the basement membrane (*) in these males but aggregates of Sertoli cells will bundle together (*) in XOSry (A), XXSry (B) and XOSox9 (C) males. For all genotypes n is at the bottom of bar. Bars are mean ± SEM. Scale = 20 µm.
Section 4.6 Contributions

The data presented in this chapter will eventually be incorporated into a new manuscript from Monika Ward's Lab.
CHAPTER 5. TESTICULAR ABNORMALITIES IN MALES WITH Y CHROMOSOME DEFICIENCIES


Section 5.1 Abstract

We recently investigated mice with Y chromosome gene contribution limited to two, one, or no Y chromosome genes in respect to their ability to produce haploid round spermatids and live offspring following round spermatid injection (ROSI). Here we explored whether such limited Y chromosome contribution affects the normalcy of germ cells and interstitial tissue of the testis. We performed quantitative analysis of spermatogenesis and semi-quantitative analysis of interstitial tissue on Periodic acid Schiff and hematoxylin (PAS-H) stained mouse testis sections. Seminiferous epithelium of mice with limited Y gene contribution contained various cellular abnormalities, total number of which was higher than in males with an intact Y chromosome. The distribution of specific abnormality types varied among tested genotypes. Interstitial tissue quality, and testis vasculature, decreased in parallel to declining presence of Y genes. We also performed a quantitative assessment of spermatogenesis and cellular abnormalities in eosin and hematoxylin (H&E) stained human testicular biopsies, including some from azoospermic men with Y chromosome deletions. Some of the cellular abnormalities observed in mice were present in human seminiferous epithelium. Together, the data indicate that in both mice and men Y chromosome gene deficiencies are associated with cellular abnormalities of the seminiferous epithelium, support a mouse as a model for human male infertility, and validates usefulness of a classic, immunostaining independent approach for the histological analysis of testis sections.
Section 5.2 Introduction

The Y chromosome is generally considered the prime mover for establishing the male fate in the developing embryo by initiating testis formation via the Y-linked gene \textit{Sry} [24, 107, 108]. One of primary responsibilities of the fully formed post-pubertal testis is to produce sperm throughout the life of the male thus conferring fertility. This sperm producing process, spermatogenesis, also has a host of Y-linked genes at the core of its initiation and regulation [25, 105, 253, 279]. Recent work has shown that Y chromosome genes fall into two main categories, retained ancestral genes and acquired ampliconic genes, and that their roles expand beyond testis formation and spermatogenesis to encompass viability of the male and sufficient expression dosage of genes involved in basic biomolecular pathways [4, 89, 90].

In both mice and men Y chromosome deletions are associated with infertility and spermatogenesis defects, the severity of which depend on which Y genes are missing. In men, Y deletions usually occur within the azoospermic factor (AZF) regions [95]. The human Y chromosome long arm has three AZF regions (AZFa, AZFb and AZFc) [105], with AZFa typically resulting in germ cell aplasia with a Sertoli cell only (SCO) syndrome while AZFb and AZFc deletions cause varying degrees of spermatogenic failure [92, 273]. In mice, deletions within the non-pairing Y chromosome long arm (NPYq) are associated with sub- or infertility and variable sperm defects [74, 77, 79, 80].

Previously, we demonstrated that only two Y-linked genes (\textit{Sry} and \textit{Eif2s3y}) are necessary to produce male mice (X\textit{E}O\textit{Sry}, Table 9) able to initiate spermatogenesis up to the round spermatid step, and that round spermatids from these males could be injected into oocytes using a technique called round spermatid injection (ROSI) to produce viable offspring [61]. More recently we demonstrated that the function of these two essential Y chromosome genes can be replaced by
transgenic activation of their homologues encoded on other chromosomes, $Sox9$ and $Eif2s3x$, respectively [3, 274]. Males carrying a single Y chromosome gene ($X^E OSox9$ and $XOSry,Eif2s3x$, Table 9) or no Y genes whatsoever ($XOSox9,Eif2s3x$, Table 9) produced round spermatids and sired offspring after ROSI [3].
Table 9. Summary characteristics of mice used in the study.

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Y gene contribution</th>
<th>Sex determination driver</th>
<th>Spermatogenesis driver</th>
<th>Spermatogenesis progression</th>
<th>Haploid round spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY</td>
<td>Intact Y</td>
<td>endogenous Sry</td>
<td>endogenous Eif2s3y</td>
<td>normal</td>
<td>frequent</td>
</tr>
<tr>
<td>X^{EOS}Sry</td>
<td>Eif2s3y &amp; Sry</td>
<td>Sry transgene</td>
<td>Eif2s3y transgene</td>
<td>impaired</td>
<td>rare</td>
</tr>
<tr>
<td>X^{EOS}Sox9</td>
<td>Eif2s3y</td>
<td>Sox9 transgene</td>
<td>Eif2s3x transgene</td>
<td>impaired</td>
<td>rare</td>
</tr>
<tr>
<td>XO,Sry,Eif2s3x</td>
<td>Sry</td>
<td>Sry transgene</td>
<td>Eif2s3x transgene</td>
<td>impaired</td>
<td>extremely rare</td>
</tr>
<tr>
<td>XO,Sox9,Eif2s3x</td>
<td>none</td>
<td>Sox9 transgene</td>
<td>Eif2s3x transgene</td>
<td>impaired</td>
<td>extremely rare</td>
</tr>
</tbody>
</table>
In our previous work we characterized spermatogenesis efficiency in male mice with two, one, or no Y chromosome genes focusing on counts of developing germ cells [3]. Here, we report on the assessment of testicular abnormalities in the same males. We demonstrated a relationship between the cellular and tissue abnormalities, and the Y chromosome gene contribution. We also performed a quantitative assessment of spermatogenesis and cellular abnormalities in human testicular biopsies, including biopsies from azoospermic men with Y chromosome deletions, and related those findings to our mouse data. Together, our data indicate that in both men and mice Y chromosome gene deficiencies are associated with cellular abnormalities of the seminiferous epithelium. For mice we also demonstrate a link between Y chromosome gene presence and the quality of the testicular interstitium.

Section 5.3 Materials and Methods

Part 5.3.1 Chemicals

See Chapter 2, Section 3, Part 1 for more information.

Part 5.3.2 Animals

See Chapter 2, Section 3, Part 2 for more information.

Part 5.3.3 Mouse Testis Histology Analysis

See Chapter 2, Section 3, Part 3 and Part 4 for more information on testicular material collection and preparation and general histology analysis.

Analysis of seminiferous epithelium abnormalities was done concurrently with quantitative analysis of spermatogenesis progression, such that any abnormal cells matching the categories described below were quantified while germ cell and Sertoli cell counts were being done. For the
analysis of testis interstitial tissue, three testis sections (>40 µm apart) per male were first viewed at low and high magnification to get a holistic sense of the interstitial tissue and then evaluated by assigning a numerical score for: Overall Appearance, Vasculature, and Macrophage Population.

**Overall Appearance** was defined on a scale of 0-10; where 10 is an XY testis appearance, 2 is the poorest quality testis, 1 is an ovotestis, and 0 is an ovary.

**Vasculature** was defined on a scale from 0-5, with 5 representing XY vasculature and 0 representing a complete lack of testis vasculature or presence of ovarian vasculature in place of testis vasculature structures. Increases in the amount of vasculature or the luminal diameter of dominant vasculature structures (a shift to more arterioles and less capillaries) was represented by the score approaching 1.

**Macrophage Population** was defined on a scale from 0-5, utilizing interstitial areas, which were zones of interstitial tissue usually bordered at least partially by seminiferous tubules. Score of 3 representing the XY testis of moderate macrophage appearance (at least 1 macrophage per 2-3 interstitial areas), 0 representing a near complete/complete lack of macrophages, and 5 representing an extreme excess of macrophages (>10 per interstitial area in most spaces).

Part 5.3.4 Immunohistochemistry

Immunohistochemistry was performed according to a standard protocol as described before [280]. The following additions were made to suit our specific needs: addition of nuclear opening step using a proteinase K digest solution (20 µg/mL Proteinase K in 10 mM Tris pH 7.5 and 5 mM EDTA) after rehydration and before antigen decloaking; blocking for endogenous avidin and biotin using a blocking kit (Life Technologies, Invitrogen, #00-4303) immediately before primary
antibody incubation; and counterstaining with Mayer’s Hematoxylin (MHS 16-500ml, Sigma-Aldrich, USA) instead of 0.5% crystal violet. Antibodies were diluted in a 10% serum (goat serum for anti-SOX9, and rabbit serum anti-GATA4) solution of phosphate buffered saline (PBS). Sections were incubated with rabbit polyclonal anti-human SOX9 antibody (1:100, AB5535, EMD Millipore Corporation, USA) [281] to detect Sertoli cells or goat polyclonal anti-mouse GATA-4 polyclonal antibody (1:200, sc-1237, Santa Cruz Biotechnology, USA) [280] to detect spermatids. Bound primary antibodies were detected using biotinylated anti-rabbit (1:500, sc-2040, Santa Cruz), or anti-goat (1:2000, sc-2274, Santa Cruz).

Part 5.3.5 Human Testis Biopsy Analysis

Human testis biopsies were fixed tissues archived by the Department of Pathology, Mount Sinai Hospital. The biopsies were obtained using micro-TESE and processed, either immediately or after storage in liquid nitrogen, for histological assessment using standard procedures. After fixation samples were embedded, sectioned, and stained with Hematoxylin and Eosin (H&E). For quantitative analysis of spermatogenesis suitable longitudinal sections were selected for each biopsy. Cellsens (Olympus) was used to calculate the surface area (μm²) of the seminiferous epithelium and cells were counted within the selected area. Each cell was classified as a specific cell type except diplotene which was omitted with the assumption that it was counted with pachytene spermatocytes. The cell counts data were expressed as cells per measured surface area ratios, which were then converted into cells per standard area (10,000 μm²) ratios.

Part 5.3.6 Statistics

Student's t-test was used for all analyses.

Section 5.4 Results
Part 5.4.1 Spermatogenesis Progression in Males with Limited Y Chromosome Contribution

Histological assessment of spermatogenesis was performed on testis sections from male mice with limited Y chromosome gene contribution (Table 9). As previously reported transgenic addition of Sry or Sox9 was sufficient to initiate and maintain male development while addition of Eif2s3y or Eif2s3x allowed for spermatogonial proliferation and differentiation in XO sex chromosome context [3, 61]. Males with Y chromosome contribution limited to two (X^EOSry), one (X^EOSox9 and XOSry,Eif2s3x), and no (XOSox9,Eif2s3x) Y chromosome genes had fewer spermatogonia than XY controls and although spermatogenesis was initiated, it did not progress efficiently resulting in a limited population of round spermatids (Table 10). These round spermatids never developed to spermatozoa, arresting at a round spermatid step. Furthermore, males transgenic for Eif2s3x had significantly depleted round spermatid populations compared to males transgenic for Eif2s3y (Table 10). The counts of testicular cells performed on PAS-H stained sections [3] were verified using immunohistochemistry with anti-SOX9 antibody for Sertoli cell detection and anti-GATA4 antibody for spermatid detection (Fig. 48). The counts of Sertoli cells in XY control males and the counts of spermatids in males with limited Y gene contribution were similar when defined with the two approaches (Fig. 48 and Fig. 49).

Part 5.4.2 Classification of Seminiferous Epithelium Abnormalities

Classic murine spermatogenesis staging relies on Period Acid Schiff (PAS) to identify the morphology of the acrosome. PAS staining can also be used to evaluate germ cell degeneration of testicular cells [282]. Here, we performed spermatogenesis staging and germ cell counts concurrently with the abnormality identification. Nuclear morphology, nucleosomal patterning, cell size, cell location and cytoplasmic appearance were used to identify cellular abnormalities in
the seminiferous tubules. We differentiated and quantified the following cellular defects: Sloughed Sertoli cell (SS), Apoptotic cell (AC), Apoptotic cell at meiotic metaphase (AM), Hypercondensed nucleus (HN), Degenerating Nucleus (DN), Multinucleated Giant Cell (MGC), Cell Remnant (CR) and Vacuole (V) (Fig. 50).

Figure 48. Verification of germ cell counts by immunohistochemistry. Testicular sections from selected males for which quantitative analysis of spermatogenesis progression was performed after PAS-H staining (Table 9, [3]) were used for immunostaining. A: SOX9 antibody was used to detect Sertoli cells in XY males. B-C: GATA4 antibody was used to identify spermatids in two males with limited Y chromosome contribution, X^6Osry (B) and XOSox9,Elf2s3x (C). D: Comparison of cell counts in testis sections stained with PAS-H and IHC revealed no differences between the two methods. Graphs bars are average ± SEM with n=3 males per genotype; for each male 60 tubules were included in analyses. Scale = 100 µm; scale shown in C applies to both B and C.
Figure 49. Verification of germ cell counts by immunohistochemistry. Testicular sections from selected males for which quantitative analysis of spermatogenesis progression was performed after PAS-H staining (Table 9; [3]) were examined using immunohistochemistry using SOX9 antibody to detect Sertoli cells and GATA4 antibody to identify spermatids. Comparison of cell counts after PAS-H staining and IHC revealed no differences between the two methods. Graphs bars are averages ± SEM, with n=3 males per genotype for IHC and n=3, 6, 13, and 10 for X^OSry, XOSry,Eif2s3x, and XOSox9,Eif2s3x for PAS-H; for each male 60 tubules were included in analyses. This figure is related to Fig. 56 C.
Figure 50. Types of abnormalities of seminiferous epithelium. Eight types of seminiferous epithelium defects were differentiated: sloughed Sertoli cell (SS), apoptotic cell (AC), apoptotic cell at meiotic metaphase (AM), hypercondensed nucleus (HN), decondensing nucleus (DN), multinucleated/giant cell (MGC), cell remnant (CR), and vacuole (V). Scale = 10 µm.
Table 10. Quantitative analysis of spermatogenesis progression.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spermatogonia/Sertoli cell ratio (mean ± SEM)</th>
<th>Round spermatid/Sertoli cell ratio (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XII-I</td>
<td>II-IV</td>
</tr>
<tr>
<td>XY (n=3)</td>
<td>0.41 ± 0.11</td>
<td>1.42 ± 0.13</td>
</tr>
<tr>
<td>X&lt;sup&gt;e&lt;/sup&gt;O&lt;sup&gt;Sry&lt;/sup&gt; (n=6)</td>
<td>0.36 ± 0.04</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>X&lt;sup&gt;e&lt;/sup&gt;O&lt;sup&gt;Sox9&lt;/sup&gt; (n=8)</td>
<td>0.15 ± 0.02</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>XO&lt;sup&gt;Sry, Eif2s3x&lt;/sup&gt; (n=13)</td>
<td>0.34 ± 0.03</td>
<td>0.92 ± 0.08</td>
</tr>
<tr>
<td>XO&lt;sup&gt;Sox9, Eif2s3x&lt;/sup&gt; (n=10)</td>
<td>0.28 ± 0.03</td>
<td>0.94 ± 0.08</td>
</tr>
</tbody>
</table>

For each male 10 tubules were examined per stage and the numbers of spermatogonia, round spermatids, and Sertoli cells were counted. The data are expressed as germ cell/Sertoli cell ratios. In wild-type males no round spermatids are present in stages IX-XI so those observed in the remaining genotypes represent 'delayed spermatids'. Statistical significance (t-test): * different than XY; & different than all other; a,b,c columns with different superscript letters are significantly different. The data shown in this table was published before as a supplementary material for Yamauchi et al. 2016 except for data for X<sup>e</sup>O<sup>Sry</sup> genotype for which we increased the number of males included in analysis from 3 published before to 6 [3].
Part 5.4.3 Effects of Limited Mouse Y Chromosome Gene Contribution on Incidence of Seminiferous Epithelium Defects

Wild-type XY males presented only occasional cellular abnormalities, which were limited to AC, AMN, HN, DN, and CR (Fig. 51A & D and Fig. 52). Transgenic males with limited Y gene contribution had overall significantly more abnormalities than XY (Fig. 51 D, P<0.05) and presented with all eight abnormality types (Fig. 51 B & C and Fig. 52). The defects in seminiferous epithelium distributed differently in males carrying different transgenes (Fig. 52). Although apoptotic cells were abundant in all transgenic males, as the amount of transgenes of Y chromosome origin declined apoptosis arose specifically in cells at meiotic metaphase (Fig. 52 and Fig. 53 L-O). Cells with hypercondensed nuclei were prevalent in males carrying the Eif2s3y transgene but their frequency decreased in males with the Eif2s3x transgene (Fig. 52).

Part 5.4.4 Effects of Limited Mouse Y Chromosome Gene Contribution on Testis Vasculature

With the decreasing Y chromosome gene number, a decline in the general quality of the interstitial tissue surrounding the seminiferous tubules was observed. In normal testes interstitial tissue contains the steroidogenic Leydig cells and macrophages along with capillaries and other vasculature structures (Fig. 54). In XY males this region is typically a small zone surrounded by seminiferous tubules (Fig. 53 A & F). Overall, in transgenic males, there was an inverse relationship between the number of Y genes and the size and general quality of the interstitial tissue regions (Fig. 53 B-E & G-J and Fig. 55). At low magnification patches of oversized interstitial tissue were apparent in all males with fewer than 2 Y chromosome genes (Fig. 53 C-E). XOSox9,Eif2s3x males lacking all Y chromosome genes had the most extreme phenotype
evidenced as large patches of interstitial tissue (Fig. 53 E & J). In some regions of XOSox9, Eif2s3x testis sections there were areas where instead of seminiferous tubules surrounding interstitial tissue the roles reversed and interstitial tissue instead surrounded seminiferous tubules (Fig. 53 E). Vasculature was the most prominent component of the interstitial tissue following the trend in decreasing quality with waning Y chromosome originating transgenes. As evidenced by first an increase in the number of capillaries and then a shift to a higher frequency of larger vessels (arterioles) as the number of Y derived transgenes was reduced (Fig. 53 G-J and Fig. 55). Macrophage populations were similar in wild-type and transgenic males (Fig. 53 F-J and Fig. 55).

Part 5.4.5 Differences in Human and Mouse Testes and Spermatogenesis Assessment

Mouse and human testes differ at the gross anatomy level. The seminiferous tubules of the mouse testis are generally arranged in loops that run from the superior to inferior pole of the testis (Fig. 19 A & C in Chapter 1) [29, 154-157] while the seminiferous tubules of the human testis are coiled in a series of radiating septa that have the rete testis as a focus (Fig. 19 B & D in Chapter 1) [164, 165]. Whereas a testis cross section from a mouse yields seminiferous tubules predominantly in cross section [19, 20, 29], this is not the case of a human testis cross section, which demonstrates a heterogeneous mix of seminiferous tubules in longitudinal, cross and oblique section [9, 162]. The seminiferous tubules of both species are also structurally divergent. In mice, stages of the seminiferous epithelium are arranged in tandem units along the length of mouse seminiferous tubules and in cross section typically demonstrate one stage per cross section (Fig. 22 A & B in Chapter 1) [19, 20, 29, 159]. In man, the stage arrangement is haphazard and occupies small independent zones (Fig. 22 C in Chapter 1) [158, 178], and a single human seminiferous tubule cross section typically represents 2-4 stages (Fig. 22 D in Chapter 1) [162, 179].
Figure 51. Males with limited Y gene contribution have increased incidence of seminiferous epithelium defects. Four types of males with limited or no Y gene complement (tested; \(X^e\text{OSry}\), \(X^e\text{OSox9}\), \(X\text{Sry},E\text{if2s3x}\) and \(X\text{OSox9,E}\text{if2s3x}\)) were compared to wild-type XY males (control). A-C: Exemplary seminiferous tubule cross sections showing few cellular defects in XY (A) and abundance of abnormalities in \(X^e\text{OSry}\) (B) and \(X\text{OSox9,E}\text{if2s3x}\) (C) males. Tubules stages are IX-X in A & B, and XI in C. D: Quantification of abnormalities in genotypes of interest. Bars are averages ± SEM with the number of males shown under the X axis. For each male 60 tubules, 10 of each stage cluster (XII-I, II-IV, V-VI, VII-VIII, IX-X, XI), were scored. Statistical significance (t-test, P<0.05): a different than all other. Scale = 100 µm. AC, apoptotic cell; DN, decondensing nucleus; HN, hypercondensed nucleus; MGC, multinucleated giant cell; SS, sloughed Sertoli; V, vacuole. For more information about abnormality types see Fig. 50.
Figure 52. Distribution of different abnormality types. Four types of males with limited or no Y gene complement (tested; X^E'O^E'Sry, X^E'O^E'Sox9, X^E'O^E'Sry, Eif2s3x and X^E'O^E'Sox9,Eif2s3x) were compared to wild-type XY males (control). Eight different abnormality types were differentiated (AC, apoptotic cell; AM, apoptotic cell at meiotic metaphase; DN, decondensing nucleus; HN, hypercondensed nucleus; MGC, multinucleated giant cell; CR, cell remnant; SS, sloughed Sertoli; V, vacuole) and their frequency is shown as a percentage of all defects. Graphs are average ± SEM with number of males shown under the X axis. For each male sixty tubules, ten of each stage cluster (XII-I, II-IV, V-VI, VII-VIII, IX-X, XI), were scored. Statistical significance (t-test, P<0.05): a different than all other; b different than all other except XY; c different than XY; d different than others except each other, or as indicated by horizontal line. For more information about abnormality types see Fig. 50.
Figure 53. Interstitial tissue abnormalities and apoptosis in males with limited Y chromosome complement. Four types of transgenic males with limited or no Y gene complement (tested; X^E OSry, X^E OSox9, XOSry,Eif2s3x and XOSox9,Eif2s3x) were compared to wild-type XY males (control). A-E: In testis cross-section panoramics X^E OSox9, XOSry,Eif2s3x and XOSox9,Eif2s3x males had interstitial tissue of poorer quality when compared to XY and X^E OSry, evidenced as an increase in its amount (*). F-J: A specific quality change was observed in interstitial tissue vasculature with XY and X^E OSry showing normal vasculature size and normal capillary (Ca) number, X^E OSox9 presenting with abundant capillaries, and XOSry,Eif2s3x and XOSox9,Eif2s3x males having arterioles (Ar) instead of capillaries. Macrophages (Ma) and Leydig cells (Le) were present in all males, with increase in Leydig cell in par with increase in interstitial tissue volume. K-O: Representative tubule cross-sections (K-O) featuring lack of cellular defects in XY, presence of apoptotic cells (AC) in X^E OSry and X^E OSox9, and abundance of apoptotic cells at meiotic metaphase (AM) in XOSry,Eif2s3x and XOSox9,Eif2s3x males. Tubules stages in K-O are XII-I (K, N, O), IX-X (L), XI (M). Scale = 400 µm (A-E, shown in E) and 100 µm (F-O, shown in O).
Figure 54. Interstitial tissue appearance. An example of typical interstitial tissue appearance with Leydig cells (L), macrophages (M) and capillaries (C) observed in XY testis. Scale = 20 µm.
Figure 55. Semi-quantititative interstitial tissue assessment in males with limited Y chromosome complement. Four types of transgenic males with limited or no Y gene complement (tested; XEOSry, XEOSox9, XOSry,Eif2s3x and XOSox9,Eif2s3x) were compared to wild-type XY males (control) were compared. Interstitial tissue was assessed using Overall Score (scale 0-10; 10 = XY testis, 2 = poorest quality testis, 1 = ovotestis, and 0 = ovary), Vasculature Score (scale 0-5; 5 = XY and 0 = complete lack of vasculature/ovary vasculature structure), Macrophage Score (scale 0-5; 3 = XY phenotype with moderate macrophage appearance, at least 1 macrophage per 2-3 interstitial areas; 0 = near complete/complete lack of macrophages, and 5 = extreme excess of macrophages, >10 per interstitial area in most spaces). Bars are averages ± SEM with the number of examined males shown under the X axis; for each male 3 sections were analyzed. Statistical significance: a different than XY and XEOSry; b different than XOSox9,Eif2s3x.
For histology assessment involving staging, PAS-H staining which recognizes acrosome is used for mouse testis. The human acrosome is too delicate to survive the fixation, embedding and sectioning process, so instead of PAS-H, H&E is commonly used [162, 174]. Human spermatogenesis staging is therefore highly dependent on germ cell identification based on nuclear morphology and cellular associations and lacks determination based on acrosome morphology as is used with mice [1, 8, 162]. Human spermatogenesis is classified as only 6 stages (I-VI) and there are 6 steps of spermatid development (Sa, Sb1, Sb2, Sc, Sd1 and Sd2) (Fig. 19 A in Chapter 1) while in mice 12 spermatogenesis stages (I-XII) and 16 steps (1-16) of spermatid development are recognized (Fig. 19 B in Chapter 1).

In addition to anatomical, structural and histological differences between mouse and human testes, testis biopsy offers significantly less tissue to work with than a testis from an orchidectomy. Over the decades a few different methods for quantitative analysis of a testis biopsy have been demonstrated [1, 2, 8, 17, 181, 183]. We opted to use the system developed by Rowley and Heller as our model for assessment in testis biopsies from normal and infertile men [8]. To be considered suitable for analysis the tubules needed (1) a clear lumina; (2) the germinal epithelium needed to be minimally thick or at least a constant thickness; (3) incomplete or mixed stages were acceptable. The tubules were considered not suitable for analysis when (4) they were tangentially or partially cut; (5) more than one layer of spermatogonia was present; and (6) the basement membrane was wide. Based on these criteria, when all tubules were scored as unsuitable the entire biopsy had to be excluded from the analysis. The exceptions for (1) and (6) were made when assessing biopsies from infertile men because sloughed cells could fill the lumen and the basement membrane could be wide due to hyalinization. This system was used to determine if biopsies and
specific tubules were suitable for our quantitative analysis (Fig. 56 and Fig. 57 A). Once deemed suitable our assessment deviated from that of Rowley and Heller as we quantified germ cells by surface area of seminiferous epithelium in the selected tubule (Fig 57 A & B) instead of by Sertoli cell or length of the selected tubule.

Figure 56. Suitability of testicular biopsies for quantitative histological assessment. A-D: Exemplary photos of sectioned testicular biopsies. Biopsy sections from normal (A) and infertile (B) men with all tubules deemed unsuitable; these samples had to be excluded from the analysis. Biopsy sections from normal (C) and infertile (D) men containing both suitable (bottom insets) and unsuitable (top insets) tubules. Panel D shows a biopsy with sparsely scattered tubules; although there are multiple sections including some scored as suitable they are of the same few tubules. Use of the same tubule in different sections for cell counts is not compatible with quantitative analysis which resulted in exclusion of this sample. Scale = 400 µm (panels) and 100 µm (insets).
Figure 57. Schematic of an approach for quantitative analysis of human spermatogenesis. A: Diagrammatic representation of the approach. Testis biopsy was processed using a standard protocol, with tissue fixed in Bouin’s fixative, sectioned at 5 µm, and stained with H&E. All obtained sections were examined microscopically and the longitudinal sections of seminiferous tubules suitable for quantitative analysis were selected. The surface area of the selected tubules was then identified and measured, and the cells were counted. The cell counts were expressed as per area. B: Exemplary tubule suitable for spermatogenesis analysis. The surface area of the of the seminiferous epithelium within which cells were counted was calculated by subtracting the area consisting of lumen (2) from the total area (1) using cellSens (Olympus). Scale = 50 µm.
Part 5.4.6 Human Spermatogenesis Assessment in Testis Biopsies from Normal and Infertile Men

Thirty-seven biopsies were subjected to evaluation, 16 from normal control and 21 from azoospermic infertile men (Table 11). Among the infertile men 12 had changes within the AZF region (6 AZFc deletions, 3 AZFc gains, 1 combined AZFb/AZFc deletions, 2 combined AZFa/AZFb/AZFc deletions). As expected, normal biopsies exhibited seminiferous tubules with active and complete spermatogenesis (Fig. 58 A). Infertile men with deletions in the AZFa,b and c regions exhibited seminiferous tubules that lacked germ cells and resulted in a Sertoli cell only (SCO) phenotype (Fig. 58 B). Infertile men with deletions only in the AZFc region exhibited hypospermatogenesis, which was often paired with a maturation arrest (Fig. 58 C). There was also a subset of cases who lacked AZF deletions but exhibited hypospermatogenesis, which was often paired with a maturation arrest similar to AZFc deletion cases (Table 11 and Fig 58 D).

For quantitative analysis all biopsies were first assessed for their suitability; 10 normal biopsies and 9 biopsies from infertile men were deemed suitable (Table 11, Fig. 56). Among the suitable biopsies from infertile men, 6 had SCO and were therefore excluded from the quantitative analysis. For the remaining 3 biopsies from infertile men and 10 normal biopsies, Sertoli cells and germ cells were counted within a specific area and the counts were expressed per area. In normal biopsies quantitative analysis revealed an approximate 1:1:2:2:3 ratio for Sertoli cells, spermatogonia, spermatocytes, round spermatids and elongated spermatids respectively in normal biopsies (Fig. 59). This was not the case for hypospermatogenic cases, which had a 2:1:1:0:0 ratio with significantly fewer spermatocytes, round spermatids and elongated spermatids per 10,000
µm² when compared to normal cases (P < 0.05) (Fig. 59). When individual testicular cell populations were compared the hypospermatogenic cases had a clear maturation arrest before reaching the spermatid step and a significant decrease in pachytene spermatocyte population when compared to normal cases (Fig. 60, P < 0.05).

Figure 58. Exemplary images of seminiferous tubules from human biopsies. A: Complete spermatogenesis with organized germ cells and good quality seminiferous epithelium. B: Complete germ cell aplasia and SCO phenotype in an infertile man with deletions in the AZFa, AZFb and AZFc regions. C: Hypospermatogenesis with an arrest at the spermatocyte stage seen in an infertile man with a deletion in the AZFc region. D: Hypospermatogenesis with an arrest at the spermatocyte stage in an infertile man for whom no deletions in the AZF regions were detected. Scale = 50 µm.
Figure 59. Cell counts in human testis biopsies. The numbers of Sertoli and germ cells were quantified for normal (n=10) and hypospermatogenic (n=3) human testis biopsies. The counts were expressed per surface area. The surface area examined was as follows: range 34,966 - 122,790 µm²; mean ± SEM 76,906 ± 7,380 µm² for normal, and range 29,677 - 50,503 µm², mean ± SEM 36,698 ± 6,903 µm² for hypospermatogenic. For details on the surface measurement see Fig. 57. For examples of cell types see Fig. 8. Bars are mean ± SEM. Statistical significance (t-test, P<0.05): a different than the same cell type in normal.
Figure 60. Cell counts in human testis biopsies. The numbers of Sertoli and germ cells were quantified for normal (n=10) and hypospermatogenic (n=3) human testis biopsies. The counts were expressed per surface area. The surface area examined was as follows: range 34,966 - 122,790 µm²; mean ± SEM 76,906 ± 7,380 µm² for normal, and range 29,677 - 50,503 µm², mean ± SEM 36,698 ± 6,903 µm² for hypospermatogenic. For details on the surface measurement see Fig. 57. Ad, Ap and B are type A dark, type A pale and type B spermatogonia. Pl, L, Z, P, M, and 2º are preleptotene, leptotene, zygotene, pachytene, meiotic metaphase, and secondary spermatocytes, respectively. Sa, Sb1, Sb2, Sc, Sd1 and Sd2 are steps of spermatid development. D, and meiotic anaphase (A) spermatocytes were counted together with pachytene and meiotic metaphase spermatocytes, respectively. For more information about germ cell types see Fig. 24 A in Chapter 1. Bars are mean ± SEM. Statistical significance (t-test, P<0.05): a different than the same cell type in normal.
Table 11. Summary characteristics of human biopsy samples used in the study.

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Part 5.4.7 Cellular Abnormalities of the Seminiferous Epithelium in Testis Biopsies from Normal and Infertile Men

Human biopsies were stained with H&E and classification of abnormalities was based on cellular morphology and location as well as nuclear morphology and patterning. We differentiated and quantified six cellular defects: Sloughed Sertoli cell (SS), Apoptotic cell (AC), Hypercondensed nucleus (HN), Degenerating Nucleus (DN), Multinucleated Giant Cell (MGC), and Vacuole (V) (Fig. 61 A). The categories used with men were relatively similar to those described for mice (Fig. 50 vs. Fig. 61 A). Normal and hypospermatogenic men demonstrated different abnormality distributions (Fig. 61 B). The majority of the abnormalities in normal cases were either hypercondensed nuclei or vacuoles. For hypospermatogenic cases decondensing nuclei and vacuoles made up the majority of abnormalities. Overall normal biopsies had significantly less abnormalities than those from hypospermatogenic men (mean ± SEM; 0.38 ± 0.09/10,000 µm² vs. 2.64 ± 0.55/10,000 µm², P < 0.001).

Section 5.5 Discussion

Here we present the comprehensive histological assessment of testes from mice with limited and no Y chromosome contribution [3, 61] (Table 9). We observed an inverse relationship between the number Y chromosome derived transgenes and the incidence and severity of testicular abnormalities. We also noted differences between transgenic mice employing two different spermatogenesis drivers, Y chromosome derived Eif2s3y and its X chromosome derived Eif2s3x. Finally, we related our mouse data to quantitative assessment of human testis biopsies from fertile and infertile men.
Figure 61. Distribution of different abnormality types in human biopsies. A: Examples of abnormality types. Six different abnormality types were differentiated (AC, apoptotic cell; DN, decondensing nucleus; HN, hypercondensed nucleus; MGC, multinucleated giant cell; SS, sloughed Sertoli; V, vacuole). Cellular abnormality identification in human spermatogenesis done using H&E stained sections shared key traits with mouse spermatogenesis evaluation done using PAS-H stained sections (see Fig. 50) with exclusions and additions, as follows: SS: the cell body must be obviously detached or part of an aggregate of Sertoli cells in a bundle/nodule and the distance of the nucleus from the basal lamina is no longer an indicator; AC: pink to fuchsia cytoplasmic color is not an indicator; DN: cellular ballooning and cytoplasmic halo are not indicators; HN: when HN is similar in size to a nucleus of late spermatid, cell location is considered (i.e. occurrence near basal lamina being indicative of abnormality); MGC: cells with a single visible nucleus that are well beyond normal size and cells with clearly visible multiple nuclei that are of relatively normal size for the specific cell type are not included in this category. Scale: SS = 10 µm, all other = 5 µm. B: The distribution of abnormality types was evaluated for normal and hypospermatogenic human testis biopsies. The frequency of abnormality types is shown as an average percentage of all defects, with n=10 for normal and n=3 for hypospermatogenic. The abnormalities were counted and counts expressed per surface area. The surface area examined was as follows: range 34,966 - 122,790 µm²; mean ± SEM 76,906 ± 7,380 µm² for normal, and range 29,677 - 50,503 µm², mean ± SEM 36,698 ± 6,903 µm² for hypospermatogenic. For details on the surface measurement see Fig. 57.
Mice with the Y chromosome contribution limited to two, one or no Y genes were previously shown to have meiotic and post-meiotic spermatogenesis arrests [3, 61]. The meiotic arrest was incomplete with a limited population of spermatocytes proceeding to round spermatid steps. When spermatogenesis progression efficiency was quantified as germ cell/Sertoli cell ratio on PAS-H stained testicular sections, all transgenic males had fewer spermatogonia and round spermatids than wild-type males. Moreover, males with spermatogonial proliferation and differentiation driven by the X chromosome derived Eif2s3x transgene had fewer spermatids than males with the Y derived transgene Eif2s3y [3, 61]. With histological assessment done solely on conventionally stained sections there are often concerns regarding proper cell recognition. Here, to address this, we verified previous PAS-H Sertoli cell and round spermatid counts using immunostaining. This analysis corroborated the differences between the Eif2s3x and Eif2s3y transgenics in respect to the efficiency of meiotic progression and reinforced the appropriateness of PAS-H staining for testicular cell identification.

In addition to poor spermatogenesis efficiency, males with limited Y chromosome contribution had severe testicular abnormalities. We quantified these abnormalities on PAS-H stained sections. PAS-H staining has been previously used to evaluate germ cell degeneration [282] and allowed us to distinguish several seminiferous epithelium defects, such as apoptotic cells, hypercondensing and degenerating nuclei, multinucleated giant cells, and cell remnants. Some of these testicular defects were previously described by others [283-288]. In these studies, usually only one specific abnormality type was described and linked to the perturbation of one or two spermatogenesis genes. Mice with limited Y gene contribution presented with an entire spectrum of defects, with the distribution variable among the genotypes.
It has been previously shown that meiotic arrest can be accompanied by increased incidence of apoptotic germ cells [287, 289, 290]. Here, all transgenic males had significantly more cellular apoptosis when compared to XY controls. Testicular apoptosis was reported before for 4-7 week old mice with Eif2s3y and Sry as the sole Y genes [25, 35, 243] and our analysis of this genotype performed with mature males is in agreement with the previous data. Interestingly, when we compared transgenics to each other, we observed that males carrying the Eif2s3x transgene had an abundance of apoptotic germ cells specifically at meiotic metaphase; that was not observed in Eif2s3y transgenics. Separation of homologous chromosomes at anaphase I is governed by the spindle checkpoint with meiotic arrest and apoptotic elimination of cells with univalent chromosomes [291, 292]. In our transgenic mice some cells were able to leak through this arrest evading the MI spindle checkpoint, with the efficiency of this escape different between the Eif2s3y and Eif2s3x transgenics. Thus, poorer progression through meiosis and by extension significantly smaller round spermatid populations in Eif2s3x transgenics might result from germ cell apoptosis specifically at meiotic metaphase which further diminishes the already limited population of spermatocytes that leak through the meiotic arrest.

Hypercondensed nuclei result from chromatin condensation and in normal spermatogenesis chromatin condensation is a critical part of spermatid elongation. In mice with limited Y gene contribution, cells with hypercondensed nuclei were frequently observed. Since spermatids from these males arrest at step 7 of development and never elongate, the affected cells must represent round spermatids or earlier germ cell stages. Presence of hypercondensed nuclei in cells other than elongated spermatids may be indicative of an attempt by these cells to undergo spermiogenesis and condense their chromatin prematurely. We previously reported that in males lacking the Y chromosome and carrying instead the sex reversal factor Sxr\textsuperscript{b} (encoding for Sry,
H2al2y, Prsly, Teyorf1, Rbmy gene cluster and Zfy2/1 fusion gene) diploid cells can undergo elongation and develop into sperm [248]. In this same study we have shown that round spermatids may undergo chromatin condensation without preceding spermatid elongation. A similar phenomena was also reported for HSP70-2 null mice, which have a leaky meiotic arrest at pachytene with limited populations of germ cells progressing to form an acrosome and begin nuclear condensation sometimes after completing meiosis but at other times while still diploid [293]. Here, the genotype comparison revealed that cells with hypercondensed nuclei were more abundant in the Eif2s3y transgenics than in the Eif2s3x transgenics. Since the former males have more spermatids, the increase in the number of cells developing hypercondensed nuclei might be simply reflective of spermatid abundance. This goes in par with the Eif2s3y transgenics having better progression through meiosis and better spermatogenesis overall. In XY males hypercondensed nuclei in cells other than elongated spermatids were rarely observed.

Limited Y chromosome gene contribution affected not only germ cells but also interstitial tissue. Interstitial tissue of the testis contains Leydig cells, macrophages, myoid cells and vasculature [294]. A major role for this tissue is the steroidogenic regulation of spermatogenesis in the seminiferous tubules as well as delivery of other standard blood components [132, 295]. We noted that as the number of Y derived transgenes decreased the regions of interstitial tissue increased in size transitioning from islands of interstitial tissue into large patches. As these patches increased in size they eventually began to surround tubules.

In both mice and men impaired spermatogenesis has been previously linked with interstitial hyperplasia and Leydig cell micronodule formation, which can be indicative of testicular dysgenesis syndrome [275, 296, 297]. Here, in most of the cases, interstitial tissue increase appeared to be cellular and not presenting with excessive connective tissue. The increase in size
of interstitial tissue may impair spermatogenesis through an overabundance of the steroidogenic Leydig cells. Testosterone regulation of spermatogenesis is a balancing act, and both too much and too little decreases spermatogenesis efficiency [296, 298-302].

Testis vasculature is known to deliver oxygen, nutrients and gonadotropins to the testis as well as removing metabolic waste and transporting testosterone systemically [303, 304]. Vasculature also plays an important role in guiding testis morphogenesis [305, 306]. Issues with testicular vasculature such as varicocele, atherosclerosis and impaired venous drainage have been shown to affect fertility [303, 307-309]. In mice with limited Y chromosome gene contributions we observed a deviation from a typical XY vasculature. Similarly, as it was with general interstitial tissue quality decrease, vasculature impairment became pronounced in parallel to decrease in the amount of Y derived transgenes. Sox9 is known to have an inhibitory interaction with Wnt4, and by extension β-catenin, during normal testis development [310-312]. Our Sox9 transgenic males (especially males lacking all Y genes) could have impairment in vasculature development during fetal life as has been observed before in mice with perturbation of Wnt and Sox9 [274, 312-314]. There is a growing body of evidence that vasculature in concert with Sertoli cells are involved in regulating the spermatogonial stem cell niche [178, 180, 315-318]. Thus, the changes in vasculature observed here may contribute to the spermatogonia reduction in transgenic males.

Macrophages are part of cellular maintenance of immunoprivilege in the testis and are associated with Leydig cells as part of Leydig cell steroidogenesis [319-322]. Testicular macrophages play roles in vascularization and morphogenesis during fetal development and the putative sub-class of peritubular macrophages (as oppose to putative interstitial macrophages) was proposed to be linked to spermatogonial differentiation in adults [294, 323]. In our study we did not observe changes in macrophage population between XY and transgenic males. This suggests
that Y chromosome encoded regulators of testicular cell development are not involved in regulation of macrophages. However, this will need to be investigated further as we have not examined putative peritubular macrophages because their morphology (long, slender and flattened) makes it difficult to distinguish them from peritubular myoid cells without immunofluorescent markers [294].

We observed differences in both the efficiency of spermatogenesis progression and the type and abundance of testicular abnormalities between *Eif2s3y* and *Eif2s3x* transgenics. *Eif2s3y* and *Eif2s3x* belong to a family of eukaryotic translation initiation factors (eukaryotic translation initiation factors 2 subunit 3 (*Eif2s3*) protein family). The two genes share high sequence identity, 98% amino acid identity, despite only 86% nucleotide identity, an indication of strong selective constraint on both proteins [126]. Both genes are ubiquitously expressed with strong expression in germ cells, and represent a typical ancestral, single copy X-Y homologous gene pair, in which the X gene escapes X chromosome inactivation, and the maintenance of two doses of *Eif2s3* gene product provides a selective advantage in both sexes [4, 89]. Such genes are hypothesized to be functionally redundant and we have shown that *Eif2s3x* can indeed substitute for the *Eif2s3y* in regulating spermatogonial proliferation and differentiation but only when sufficiently overexpressed [3]. Thus, the two genes are functionally redundant but evolved distinct expression level. The global level of *Eif2s3x/y* transcripts are significantly higher in the *Eif2s3y* transgenics as compared to *Eif2s3x* transgenics and we suggested that this might be a reason for poorer spermatogenesis progression through meiosis in the latter males [3]. Here we extend this explanation to the observed differences in testicular abnormalities between the two types of transgenics.
We previously proposed that mouse models with limited Y chromosome gene contribution and associated meiotic and post-meiotic arrests can be considered models for azoospermia in humans [3, 61]. To substantiate this hypothesis, we histologically examined testicular biopsies from fertile men and azoospermic men with and without Y chromosome deletions. The azoospermic men presented with hypospermatogenesis, maturation arrest and Sertoli cell only (SCO) phenotype. The two men with combined AZFa/AZFb/AZFc deletions exhibited the expected SCO phenotype, which looked similar to our previously described XO Sry mice [3]. Men who presented with hypospermatogenesis and maturation arrest were spermatogenically reminiscent of the male mice with Y gene contribution limited to two, one or no Y genes, all of which have a leaky arrest at meiotic metaphase [3]. Our stringency parameters removed a number of the biopsy samples from our pool of assessed samples rendering statistical analysis within specific Y chromosome deficiency subsets not possible. However, when assessed qualitatively, the infertile samples deemed unsuitable allowed for some observations. Some of the excluded infertile samples were from men with AZFc deletion, and although there was not enough tissue for quantitative analysis they appeared hypospermatogenic. Interestingly two of the samples from men with a putative gain of AZFc were spermatogenically divergent, one presenting SCO and the other hypospermatogenesis (ages 35 and 37 respectively),

We expected biopsies from hypospermatogenic men to contain more abnormalities than those from fertile men. Human spermatogenesis is more prone to germ cell apoptosis than other male mammals such as mice and rats [182, 183]. In infertile men it is not just an impairment in spermatogenesis efficiency that decreases the germ cell number but also excessive apoptosis [324]. Infertile men can also present with a spectrum of cellular abnormalities as indicated in testis biopsy assessments [273, 307, 325-327]. Here, we demonstrated that many of the abnormalities we
observed in men correlated almost directly with a subset of the abnormalities found in mice with limited Y gene contribution. The histological findings for men and mice reported here lend more support to the appropriateness of mouse models for human male infertility.

While pursuing human testis biopsy analyses, we developed and described a modern adaptation to a tried and true method for spermatogenesis assessment in men [8]. The adapted method continues to utilize the limited amount of tissue present in a testis biopsy, which is more readily obtained than a testis from orchidectomy from men being screened for infertility. Further, we attempted to provide a novel amalgamation of the cellular abnormalities that can be found in the seminiferous epithelium of mice and men. This was done with color images of example cells in their most common staining environment with the addition of detailed description to guide histologists in making determinations about a suspect cell. Our adapted method can be considered a new tool at the disposal of any histologists who find themselves charged with the laudable task of doing comprehensive spermatogenesis analysis using human testis biopsies.

Testis histology assessment has changed with the advent of fluorescence and availability of molecular markers. The classic histology approach, relying on recognition of the subtleties of a cell for identification, is becoming a dying art. Here we demonstrated, in mice and human, the power of classic histology using PAS-H and H&E by doing a multi-parameter assessment to quickly gain a holistic in depth view of the testis and the spermatogenesis within, in lieu of a series of molecular investigations answering one question at a time.

Section 5.6 Contributions

Dr. Monika A. Ward – P.I., lead author

Torbjoern Nielsen – assistance with all immunohistochemistry data
Dr. Jonathan M. Riel – all expression data

Dr. Yasuhiro Yamauchi – all ART related work

Dr. Keith Jarvi – provided human testis biopsy samples
CHAPTER 6. MACROSCOPIC DEMONSTRATION OF THE MALE UROGENITAL SYSTEM WITH EVIDENCE OF A DIRECT INGUINAL HERNIA UTILIZING ROOM TEMPERATURE PLASTINATION


Section 6.1 Abstract

The male urogenital system represents a morphologically complex region that arises from a common embryological origin. However, it is typically studied separately as the excretory system is dissected with the posterior wall of the abdomen while the reproductive features are exposed with the pelvis and perineum dissection. Additionally, the reproductive structures are typically dissected following pelvic and perineal hemisection obviating a comprehensive and holistic examination. Here we performed a dissection of the complete male urogenital system utilizing a 70-year-old donor and room temperature silicon plastination. Identification of a direct inguinal hernia during the dissection facilitated a unique opportunity to incorporate a common abdominal wall defect into the plastination requiring a novel approach to retain patency of relevant structures. Results showed that the typical structures identified in medical gross anatomy were retained in addition to the hernia. Thus, the described approach and the resulting specimen provide valuable and versatile teaching tools for male urogenital anatomy.

Section 6.2 Introduction

In the adult male the unity between the renal and genital systems and their supporting structures is somewhat subtle. However, the integration of these two systems is quite clear during the embryonic and fetal periods. There is a close developmental association between embryonic
kidneys and primitive gonads. Both originate from the intermediate mesoderm in the pelvic region. However, after forming, the embryonic kidneys and testes migrate in opposite directions as their development continues [328, 329]. Between gestational week (gw) 6 and 9 the kidneys ascend into the abdominal cavity [328, 330]. By gw 6, in the male fetus, the primitive gonads are directed towards the testis fate by the Y chromosome, and specifically Y-encoded SRY [24, 329]. The testes descend abdominally between gw 8 to 12 [329]. Once the testes enter the inguinal canal around gw 24, they contribute to the formation of the canal as they migrate inferomedially to enter the scrotum by gw 35 [331]. The common developmental origin of the kidneys and testes leads to common excretory sites for both systems while their divergent migration patterns result in long testicular vasculature extending from upper-mid abdomen to scrotum [328, 329]. In the adult male, the urethra is the common exit site for products of the kidneys and testes with their accessory glands. There is also a hormonal relationship between these two regions. From an endocrine perspective, the primary source of male testosterone are the Leydig cells of the testis [332]. However, the adrenal glands that are positioned superior to the kidneys also serve as a source of androgens in the adult [333, 334].

The common developmental origin of the testes and kidneys results in a persistent link in the adult. Because of this inherent association it seems logical to showcase these two systems in a single urogenital dissection. The involvement of the endocrine system and influence of migration on the vascular systems of these organs during development also makes a clear case for maintaining pertinent vasculature with a urogenital dissection. However, general instructional dissection is usually performed regionally such that the kidneys and adrenal glands, and the pelvis and perineum are investigated separately [335]. This type of division precludes observing the connectivity of structures in these two regions. For example, the typical procedure for viewing the
erectile bodies of the penis is achieved through cross-sectional excision the body [335]. This approach obscures the longitudinal spatial relationships between the corpus spongiosum and the corpora cavernosa, particularly at the dorsal base of the glans. Likewise, the testes and accessory glands are often dissected as individual components so that the concept of their inherent connectedness is lost to the dissector and viewers.

The other common method for viewing the male genital system follows transverse sectioning at approximately L5 (lumbar vertebra 5) with subsequent hemisectioning of the pelvis. While a hemisection allows the observer an excellent in situ view of the main focal points of the male genitalia, the kidneys are often missing from this approach, as well as the vasculature of the testis. At the same time the inclusion of the bones and ligaments of the pelvis obscures spatial relationships between the bladder, prostate, urethra, erectile bodies, and muscles at the bulb and crura of the penis.

A dissection that retains the entire urogenital system and its associated vasculature as one entire unit would allow for a readily apparent comprehensive understanding of the interlinking between the renal and male reproductive systems along with the impact their embryological development has on the accompanying vasculature in the adult.

Here, our first goal was to perform a dissection that maintained connections between urogenital components so that critical spatial relationships between distant regions would be readily apparent. Our second goal was to eliminate non-urogenital structures to facilitate a 360-degree view of the entire system while avoiding organ transection in an effort to maintain normal morphology. Our third goal was to increase the instructional collection of anatomical plastinations for educational purposes among medical and allied medical students at our institution. During the dissection, a left direct inguinal hernia was discovered providing a unique opportunity to
successfully preserve a hernia and its patency in the human male. To our knowledge this study is the first description of plastination of a direct inguinal hernia except what seems to be an anecdotal observation of a hernia at a plastination demonstration [336, 337]. Thus, our fourth and final goal was to devise a method to preserve the direct hernia so that it could be demonstrated within the final plastinated specimen.

Section 6.3 Materials and Methods

Part 6.3.1 Anatomical Specimen

All procedures were performed in accordance with protocols approved by the University of Hawai‘i Biosafety Committee. Human anatomical material was utilized following standard operating procedures of the JABSOM Willed Body Program [338].

Part 6.3.2 Embalming

This part can be found in Chapter 8, Section 3, Part 1.

Part 6.3.3 Dissection

The dissection protocol was derived from several sources including: Clark’s Anatomy and Physiology, Moore’s Clinically Oriented Anatomy, Gilroy’s Atlas of Anatomy, Tank’s Dissector, and The Physiology of Reproduction [164, 335, 339-342].

6.3.3.1 Cutaneous Tissue Removal

Skin was removed from the abdominal, anterior and lateral pelvic, and thigh regions but the skin and fascia of the penis, scrotum and perineum were retained.
6.3.3.2 Excising the Abdomen

An excision was made along the midline of the abdomen that ran from xiphoid process to pubic symphysis. The superior cut was extended laterally following the path of the costal margin. The inferior cut was extended laterally parallel but superior to the inguinal canal medially and superior to the inguinal ligament laterally.

6.3.3.3 Preparing the Abdomen

Once the abdomen was opened, the alimentary canal and associated organs were removed from the abdominal cavity. The abdominal aorta and inferior vena cava were isolated. Both right and left kidney and adrenal glands were identified and the left and right renal arteries and veins were traced back to the abdominal aorta and inferior vena cava, respectively.

6.3.3.4 Kidney and Vessel Release

The kidneys, renal vessels and ureters were manually released from the renal capsule and surrounding fascia while the adrenal attachment with the kidney was preserved. The right and left testicular veins were identified and retained at their origins. A pair of inferior incisions were performed along the internal and external iliac arteries and veins approximately 6 cm distal to their bifurcation at the common iliac artery and vein, respectively. Remaining vessels were removed.

6.3.3.5 Detachment of the Rectum and Anus

The legs were maximally abducted and a superficial incision was made that encircled the posterior scrotum. Externally the skin of the perineum was carefully reflected, up to the perimeter of the anus. Internally the connective tissue surrounding the anus and rectum was excised and both structures were removed from the perineum.
6.3.3.6 Degloving the Penis

The skin around the root of the penis was reflected, except for skin of the scrotum. The penis was degloved by cutting the fascia (Buck’s fascia) separating the skin from the erectile bodies; attachment to the scrotum was retained to facilitate removal of scrotal skin subsequently. Complete degloving was achieved by releasing the foreskin.

6.3.3.7 Removing the Scrotum and Freeing the Testes

The skin of the scrotum was removed from the testes by excising the underlying dartos fascia. Care was taken to preserve the testes and spermatic cord structures including the pampiniform plexus, epididymides and ductus deferentes. Palpation was then used to locate the spermatic cord inside the inguinal canal. Palpation along the path of the spermatic cord was also used to bluntly check for the presence of any hernias. Once each testis was released, the spermatic cord was followed superiorly and loosened manually.

6.3.3.8 Removal of the Urogenital System and Vasculature from the Body

The testes and spermatic cord were reflected superiorly into the abdominal cavity. Bone cutters were used to excise the left and right superior pubic and ischiopubic rami. The entire urogenital system and vasculature were then removed from the abdominal and pelvic cavities.

6.3.3.9 Bone Removal and Perineal Muscle Dissection

The bulbospongiousus muscle and ischiocavernosus muscles were separated from the ischiopubic ramus as completely as possible, while simultaneously maintaining their connection to the bulb and crura of the penis respectively. The remaining portion of the deep transverse perineal muscle between the base of the penis and prostate was retained in an effort to protect the bulbo-
urethral (Cowper’s) glands and the intermediate part of (membranous) urethra within. The lateral puboprostatic ligament was identified and preserved. All excess tissue and muscle fragments were removed from the region so that relevant structures including the bulb and crura of the penis with bulbospongiousus, ischiocavernosus, deep transverse perineal, and external urethral spincter muscles, prostate, urinary bladder, ureters, seminal vesicles and ductus deferentes were all clearly visible.

### 6.3.3.10 Dissection of the Body of the Penis

The remaining fascia was removed from the body of the penis. Care was taken to preserve the deep dorsal dorsal vessels of the penis. The fascia between the glans of the penis and corpora cavernosa was teased away facilitating separation of the corpora cavernosa from corpus spongiosum and glans.

### 6.3.3.11 Demonstration of Accessory Glands

Adipose and minor tissue remnants were removed from the surface of the bladder, seminal vesicles, and prostate. At the apex of the prostate, the deep transverse perineal muscle was delicately dissected revealing the bulbo-urethral glands and membranous urethra.

### 6.3.3.12 Inguinal Hernia

During the dissection a direct inguinal hernia on the left side was discovered at the inferomedial margin of the medial inguinal fossa (Hesselbach’s triangle). The protocol was modified slightly to preserve this pathology. The left spermatic cord and inguinal canal were preserved and remained attached to the lateral side of the herniated abdominal peritoneum. The medial side of the hernia was kept in contact with the left lateral portion of the bladder.
6.3.3.13 Varicocele

During the dissection a grade II varicocele was discovered [343-345]. The protocol was modified slightly to preserve this pathology. The internal spermatic fascia was preserved and remained around the spermatic chord and testis to retain the structure of varicose pampiniform plexus.

Part 6.3.4 Plastination

Following dissection, the urogenital specimen was rinsed overnight in running tap water. A Foley Catheter (18 Fr. 5cc) was introduced distally into spongy urethra. The specimen was placed in a chemically resistant bucket with a sealable lid (Gamma lids). Dehydration was performed in an explosion-proof freezer (-25°C, Lab-Line Frigid Cab) in ≥97.5% acetone bath. The percentage of acetone was checked weekly and adjusted to 97.5% after the 1st and the 2nd week and to 99.5% after the 3rd week. After greater than 99% of acetone was achieved, the specimen was considered dehydrated and transferred to room temperature. Degreasing occurred during submersion in acetone (>99% and fresh 100%, 4 weeks each) at room temperature. For impregnation, the specimen was removed from the acetone and submerged into a bath of PR10 silicone solution, NCS10 polymer/NCSVI cross-linker (Silicones, Inc.) diluted 100:8 in water, and placed in a medium sized vacuum chamber attached to an oil-free two-stage vacuum pump (Labport, KNF, Neuberger) at room temperature. Forced impregnation (2 cm Hg) was achieved after 48 hr. Following impregnation, the specimen was removed from the vacuum chamber, placed on a wire rack, blotted with a paper towel, and allowed to drain for two days. The specimen was lightly coated with NCSIII (catalyst), wrapped in plastic foil and monitored daily. Curing was successfully achieved 30 days after impregnation.
Section 6.4 Results

Part 6.4.1 General Appearance

The new dissection protocol and the established plastination process used at our institute (see Methods and Materials, and [346] for more details) yielded a good quality specimen demonstrating the male urogenital system and associated vasculature (Fig. 62 and Fig. 63). The plastination was durable, and smaller vessels maintained their general shape (Fig. 64 top & bottom right). The placement of a catheter in the urethra before plastination successfully kept the spongy urethra patent within the corpus spongiosum, which maintained shape (Fig. 3, bottom left). The relationship of the erectile bodies and the muscles at their proximal end was demonstrated while maintaining the overall structure of the penis (Fig. 64 bottom left).
Figure 62. Anterior view of the urogenital system plastination. The structures shown represent a subset of structures required to be identified by medical students performing dissections in Gross Anatomy Laboratory listed in Table 12. A: abdominal aorta; B: left renal artery; C: left kidney; D: left renal vein; E: left testicular vein; F: abdominal aorta aneurysm; G: left ureter; H: left common iliac artery; I: left external iliac artery; J: spermatic cord; K: anterior scrotal artery/vein, ilio-inguinal nerve; L: spermatic fascia/parietal layer tunica vaginalis; M: bladder; N: prostate; O: corpus cavernosum; P: glans penis; Q: corpus spongiosum and spongy urethra patent within; R: seminal vesicle; S: ampulla of ductus deferens; T: ductus deferens; U: testis/visceral layer tunica vaginalis/tunica albuginea; V: epididymis; W: pampiniform plexus; X: right common iliac artery aneurysm; Y: right ureter, Z: right testicular artery/vein; AA: right kidney; AB: right adrenal gland.
Figure 63. Posterior view of the urogenital system plastination. The structures shown represent a subset of structures required to be identified by medical students performing dissections in Gross Anatomy Laboratory listed in Table 12: A: right renal artery; B: right kidney; C: inferior vena cava; D: abdominal aorta aneurysm; E: right common iliac artery aneurysm; F: left internal iliac artery; G: median umbilical ligament of bladder; H: fundus of bladder; I: epididymis; J: ductus deferens; K: ejaculatory duct; L: glans penis; M: corpus spongiosum with spongy urethra patent within; N: corpus cavernosum; O: membranous urethra; P: prostate; Q: seminal vesicle; R: left external iliac artery; S: left testicular artery/vein; T: left common iliac artery; U: left ureter; V: left renal vein; W: left renal artery.
Figure 64. Higher magnification of selected structures. Top Left Panel: The superior portions of testicular vasculature as they relate to renal structures. Top Right Panel: A lateral view of the right testis showing the scrotal contents viewable when the spermatic fascia is removed. Bottom Left Panel: The penis: the erectile muscles, erectile bodies and deep venous structures superficial to the corpora cavernosa. Specifically highlighting the association of the corpus spongiosum and the glans penis as the spongy urethra traverses the center of both structures. Bottom Right Panel: Male reproductive glands and associated structures. Indicated structures: A: left testicular artery; B: left renal vein; C: left testicular vein; D: left kidney; E: left ureter; F: right testicular vein; G: right testicular artery; H: testicular artery; I: caput epididymis; J: testis; K: caudate epididymis; L: ductus deferens; M: corpus epididymis; N: pampiniform plexus; O: deep dorsal vein of the penis; P: corpus cavernosum; Q: glans penis; R: corpus spongiosum; S: bulbospongiosus muscle (green pin head); T: ischiocavernosus muscle (blue pin head); U: right ejaculatory duct; V: membranous urethra; W: prostate; X: left seminal vesicle.
Part 6.4.2 Male Genital Organs and Glands

The vasculature of the testis at its superior pole retained shape. All three segments of the epididymis were clearly visible as they follow the curvature of the posterior testis and direct sperm to the ductus deferens. The seminal vesicles and prostate along with the ejaculatory duct between them were maintained (Fig. 64 top right). Most likely due to its small size and delicacy, the bulbo-urethral gland was inadvertently removed with the external urethral sphincter muscle during dissection to reveal the membranous urethra (Fig. 64 bottom right).

Part 6.4.3 Pathologies

The direct inguinal hernia remained patent after plastination (Fig. 65). The abdominal peritoneum formed a thickened outer sheath and protruded laterally and inferiorly; however, it remained distinct from the inguinal canal and its contents (Fig. 65 left). The herniated peritoneal pouch retained a position lateral to the urinary bladder and medial to the deep inguinal ring and protruded into the medial inguinal canal adjacent to the spermatic cord as the spermatic cord approached the superficial inguinal ring (Fig. 65 left). The hernia also projected anteriorly towards the anterior abdominal wall, but remained posterolateral to the rectus abdominus muscle (Fig. 65 right). Thus, it protruded slightly medial to the medial inguinal fossa (Hesselbach’s triangle). Distortion and closure of the hernia during plastination was prevented possibly due to the abnormal thickening of the peritoneum as well as the retention of its connections extraperitoneally (Fig. 65 right). The retention of the spermatic fascia also preserved the anterior scrotal artery and vein (Fig. 62).
Figure 65. Demonstration of direct inguinal hernia and validity of the technique used to preserve pathology. **Left Panel:** Left anterior view demonstrating path of abdominal peritoneum herniation (pink) in relation to spermatic cord (green) and deep inguinal ring (blue). **Right Panel:** Left superior view demonstrating location of abdominal peritoneum herniation (needle) in relation to inferior epigastric artery and rectus abdominus muscle (green pin head). **Indicated Structures:** A: ductus deferens; B: testicular artery and vein; C: bladder; D: rectus abdominus muscle (green pin head); E: ureter; F: inferior epigastric artery.
After dissection it was obvious that a large abdominal tumor had caused extensive deformation of the abdominal aorta. The tumor and associated fibrous tissue covered the length of the abdominal aorta and common iliac arteries while further binding these vasculature structures to a fibrous mass that encased parts of the stomach, pancreas and large intestine. The same region also seemed to have suffered an abdominal aortic aneurysm (Fig. 62 and Fig. 63). Despite these obstacles, the vasculature was maintained, though it appeared somewhat morphologically abnormal. The origins of the right and left testicular arteries and termination of the right and left testicular veins were intact and visible (Fig. 64 top left).

The dissection of the grade II varicocele was done on a second permanent willed body donor. Unfortunately, it was not possible to plastinate the dissection in time for publication in the manuscript this chapter of the dissertation is taken from. However, varicoceles are a common male urogenital pathology and have a link to male infertility. Therefore, the author feels it is prudent to insert the unpublished results of his second urogenital dissection.

Clinically varicoceles are categorized into three grades: grade I varicoceles are palpable only from a standing position and only during Valsalva’s maneuver; grade II varicoceles are palpable while standing without Valsalva’s maneuver; and grade III varicoceles are visible through scrotal skin upon initial examination without palpation [343-345]. Valsalva’s maneuver increases intra-abdominal pressure by having the patient forcibly exhale against a closed glottis (cough) [343]. Although the standard clinical exam was not possible during dissection it was clear that the varicocele was grade II. The varicocele was not visibly evident through the scrotum but it was easily palpable with the cadaver in the supine position before the scrotum was reflected. The decision to retain all of the connective tissue of the internal spermatic fascia kept the varicocele in
a more natural state. Upon palpation both venous engorgement and torsion are obvious (Fig. 66). Currently the specimen has been fully dehydrated and fully degreased while the pathology continues to be in excellent condition for educational purposes.

Figure 66. Demonstrating change in morphology due to varicocele using left and right testis from single donor. Left Panel: right healthy normal testis and spermatic cord encased in spermatic fascia. Right Panel: pathologic left testis and spermatic cord due to grade II varicocele encased in spermatic fascia. The region superior to the testis has become engorged with the enlarged and tortuous pampiniform plexus. The spermatic fascia superficial to the testis has a corrugated appearance due to the excess vasculature between the fascia and testis surface. Scale = 2 cm.
Part 6.4.4 Efficacy as a Teaching Tool

The plastination was assessed for instructional relevancy by determining the number of structures that could be identified compared to a standard list of structures required for observation by medical students during gross anatomy dissection of the Abdomen and Pelvis & Perineum (Table 12). Typically, many prosections and plastinations are necessary to cover all of the structures on the list. However, over 50% of the structures on the list were identified on the single plastinated specimen generated in this study. Over one-third of the structures absent from the specimen were either nerves that were not on the dissection or structures that would require transecting part of the dissection to be visible. The latter was specifically not performed to maintain the general morphology of the structures that were highlighted in the dissection.
### Table 12. Efficacy of specimen as teaching tool.

<table>
<thead>
<tr>
<th>Male Urogenital Relevant Structures from MS1 Identify List&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Structure Present (Figure #)</th>
<th>Structure Not Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland (1)</td>
<td>Artery of the bulb (penis/vestibule)</td>
<td></td>
</tr>
<tr>
<td>Ampulla of ductus deferens (1)</td>
<td>Artery to ductus deferens</td>
<td></td>
</tr>
<tr>
<td>Bulbospongious m (3 Bottom Left)</td>
<td>Bulbo-urethral glands</td>
<td></td>
</tr>
<tr>
<td>Corpus cavernosum (3 Bottom Left)</td>
<td>Deep artery of the penis</td>
<td></td>
</tr>
<tr>
<td>Corpus spongiosum (3 Bottom Left)</td>
<td>Deep transverse perineal m</td>
<td></td>
</tr>
<tr>
<td>Deep dorsal vein of the penis (3 Bottom Left)</td>
<td>Dorsal artery of the penis</td>
<td></td>
</tr>
<tr>
<td>Ductus deferens (1, 2, 3 Top Right, 4)</td>
<td>Dorsal nerve of penis/clitoris</td>
<td></td>
</tr>
<tr>
<td>Ejaculatory ducts (3 Bottom Right)</td>
<td>Ganglion of sympathetic trunk</td>
<td></td>
</tr>
<tr>
<td>Epididymis (1, 3 Top Right)</td>
<td>Internal pudendal artery</td>
<td></td>
</tr>
<tr>
<td>Fundus of bladder (2)</td>
<td>Internal urethral orifice&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Glans penis (1, 2, 3 Bottom Left)</td>
<td>Pelvic splanchnic nerves</td>
<td></td>
</tr>
<tr>
<td>Internal iliac artery (2)</td>
<td>Posterior scrotal/labial nerve</td>
<td></td>
</tr>
<tr>
<td>Ischiocavernosus m (3 Bottom Left)</td>
<td>Prostatic urethra&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Kidney (1, 2)</td>
<td>Prostatic duct&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Median umbilical ligament of bladder (2)</td>
<td>Prostatic plexus of veins</td>
<td></td>
</tr>
<tr>
<td>Membranous urethra (3 Bottom Right)</td>
<td>Prostatic capsule</td>
<td></td>
</tr>
<tr>
<td>Pampiniform plexus (1, 3 Top Right)</td>
<td>Prostatic utricle</td>
<td></td>
</tr>
<tr>
<td>Penis (3 Bottom Left)</td>
<td>Rete testis&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Prostate (1, 2, 3 Bottom Right)</td>
<td>Scrotum</td>
<td></td>
</tr>
<tr>
<td>Renal artery (1, 2)</td>
<td>Seminal colliculus</td>
<td></td>
</tr>
<tr>
<td>Renal vein (1, 2, 3 Top Left)</td>
<td>Seminiferous tubules&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle (1, 2, 3 Bottom Right)</td>
<td>Sphincter urethrae&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Spermatic cord (1, 4 Left)</td>
<td>Spongy urethra&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Testicular artery (1, 3 Top, 4)</td>
<td>Trigone of bladder&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Testicular vein (1, 3 Top, 4)</td>
<td>Tunica dartos</td>
<td></td>
</tr>
<tr>
<td>Testis (1, 3 Top Right)</td>
<td>Ureteric orifice&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tunica albuginea (1)</td>
<td>Urethral artery</td>
<td></td>
</tr>
<tr>
<td>Tunica vaginalis (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ureter (1, 2, 3 Top Left, 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary bladder (1, 4 Left)</td>
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<td></td>
</tr>
<tr>
<td><strong>Total 30/57</strong></td>
<td><strong>Total 27/57</strong></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Structures present on specimen were compared to the male urogenital relevant vocabulary list of structures typically required to be identified by medical students performing dissections in Gross Anatomy laboratory.

<sup>2</sup> Internal structure that requires transection of the prossection for viewing (in general transection was counter to the purpose of this dissection, see Results, Efficacy as a Teaching Tool).
Section 6.5 Discussion

Here, we detailed an alternative way of dissecting the renal and male genital systems. Dissection was followed by a plastination method that can be used to preserve the specimen for years of instructional use. We produced a complete urogenital dissection that has been removed from the abdomen and pelvis in such a way that connections between components are readily apparent in a 360-degree view. This represents a valuable alternative to regional dissections, which treat the component parts as separate entities; or pelvic hemisection, which can at times omit components or mute their spatial relationships.

Thorough dissection of the erectile bodies after removal of the fascia encasing them (Buck’s Fascia) was a conscious dissection decision. This approach allowed for a much better understanding of the continuity between the corpus spongiosum and glans, while concurrently demonstrating the termination of the corpora cavernosa distally at the proximal end of the dorsal glans. These relationships cannot be studied when the penis is simply excised cross-sectionally midway along the body.

Tissue dehydration occurs as a result of the plastination process thus raising concerns regarding the retention of urethral morphology. In an attempt to overcome this problem catheterization was performed. However, success was limited since the proximal portion could not be reached and the urethra lacked patency at its origin. Future efforts will utilize smaller tubing or perhaps something solid such as a rounded flexible plastic rod so that the entire length of the urethra can be catheterized with ease.
The demonstration of bladder morphology did not achieve expectations since the bladder became compacted unexpectedly during plastination. Despite this compaction, structures such as the fundus and median umbilical ligament of the bladder were visible. In future attempts, we will consider packing or inflating the bladder, via a small incision, to maintain its shape.

Preservation of the testicular vasculature all the way to arterial origin at the abdominal aorta, and venous termination at either the inferior vena cava or the left renal vein was another significant component of the dissection. By not transecting the spermatic cord or its contents, similarities and differences between testicular vasculature could be observed. Specifically, the strong 90-degree angle of the left testicular vein at the left renal vein is anatomically divergent from the much more acute angle the right testicular vein forms with the inferior vena cava. This left-right disparity is linked to a higher prevalence of varicocele, a common male reproductive pathology affecting the pampiniform plexus, on the left side [347].

The fortuitous finding of a direct inguinal hernia necessitated divergence from our original dissection agenda. The presence of the direct inguinal hernia gave us an excellent opportunity to demonstrate a common male pathology from an anatomical viewpoint. When strategizing how best to preserve the pathology, the decision was made to forgo transection of the testis ipsilateral to the direct inguinal hernia. The intention was that by retaining the spermatic fascia and tissue around the inguinal canal, the identifying features of the direct inguinal hernia would remain readily apparent after plastination.

About 75% of abdominal hernias in adults occur in the inguinal canal. A recent study found there were 770,000 cases in the United States during 2003, accounting for about 5% of Americans with a 9:1 male to female ratio [348-350]. Examining for the presence of a hernia is typically performed as part of a routine physical for males and about 25% of American men are expected to
have a medically recognizable inguinal hernia [351]. Due to this prevalence, hernia repair has become the most common routine surgical procedure for general surgeons and is divided between two surgical treatments: mesh-free repair or tension-free mesh repair [348, 352, 353].

According to a recent randomized study of men who had asymptomatic or minimally symptomatic inguinal hernias found that 53% were indirect (i.e. entering the inguinal canal laterally via the spermatic cord and deep inguinal ring) and 41% were direct [354]. Generally, indirect inguinal hernias are more prevalent in young men and boys while direct inguinal hernias are more prevalent in older men [355, 356]. Indirect inguinal hernia at a young age is most often due to persistent patency of the of processus vaginalis after development [355, 357], while direct inguinal hernia in the elderly is thought to be linked to compromised integrity of the abdominal wall at the medial inguinal fossa (Hesselbach’s triangle) [356, 358, 359]. From a surgical perspective treatment of either kind of inguinal hernia is the same, but classification distinguishing between direct and indirect inguinal hernia, is still a dominant part of diagnosis [360]. At the same time there is a growing recommendation to not perform a preoperative diagnosis of direct versus indirect inguinal hernia, as the preoperative diagnosis often does not match the intraoperative findings [361, 362].

The dissection and plastination technique described above allowed for observation of a clear and classic direct inguinal hernia in the context of surrounding structures and reproductive organs often affected by its presence. Although distinction between direct and indirect inguinal hernia may not be a critical part of preoperative diagnosis, intraoperative distinction is vital for treatment at the correct location. Further, there is still a great deal to be learned about inguinal hernias, especially in light of the continued discourse on proper treatment in regards to hernia recurrence and pain management of this common pathology [360, 363]. In future plastination
work, it could be prudent to pack a hernia with material that can be removed after plastination to ensure patency of the specimen.

Globally varicoceles are a fairly common pathology of the male reproductive tract. About 15% of the male population has some grade of varicocele either unilaterally or bilaterally, though varicoceles are significantly more common on the left side when unilateral [307, 347, 364]. The occurrence increases to 40% when only infertile men are considered, but drops down to 4.3-13.3% when specifically, azoospermic men (1% of the total male population, 10-15% of the total infertile male population) are focused on [307, 364]. This drop in occurrence with azoospermia may be due to the putative role varicoceles play in hypospermia [211, 307]. The most common theories on varicocele caused infertility center around hypoxia, venous hypertension, temperature elevation and excess oxidative stress in and around the testis [307, 308]. The more common occurrence of left unilateral varicocele (including the specimen discussed above) is thought to be due to the lower quality flow of venous return from the left testicular vein due to its strong 90° intersection with the left renal vein. The right testicular vein has a more acute angle as it enters the inferior vena cava which is more conducive to quality venous flow [347].

Originally one testis was to be transected from pole to pole after plastination to demonstrate the internal structures. The unexpected appearance of a direct inguinal hernia, which we opted to feature in our dissection, necessitated retaining the spermatic fascia and connective tissue of the spermatic cord of the ipsilateral testis. In the future, one testis will be kept intact while the other will be transected from pole to pole, barring the presence of another pathology.

We have previously demonstrated the general effectiveness of plastinations as teaching tools [365]. The specimen detailed in this manuscript represents a teaching tool with high impact in terms of medical education at our institution. This single specimen allows for viewing of the
collection of anatomical structures, investigation of which would usually require two or more specimens. The specific vantage points that are offered by the specimen described here are rarely available to students, who instead work with specimens offering an obstructed view resulting from hemisectioning or visually lacking the relation between urogenital structures as occurs during regional dissection.

To summarize, the new dissection approach resulted in an excellent complete male urogenital dissection with vasculature along with an example of a common male pathology, the direct inguinal hernia. The final plastination enables a viewing perspective of the system from any orientation without obstruction while also enforcing the idea that it is a very connected system despite the spatial separation of its integral parts.

Section 6.6 Contributions

Dr. Monika A. Ward – P.I.

Dr. Scott Lozanoff – intellectual contribution

Mr. Steven Labrash – embalming, plastination guidance

Philip and Phillip – the altruistic individuals who donated their bodies for the advancement of medical education and research
CHAPTER 7. CONCLUSION

Section 7.1 Closing Remarks

A dangerous misinterpretation of this dissertation would read it as death knell for the Y chromosome and men as we know them. If the reader bears in mind just how much is going wrong in the testis of mice with limited or no Y genes, especially males lacking all Y chromosome genes, it becomes obvious that although you can substitute for the key Y genes the substitution is not equal. It is also important to bear in mind that round spermatid injection (ROSI), which was used to verify the ability to sire offspring for a number of the mice with limited or no Y genes, is still an experimental assisted reproductive technique for humans.

What is truly fascinating, especially in terms of gene substitutions, is how far along male processes can get with so little. The power of just a handful of genes to take the masculinizing process so far is a testament to the resilience of men as a gender and sex, and a slap in the face to the blasphemy related to the Y chromosome disappearing and taking men along with it, in the future. The war song of this dissertation is „we will endure”. The Y chromosome may be small but it is still mighty and has a plan B.

The work here with Y-link gene substitution shows how there are back up systems in place to ensure some level of maleness remains in the event the Y does totally erode. The work on Eif2s3y and Eif2s3x is a beautiful example of an X-Y gene pair that work together in harmony instead of being locked in an age old battle of the sex chromosomes exemplified by Sly and Slx/Slxl1. Less ethereally, this dissertation brings new knowledge about testicular abnormalities to the world of histology and new methods for human and murine histological testis assessment and human male urogenital dissection. All of this is in an effort to better understand what makes one a
man, both genetically and anatomically, and how this age old question fits into the context of our current era where male fertility is declining and it is not always clear what it means to be a man.

A key line item in the list of future directions for this work is a full investigation into endocrine function in mice with limited and no Y genes. The hypothalamic-pituitary-testis axis is the hormonal regulator of spermatogenesis and it would be very informative to know if there are divergences in gonadotropin releasing hormone, luteinizing hormone, follicle stimulating hormone and testosterone in mice with limited to no Y genes when compared to XY mice. Testosterone is a critical piece of the story, in light of the interstitial tissue differences in the testes of some mice with limited and no Y genes, as testosterone is produced by the steroidogenic Leydig cells of the testis interstitium.

The translational aspect of this work is just beginning to blossom. More human AZF testis biopsies are needed to draw stronger conclusions about the parallels between mice with limited and no Y genes, and AZF phenotypes observed in men. A further translational direction is gene therapy. The substitutability of Y-linked genes has been demonstrated in the context of spermatogenesis initiation with mice. Potentially this could be applied to SCO syndrome men as a way to correct their infertility, ultimately, giving hope to men afflicted with infertility is the goal of this research.
Section 8.1 Supplements to Chapter 2

Part 8.1.1 from Part 2.3.5 Round Spermatid Injection (ROSI)

All ROSI offspring were derived using spermatids from males on partial (transgenics) or full MF1 (XY) genetic background and oocytes from B6D2F1 females. The metaphase II (MII) oocytes for ROSI were collected from superovulated (5 iu eCG and 5 iu hCG given 48 hours apart) female mice and incubated at 37°C, 5% CO₂ until injection. Testicular sperm suspension was diluted with HEPES-CZB containing 1% (w/v) polyvinyl pyrrolidone (PVP) on the injection dish. Spermatids were injected individually into the oocytes. The total duration of ROSI was no longer than 1 hour. The oocytes were activated shortly after injection by incubation in Ca²⁺-free CZB medium supplemented with 2.5 mM SrCl₂ at 37°C, 5% CO₂ for 4 hrs, after which time they were transferred into standard CZB medium for subsequent culture. At ~6-8 hrs after injection the oocytes were assessed for polar body extrusion and pronuclei development. Normally fertilized oocytes exhibiting two pronuclei (PN) and extruded second polar body (PBII) as well as abnormally fertilized oocytes (with deviations from normal in regard of pronuclei and extruded polar body number) were differentiated. Twenty four hours after injection the oocytes in each group were scored for cleavage. Embryo transfer was performed with the 2-cell embryos derived from normally fertilized oocytes. Surrogate mothers were subjected to caesarian section on day 20 of pregnancy to allow for scoring of the numbers of fetuses and implantation sites.

Part 8.1.2 from Part 2.3.6 ROSI Progeny Analysis

All offspring were raised by foster mothers until weaning, allowed to mature, and bred (if applicable). All offspring were genotyped by PCR to identify presence of Eif2s3y, Eif2s3x, Sox9,
and Sry transgenes and the sex chromosomes. Some of the offspring from XOSox9, Eif2s3x males were also tested for a battery of other Y chromosome genes to show that than none were present. The overall health and well-being of some of the ROSI progeny were monitored carefully during the first 8 months of age, which included monthly body weight measures. Some of the ROSI offspring were maintained beyond 8 months of age, with the oldest progeny being now at 20 months of age. None from the mice that were maintained died prematurely or developed tumors.

Part 8.1.3 from Part 2.3.7 Transgene Copy Number

Genomic DNA for Eif2s3x transgene copy number assessment was isolated from mouse tails using phenol chlorophorm extraction and ethanol precipitation. 5 ng/µl of DNA was used to amplify single copy X chromosome genes Eif2s3x, Amelx and Prdx4 by qPCR using Power SYBR Green PCR Master Mix on QuantStudio 12K Flex machine (Applied Biosystems). The following conditions were used: 95°C for 10 min, followed by 37 cycles of 95°C for 10 sec and 60°C for 60 sec. Eif2s3x was amplified with cESXs primers, which recognize both endogenous and transgenic Eif2s3x and not Y chromosome gene Eif2s3y. XO samples were used as a reference control since they have a single X chromosome and are expected to show single copies of Eif2s3x, Amelx and Prdx4, and no Eif2s3x transgene. XX and XY samples were included to verify primer specificity. Amelx and Prdx4 were run for each sample as quality and copy number controls. A minimum of three samples per genotype were tested in quadruplicate per assay. Copy number estimation for each gene was calculated with the ΔΔCt method using Atr as a housekeeping gene. Briefly, ΔCt values were calculated as difference between tested gene and Atr. ΔΔCt values were calculated by subtracting ΔCt of tested gene from the reference samples. The copy numbers were calculated by raising 2 to the power of ΔΔCt (2^{ΔΔCt}). Eif2s3x transgene copy number was inferred from a
difference in *Eif2s3x* amplification readout between XO and transgenic samples. Primer sequences are shown in Table 12.
Table 13. List of primers from work in Chapter 2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ID</th>
<th>Primer sequence</th>
<th>Amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>qPCR primers for sex chromosome copy number estimation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eif2s3x (sp)*</td>
<td>Eif2s3x-sp-CNAFP</td>
<td>CAATGTGGCGAGATCCTGTC</td>
<td>87 bp</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>Eif2s3x-sp-CNARP</td>
<td>TTTTCTCTCCGAGCAAGATG</td>
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<tr>
<td>Prdx</td>
<td>Prdx4-F</td>
<td>CATGATATCCACTGAAAGCTAC</td>
<td>82 bp</td>
<td>[244]</td>
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<td>Prdx4-R</td>
<td>GAGACAGTGTATCTATCCTCTG</td>
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<td>Amelx</td>
<td>Amelx-F</td>
<td>GTGGTGTTGGAGCTAGGAGG</td>
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<td>Amelx-R</td>
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<td>Atr</td>
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<td>GGGATTTTACACACCCGTCT</td>
<td>143 bp</td>
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<td>Atr-WT R1</td>
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<td><strong>Standard genotyping PCR primers</strong></td>
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<td>Myog</td>
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<td>Om1b</td>
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<td>Zfy</td>
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<td></td>
<td>oMJ275</td>
<td>aacagcaagttggactacag</td>
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<tr>
<td>Eif2s3y</td>
<td>gESYsF1</td>
<td>ATAGATCCGCCAGAAGACAAAG</td>
<td>843 bp</td>
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<td>gESYsR1</td>
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<td>Sry (YTdym1)</td>
<td>Mutry3</td>
<td>GTTGTCTCAAGCTCACTCTC</td>
<td>204 bp</td>
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<td>Mutypr1</td>
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<td>Ssyt1</td>
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<td></td>
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<td>Eif2s3y tg**</td>
<td>EyTspF8</td>
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<td>782 bp</td>
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<td>300 bp</td>
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<td></td>
<td>Sox9-SS2</td>
<td>GCTGAGCCGGTTGACGCG</td>
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<tr>
<td>Eif2s3x tg**</td>
<td>pTARBAF7</td>
<td>GGTGATGCGGTATTCTCTC</td>
<td>87 bp</td>
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<tr>
<td><strong>Real-time RT-PCR primers</strong></td>
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<tr>
<td>FoxL2</td>
<td>FoxL2.F</td>
<td>AGGGAGAGAATAAACATTCATGG</td>
<td>63 bp</td>
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<td></td>
<td>FoxL2.R</td>
<td>TGGGACCTCAACCCCGGACTAC</td>
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<tr>
<td>Rsopo</td>
<td>Rsopo1_F</td>
<td>CGACATGAAACAAAGTCA</td>
<td>82 bp</td>
<td>[367]</td>
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<td></td>
<td>Rsopo1_R</td>
<td>CTCTGACATCGGTTGCAAG</td>
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<td>Wnt4</td>
<td>Wnt4_F</td>
<td>CGAGAGATGCGAATTACAGT</td>
<td>138 bp</td>
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<td>Wnt4_R</td>
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<td>Actin-R</td>
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<td>Sdha</td>
<td>Sdha-F</td>
<td>TGTCTAGTTCACCCACAC</td>
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<tr>
<td>Eif2s3x***</td>
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<td>82 bp</td>
<td>[369]</td>
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<td>Eif2s3x-R2</td>
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<td></td>
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<td>eESYsF2</td>
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<td>CGCTTACGAGGAAGATGAGA</td>
<td></td>
<td></td>
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<tr>
<td>Eif2s3x/y***</td>
<td>eEIFsF2</td>
<td>GACGAAATGTTACAGAT</td>
<td>192 bp</td>
<td>[3]</td>
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<td></td>
<td>eEIFsR2</td>
<td>TACCAGCTGATACAGAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Recognize endogenous and transgenic Eif2s3x but does not recognize any of the Eif2s3x retrogenes or pseudogenes; ** Recognize specifically transgene. *** Recognize endogenous and transgenic Eif2s3x transcripts but will also pick up genomic Eif2s3x X-linked predicted pseudogene (MGI: Gm2223 or VegaSanger: RP23-191G17.4), if it is expressed.
Part 8.1.4 from Part 2.3.8 Real-Time RT-PCR

For real-time reverse transcriptase polymerase chain reaction (RT-PCR), total testis RNA was extracted using Trizol and DNase I treatment (Ambion, Austin, TX, USA), and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer’s guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI QuantStudio 12K Flex machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 40 PCR cycles (10 s at 95°C and 60 s at 60°C).

The analysis of ovarian markers (Fig. 35 in Chapter 2) was performed using primers for FoxL2, Rspo1, Wnt4 with Actin and Sadha as loading controls. For analysis of Eif2s3 expression (Fig. 36 H-J and Fig. 37 in Chapter 2), three types of PCR reactions were performed: (1) ‘Eif2s3x’ amplifying endogenous and transgenic Eif2s3x transcripts; (2) ‘Eif2s3y’ amplifying endogenous and transgenic Eif2s3y transcripts; and (3) 'Eif2s3x/y' amplifying transcripts from both Eif2s3x and Eif2s3y, both endogenous and transgenic.

At least three mice per genotype were analyzed, all reactions were carried out in at least triplicates per assay, and two ubiquitously expressed genes (Actin and/or Sdha) were used as loading controls. ΔCt value for each individual sample was calculated by subtracting the average Ct of loading control(s) from the average Ct of a tested gene. ΔΔCt value was calculated by subtracting the ΔCt of each tested male from the average ΔCt of control samples (XO Sry or XY males in Eif2s3 expression analysis and XX in ovarian marker analysis), which served as
references. The data were expressed as a fold value of expression level. For \( Eif2s3x/y \) specific and
global expression analysis PCR primer efficiencies were taken into consideration. A 5x serial
dilution of cDNA template was used to obtain the efficiency (E) for each primer pair. The average
CT values were plotted against \( \log_5 \) cDNA concentrations, and the slope (a) was obtained to
calculate (E) which equals to \( 5^{-1/a} \). Each plot yielded a trend line that had an \( R^2 \) value greater than
0.99. The fold expression was calculated using the formula \( E^{\Delta Ct} \).

Part 8.1.5 from Part 2.4.1 and 2.4.2 Aggregated ART and Fertility Analysis

We next tested for spermatid function in assisted reproduction. Round spermatids could be
found in testicular cell suspensions from 8 out of 10 \( X^E OSox9 \) males (Table 14). Following
spermatid injection (ROSI) the oocytes were successfully fertilized, and when the resulting 2-cell
embryos were transferred into the oviducts of recipient females, live offspring were obtained from
7 out of 8 males. ROSI efficiency, measured as a proportion of offspring developed from
transferred embryos (15.7%, Table 14), was similar to that reported previously for \( X^E OSry \) (9.1%,
[61]) and that of \( XY \) controls (25%).

Out of 31 \( XOSry, Eif2s3x \) males that provided testes for ART trials 17 had round spermatids
identifiable in testicular cell suspension; these cells were used for injections followed by embryo
transfer. Live offspring were obtained from 16 males, with an efficiency comparable to that
obtained with \( XY \) control (22.5% vs. 25%, Table 14).

In ART trials, 47 \( XOSox9, Eif2s3x \) males were examined, and 13 scored as having ongoing
spermatogenesis. Round spermatids from these males were used for injections, and yielded
zygotes with two well-developed pronuclei, which subsequently cleaved to normal 2-cell embryos
(Fig. 67). Embryos from 11 males were used for transfer, 10 resulted in pregnancy, and 9 yielded
offspring (20.7%, Table 14). Among the males that yielded progeny there were F1, F2, and F3
generation ROSI offspring derived from the first XOSry,Eif2s3x male to produce round spermatids (Fig. 68).
Figure 67. Round spermatid injection (ROSI) with spermatids from males with a single or no Y chromosome genes. (A) Example of testicular cell suspension from XO Sox9, Eif2s3x male, with injection pipette visible on the right and inset (x3 magnification) showing a single round spermatids; (B) 6 hours after injection injected oocytes developed two pronuclei (arrowheads) and extruded a second polar body (arrow). (C) 24 h after injection fertilized oocytes cleaved and became 2-cell embryos. Scale = 50 µm.
Figure 68. Three generations of XOSox9,Eif2s3x males derived with ROSI. The albino male was the first XOSox9,Eif2s3x male, generated by breeding, who successfully sired first ROSI offspring. His XOSox9,Eif2s3x son (F1 ROSI) then sired XOSox9,Eif2s3x (F2 ROSI), which then subsequently sired XOSox9,Eif2s3x (F3 ROSI). All males had no Y chromosome genes.
Table 14. The results of round spermatid injection (ROSI) with spermatids from males with a single or no Y chromosome genes.

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Y gene contribution</th>
<th>Males with round spermatids % (No)</th>
<th>Males yielding progeny</th>
<th>Live offspring % (no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X&lt;sup&gt;0&lt;/sup&gt;Sry</td>
<td>Eif2s3y &amp; Sry</td>
<td>100 (4/4)</td>
<td>3/4</td>
<td>9.1 (12/132)</td>
</tr>
<tr>
<td>X&lt;sup&gt;0&lt;/sup&gt;Sox9</td>
<td>Eif2s3y</td>
<td>80 (8/10)</td>
<td>7/8</td>
<td>15.7 (18/115)</td>
</tr>
<tr>
<td>XO,Sry,Eif2s3x</td>
<td>Sry</td>
<td>55 (17/31)</td>
<td>16/17</td>
<td>22.5 (57/253)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>XO,Sox9,Eif2s3x</td>
<td>none</td>
<td>27 (13/48)</td>
<td>9/10</td>
<td>20.7 (46/222)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>XY control</td>
<td>intact Y</td>
<td>100 (6/6)</td>
<td>6/6</td>
<td>25.0 (22/88)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

X<sup>0</sup>Sry data (shaded) were published before and are shown here for comparison only.

<sup>a</sup> Refers round spermatids identifiable in live testicular cell suspension.

<sup>@</sup> Out of males, which yielded embryos used for embryo transfer

<sup>#</sup> Percentage calculated from embryos transferred.

Statistical significance (Fisher's Exact Test): <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs. X<sup>0</sup>Sry.

No differences were noted between males with a single or no Y genes and wild-type control.
Part 8.1.6 from Part 2.4.4 Healthy and Normal Progeny from Males with a Single or with No Y Chromosome Genes

Altogether, 18, 46, and 41 offspring were produced from $X^E$OSox9, XOSry,Eif2s3x, and XOSox9,Eif2s3x, respectively. All ROSI offspring were genotyped and the genotypes and their frequency met expectancy (Fig. 69). For some offspring from XOSox9,Eif2s3x males we assayed for presence of Y chromosome genes, and none were present, as expected (Fig. 70). ROSI progeny were delivered as normal and healthy pups (Fig. 71 A). Some of them were maintained for more than 20 months and remained healthy into old age (Fig. 71 B). All male progeny were expected to be infertile due to their sex chromosome gene content and thus were not bred. Female progeny were efficient and successful breeders (Fig. 71 C-E). Some of the ROSI progeny were weighed monthly between 3 and 8 months of age to assess their growth, and all developed normally (Fig. 71 F and Fig. 72).
Figure 69. Genotypes of progeny obtained after ROSI with spermatids from males with 1 or no Y genes. Progeny obtained after ROSI from \(X^{E}O\text{Sox9}\) (A), \(XO\text{Sry,Eif2s3x}\) (B), and \(XO\text{Sox9,Eif2s3x}\) (C) males were genotyped with PCR and qPCR. The expected progeny genotypes are 4, 8 and 8 for \(X^{E}O\text{Sox9}\), \(XO\text{Sry,Eif2s3x}\), and \(XO\text{Sox9,Eif2s3x}\) males, respectively. All expected progeny genotypes were obtained from \(X^{E}O\text{Sox9}\) and \(XO\text{Sry,Eif2s3x}\) males, and were evenly distributed. Among the progeny obtained from \(XO\text{Sox9,Eif2s3x}\) one expected genotype (XX\text{Sox9}) did not appear and the male progeny genotypes were not evenly distributed. Number of progeny genotyped was n=18, n=46, n=41 for A, B, and C, respectively. Statistical significance (Fisher's Exact Test): * different than all other except XX\text{Sox9,Eif2s3x}; ** P<0.01; *** P<0.001.
Figure 70. The genotyping of the progeny obtained after ROSI from "No Y" male. D = XOSox9,Eif2s3x male dad, which provided round spermatids for ROSI. P1-P7 = ROSI offspring derived from D. +, -, and B are positive, negative, and blank control, respectively. Eif2s3x tg and Sox9 tg amplify both transgenic and endogenous Eif2s3x and Sox9, respectively. X-encoded Eif2s3x and autosomally encoded myogenin (Myog) are present in all mice while no Y chromosome genes are present in XOSox9,Eif2s3x dad and its progeny.
Figure 71. Progeny from males with a single or no Y chromosome genes. (A) Newborn pups born after ROSI with spermatids from XOSox9,Eif2s3x male. (B) 19 months old ROSI offspring derived from XOSox9,Eif2s3x male. (C) The results of breeding of female ROSI offspring. (D &E). Female ROSI offspring derived from XOSox9,Eif2s3x male with their own pups. (F) Offspring weights. ROSI offspring derived from X^{E}OSox9, XOSry,Eif2s3x and XOSox9,Eif2s3x males were weighted monthly from 3 to 8 months of age. Two controls were 'XY mating' (B6D2F1 mice derived by mating) and 'XY ROSI' (ROSI offspring derived from wild-type males). P value in bottom left corner of each graph shows the result of one-way ANOVA analysis and horizontal lines show the differences between individual groups measured by post-hoc Tukey test * P<0.05; ** P<0.01 (GraphPad Prism). The only group that differed from other were female offspring derived from X^{E}OSox9 males; this group had the smallest number of mice analyzed.
Figure 72. Offspring growths measured as an increase in body weight over time. ROSI offspring derived by injection of round spermatids from X\textsuperscript{E}O\textsubscript{Sox9}, XOSry,Eif2s3x and XOSox9,Eif2s3x males were weighted monthly from 3 to 8 months of age. The data shown are from mice, for which weights were scored for all examined months (4, 5, 6, and 7). Two control groups were included in the analysis: 'XY mating' are B6D2F1 mice derived by mating. 'XY ROSI' are offspring derived after ROSI with wild-type males. The data were analyzed with two-way ANOVA with age and group as factors, and post-hoc Bonferroni test for multiple paired comparison. Males and females from all groups increased significantly in weight over the examined period (males, P=0.003; females, P=0.006, ANOVA, age). There were no differences between groups of male offspring at any age (P=0.345, ANOVA group) but the difference was observed for females (P<0.0001, ANOVA group). Post-hoc test demonstrated that female offspring derived from X\textsuperscript{E}O\textsubscript{Sox9} males were different from offspring from 'XY mating' group at 4, 6, and 7 months (P<0.05, P<0.05, and P<0.01, respectively) and from females derived from XOSry,Eif2s3x males at 7 months (P<0.05). The number of mice examined for XY mating, XY ROSI, X\textsuperscript{E}O\textsubscript{Sox9}, XOSry,Eif2s3x and XOSox9,Eif2s3x groups was 7, 5, 6, 4, 10 (males) and 7, 5, 3, 7, 6 (females), respectively. All ROSI offspring were derived after injection of spermatids from males on partial (transgensics) or full MF1 (XY) genetic background and oocytes from B6D2F1 females. Error bars are not shown for graph.
Section 8.2 Supplements to Chapter 3

Part 8.2.1 from Part 3.3.4 Round Spermatid Injection (ROSI) and Intracytoplasmic Sperm Injection (ICSI)

Injections with testicular cells were performed as described before [7, 61]. Testes were collected twice from each male following initial semi-castration, and used for preparation of testicular cell suspensions for injections. The metaphase II (MII) oocytes for ROSI were collected from superovulated (5 iu eCG and 5 iu hCG given 48 hrs apart) female mice and incubated at 37°C, 5% CO\textsubscript{2} until injection. Testicular sperm suspension was diluted with HEPES-CZB containing 1% (w/v) polyvinyl pyrrolidone (PVP) on the injection dish. Spermatids were injected individually into the oocytes. The total duration of ROSI was no longer than 1 hour. The oocytes were activated shortly after injection by incubation in Ca\textsuperscript{2+}-free CZB medium supplemented with 2.5 mM SrCl\textsubscript{2} at 37°C, 5% CO\textsubscript{2} for 4 hrs, after which time they were transferred into standard CZB medium for subsequent culture. At ~6-8 hrs after injection the oocytes were assessed for polar body extrusion and pronuclei development. Normally fertilized oocytes exhibiting two pronuclei (PN) and extruded second polar body (PBII) were allowed to cleave and were subjected to embryo transfer. Surrogate mothers were subjected to caesarian section on day 18 of pregnancy to allow for scoring of the numbers of fetuses and implantation sites.

Part 8.2.2 from Part 3.3.5 Zygote Chromosome Analysis

Chromosome preparation and analysis were performed as previously described [80]. In wild-type males the spermatid used for injection is considered chromosomally normal when resulting zygote contains 40 normal metaphase chromosomes, 20 maternal and 20 paternal. Males
with limited Y gene complement either lack the Y chromosome or carry a minute Y*^X chromosome variant. Thus, for these genotypes lack of one chromosome in the paternal chromosome complement and the presence of one small variant were considered normal.

Part 8.2.3 from Part 3.3.7 Real-Time RT-PCR

For real-time reverse transcriptase polymerase chain reaction (RT-PCR), total testis RNA was extracted using Trizol and DNaseI treatment (Ambion, Austin, TX, USA), and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer’s guide-lines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI QuantStudio 12K Flex machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 40 PCR cycles (10 s at 95°C and 60 s at 60°C). For analysis of Zfy expression, two types of PCR reactions were performed: (1) ‘Zfy1’ amplifying only Zfy1 transcripts and (2) ‘Zfy Global’ amplifying both Zfy1 and Zfy2. Three mice per genotype were analyzed, all reactions were carried out in quadruplicates per assay, and four different loading controls, two ubiquitously expressed genes (actin and Sdha) and two spermatid-specific genes (Act and Acrv) were used. ΔCt value for each individual sample was calculated by subtracting either the average Ct or geometric mean of loading control(s) from the average Ct of a tested gene. ΔΔCt value was calculated by subtracting the ΔCt of each tested male from the average ΔCt of wild-type XY males, which served as references. The data were expressed as a fold value of expression level.

Part 8.2.4 from Part 3.4.1 Functional of Sperm from X^E Sxr^b O and X^E Sxr^b Y*^X Males in Assisted Fertilization

The presence of Sxr^b enables spermatid elongation in X^E Sxr^b O and X^E Sxr^b Y*^X males, with
occasional development of mature testicular sperm [35, 61] (Fig. 73). To test for the ability of these testicular sperm to participate in successful assisted fertilization and embryo development, we performed intracytoplasmic sperm injection (ICSI). No live offspring were obtained from $X^ESxr^bO$ males ($n=4$ males, $0/94$ fetuses from embryos transferred), while ICSI with sperm from $X^ESxr^bY^{*X}$ males yielded a single fetus ($n=5$ males, $1/84$ fetuses from embryos transferred). Thus, sperm from $X^ESxr^bO$ and $X^ESxr^bY^{*X}$ males are virtually non-functional in assisted fertilization.

A possible reason for the lack of function of these sperm could be sperm diploidy. We have previously shown that the great majority (86%) of round spermatids from $X^ESxr^bO$ males were diploid while the opposite was true for $X^ESxr^bY^{*X}$ males, in which haploid round spermatids predominated (71%) [61]. To test whether testicular sperm from these males carried doubled DNA content we performed zygotic chromosome analysis after sperm injection. This analysis demonstrated that only about one-fourth of the embryos obtained after ICSI with sperm from $X^ESxr^bO$ males were diploid (26%, 12/46, Fig. 74 A); the remaining zygotes were triploid and thus presumably derived from diploid sperm. Zygotes obtained after ICSI with sperm from $X^ESxr^bY^{*X}$ males were predominantly diploid (64%, 9/14, Fig. 74 A & B). These data support that while sperm diploidy might be responsible for the lack of ICSI success with $X^ESxr^bO$, it is not likely the case with $X^ESxr^bY^{*X}$ males. We have shown earlier that testicular sperm from males carrying the Y chromosome derived sex reversal factor $Sxr^{a}$ (Fig. 38 A & B in Chapter 3, $Sxr^{a}$, $XY^{*X}Sxr^{a}$) are haploid [80]. Thus, functional deficiency of sperm from $X^ESxr^bY^{*X}$ males is likely due to lack of one or more Y chromosome genes that are present in $XY^{*X}Sxr^{a}$ and not in $X^ESxr^bY^{*X}$.

Overall, the data demonstrate that testicular sperm from $X^ESxr^bO$ and $X^ESxr^bY^{*X}$ males are not functional in assisted fertilization and that this may reflect lack of certain Y gene/s.
Figure 73. Testicular sperm from $X^{E_{X,r} Y^X}$ males. Examples of sperm found in live testicular sperm suspension from two different $X^{E_{X,r} Y^X}$ males. Arrows show sperm with tails and arrowheads separated sperm heads, both of which could be found in testis cell suspension. Also visible is a pipette used to transfer sperm. Scale = 10 µm.
Figure 74. Zygotic chromosome analysis after ICSI with sperm from $X^E Sxr^b O$ and $X^E Sxr^b Y^* X$ males. (A) Zygote chromosome analysis after sperm injection from males with limited Y gene complement was used to assess the ploidy of sperm used for injections. The incidences of diploid and triploid zygotes generated with ICSI are shown. (B) Exemplary zygotic chromosome spreads. Injection of sperm from $X^E Sxr^b Y^* X$ males yielded zygotes containing either ~40 chromosomes (B, left panel, diploid) or ~60 chromosomes (B, right panel, triploid) indicating that injected sperm varied in respect to their chromosome number. Lower number of chromosomes (38 instead of expected 40 and 56 instead of expected 60) is either because of chromosome loss during preparation or because of oocyte hypoploidy, which is common in the mouse (our unpublished observations), or because of chromosome aberrations (fragments, breaks, translocations), which account for the lower number countable chromosomes overall. cf = chromosome fragments. Bar = 50 µm.
Part 8.2.5 from Part 3.4.3 Increases of Success with Round Spermatids Injection (ROSI) and ICSI Yielding Live Offspring with Addition of Zfj2 to XE OSry and XE Y*XSry Males

In our recent study we have shown that ROSI success with XE OSry and XE Y*XSry males was below 10% (9% and 6%, respectively) while with XE Sxr/O and XE Y*XSxr males ROSI efficiency increased by ~2-2.5 fold (20% and 16%, respectively), suggesting that a gene/s encoded within Sxr provides some benefit for assisted reproduction success [61]. Considering the Zfj2 role in meiotic progression [244] and spermatid elongation (Fig. 39 in Chapter 3) we decided to test whether Zfj2 is beneficial for germ cell function. When ROSI was performed with round spermatids from XE Z2 OSry and XE Z2 Y*XSry males (Fig. 38 B in Chapter 3) live offspring rate increased, reaching 27% and 43%, respectively (Table 15).

Sperm from XE Sxr/O and XE Sxr/Y*X males are not functional in assisted fertilization, and for XE Sxr/Y*X males it could be attributed to Y gene deficiency. To test whether Zfj2 influences ICSI outcome, we performed injections with sperm from an XE Z2 Y*XSxr male, which had both Sxr (encoding Zfj2/1 fusion gene) and the Zfj2 transgene (Fig. 1B). We had only one male of this genotype available as sperm donor for ICSI, and only 7 embryos were transferred but those yielded 2 live offspring (29%, 2/7). Encouraged by this result we moved on to test sperm from XE Z2 Y*XSry males. Out of 5 males examined, 4 had testicular sperm and yielded live ICSI offspring (Table 1). The efficiency of ICSI with sperm from XE Z2 Y*XSry males was lower than with sperm wild-type XY controls (23% vs. 57%, P<0.001). Because each XE Z2 Y*XSry and XY male provided both round spermatids (ROSI) and sperm (ICSI) for injections, we were able to perform a direct comparison of these two types of germ cells for their ability to participate in successful assisted fertilization. In XY males, as expected, the efficiency of ROSI was lower than ICSI (Table 15,
30% vs. 57%, P<0.01). Interestingly, this pattern was reversed in $X^{E,Z2}Y^*X^{Sry}$ males, in which ROSI success was significantly better (Table 15, 43% vs. 23%, P<0.01).

$X^{E,Z2}Y^*X^{Sry}$ males generate several types of gametes, which consequently lead to several possible progeny genotypes. Genotyping of all progeny obtained after ROSI and ICSI revealed that anticipated offspring types were produced and their frequency met the expectancy, with 4 predominating genotypes accounting for 87% of all genotypes and distributed within 11%-44% range each, and 4 rare genotypes, which originated from atypical segregation of sex chromosomes or from rare recombination events (Fig. 75 and Table 16).
Progeny genotype frequencies. Frequency of the offspring genotypes obtained after assisted reproduction with $X^{E,Z2}Y^{Sry}$ males. Four predominant genotypes (grey bars) and eight rare genotypes derived from rare recombination events or untypical sex chromosome segregation which appear sporadically (black bars on the right side of the dashed line) are expected from $X^{E,Z2}Y^{Sry}$ males. Four additional genotypes representing a combination of rare recombination and untypical chromosome segregation are also possible (see Table 16) but were not noted among the progeny and are not included here for simplicity. Number of genotyped progeny was 49 for ROSI and 18 for ICSI.
Table 15. The results of round spermatid injection (ROSI) and intracytoplasmic sperm injection (ICSI) with germ cells from males with limited Y gene complement.

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Y gene contribution</th>
<th>Males yielding progeny</th>
<th>Fetuses % (no)</th>
<th>Implants % (no)</th>
<th>Males yielding progeny</th>
<th>Fetuses % (no)</th>
<th>Implants % (no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XE, Z2 O Sry</td>
<td>Eif2s3y, Sry &amp; Zfy2</td>
<td>2/2</td>
<td>27 (7/26)</td>
<td>39 (10/26)</td>
<td>no sperm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XE, Z2 Y*Sry</td>
<td>Eif2s3y, Sry &amp; Zfy2</td>
<td>5/5</td>
<td>43 (48/111)</td>
<td>79 (71/90)</td>
<td>4/4</td>
<td>23 (18/79)</td>
<td>48 (38/79)</td>
</tr>
<tr>
<td>XY</td>
<td>intact Y</td>
<td>4/4</td>
<td>30 (21/70)</td>
<td>63 (44/70)</td>
<td>3/3</td>
<td>57 (32/56)</td>
<td>80 (45/56)</td>
</tr>
</tbody>
</table>

Statistical significance (Fisher's Exact Test):  a P<0.05,  b P<0.001 vs. respective category in XY control;  c P<0.05,  d <0.01 vs. ICSI within genotype.
Table 16. The genotypes of offspring obtained after ICSI and ROSI with germ cells from X<sup>E,Z2</sup>Y<sup>*X</sup>Sry males.

<table>
<thead>
<tr>
<th>Male ID</th>
<th>M1</th>
<th>M5</th>
<th>M7</th>
<th>M8</th>
<th>M10</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of pups</td>
<td>20</td>
<td>14</td>
<td>17</td>
<td>10</td>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E,Z2&lt;/sup&gt;</td>
<td>5 (25)</td>
<td>0</td>
<td>1 (6)</td>
<td>0</td>
<td>2 (33)</td>
<td>8 (12)</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E,Z2&lt;/sup&gt;Sry</td>
<td>1 (5)</td>
<td>5 (36)</td>
<td>4 (24)</td>
<td>4 (40)</td>
<td>0</td>
<td>14 (21)</td>
</tr>
<tr>
<td>XY&lt;sup&gt;*X&lt;/sup&gt;</td>
<td>4 (20)</td>
<td>3 (21)</td>
<td>5 (29)</td>
<td>3 (30)</td>
<td>1 (17)</td>
<td>16 (24)</td>
</tr>
<tr>
<td>XY&lt;sup&gt;*X&lt;/sup&gt;Sry</td>
<td>7 (35)</td>
<td>3 (21)</td>
<td>5 (29)</td>
<td>3 (30)</td>
<td>2 (33)</td>
<td>20 (30)</td>
</tr>
<tr>
<td>XY&lt;sup&gt;<em>X&lt;/sup&gt;Y</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>2 (12)</td>
<td>0</td>
<td>0</td>
<td>2 (3)</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E&lt;/sup&gt;Sry</td>
<td>2 (10)</td>
<td>1 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (4)</td>
</tr>
<tr>
<td>XX&lt;sup&gt;Z2&lt;/sup&gt;</td>
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<td>2 (15)</td>
<td>0</td>
<td>0</td>
<td>1 (17)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>XX&lt;sup&gt;Z2&lt;/sup&gt;Sry</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E,Z2&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E,Z2&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;Sry</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>X&lt;sup&gt;Y&lt;/sup&gt;</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XO</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (2)</td>
</tr>
<tr>
<td>XO Sry</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;E&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;Sry</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;Z2&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XX&lt;sup&gt;Z2&lt;/sup&gt;Y&lt;sup&gt;*X&lt;/sup&gt;Sry</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The genotypes in shaded cells are supposed to be infrequent and originate from rare recombination events, untypical segregation of sex chromosomes, or a combination of both. X chromosome in blue font is of oocyte origin. See Figure 5 for distinction between ROSI and ICSI progeny.
Part 8.2.6 from Part 3.4.4 Determination of Zfy2, and Zfy1 Role in Sperm Function with Zfy Expression Analysis

To correlate spermiogenic phenotype with Zfy expression we performed Zfy transcript quantification on whole testes from $X^EY^{*}X^{Sry}$ (no Zfy), $X^EY^{*}X^{Sxr}$ (Zfy2/1 fusion gene), $X^{E,Z2}Y^{*}X^{Sry}$ (Zfy2 transgene), with $XY^{*}X^{Sxr}$ (endogenous Zfy2 and Zfy1 and no NPYq) and XY (intact Y chromosome) serving as controls. Because Zfy2 and Zfy1 are very similar (97% and 94% for transcript and amino acid identity, respectively) we failed to design real-time PCR primers that were specific to Zfy2. We therefore quantified the expression of Zfy1, and Zfy1 and Zfy2 combined (global) (Fig. 76 and Fig. 77).

As expected, no Zfy1 transcripts were detected in $X^EY^{*}X^{Sry}$ and $X^{E,Z2}Y^{*}X^{Sry}$ males. $XY^{*}X^{Sxr}$ males had ~1.5 fold higher Zfy1 levels than XY males, due to the lack of NPYq genes known to result in the upregulation of sex chromosome genes, including Zfy [58]. Zfy1 levels in $X^EY^{*}X^{Sxr}$ males were ~10 fold higher than in XY, representing a combined effect of the NPYq absence and strong Crypt-derived spermatid-specific promoter driving expression of the Zfy2/1 fusion gene. The global Zfy expression was again higher in $X^EY^{*}X^{Sxr}$ and $XY^{*}X^{Sxr}$ than in XY, but the difference was lower in magnitude. Zfy global levels in $X^{E,Z2}Y^{*}X^{Sry}$ males were similar to those of $XY^{*}X^{Sxr}$ and lower than in $X^EY^{*}X^{Sxr}$ males. In $X^{E,Z2}Y^{*}X^{Sry}$ males the global levels were reflective exclusively of Zfy2, while in $X^EY^{*}X^{Sxr}$ males primarily of Zfy1 since the Zfy2/1 fusion gene encodes Zfy1 coding region under the control of Zfy2 promoter [50].

When the spermiogenic phenotype is viewed in the context of Zfy expression our data support that it is Zfy2, and not Zfy1 (even if present in abundance), that enables the formation of sperm functional in ICSI.
Figure 76. Zfy expression. (A) Zfy1 transcript levels in genotypes of interest (n=3 per genotype) obtained by real-time PCR. The loading controls were two ubiquitously expressed genes (actin and Sdha) and two spermatid-specific genes (Act and Acrv), and normalization was achieved by geometric averaging of these genes. (B) Zfy1 and Zfy2 (global Zfy) transcript levels were examined as in A. Values are mean ± SEM. Statistical significance: a different than all others (except zero to zero values comparison); * P < 0.05. For explanation of genotypes see Fig. 1, Table 1 and text. Primer sequences are shown in Table S2. The data normalized to individual reference genes are shown in Fig. 77.
Figure 77. *Zfy* expression expanded. *Zfy1*, and *Zfy1* and *Zfy2* (global *Zfy*) transcript levels transcript levels in genotypes of interest (n=3 per genotype) obtained by real-time PCR. The same samples were run independently with four different loading controls, two ubiquitously expressed genes (actin and *Sdha*) and two spermatid-specific genes (*Act* and *Acrv*). Values are mean ± SEM. Statistical significance: a different than all others (except zero to zero values comparison); * P < 0.05. Primer sequences are shown in Table 8 in Chapter 3.
Section 8.3 Supplements to Chapter 6

Part 8.3.1 from Part 6.3.2 Embalming

The body was washed thoroughly using antibacterial soap, with special care paid to the eyes, nose and mouth. The body was elevated above the Table surface using body blocks at critical points (neck, shoulders, lumbar region and heels) to avoid constricting arteries in the back and buttocks. Anatomical embalming was a three-day process. **Day One:** Embalming cannulas were placed in the carotid arteries directed superiorly into the head and inferiorly into the body. Injection was done at a moderate/high pressure and a slow flow rate (~6-8 oz. per min). The embalming solution consisted of: 1 L formaldehyde, 1 L of Methanol, 2.5 L of Isopropyl Alcohol, 0.5 L of Glycerin and enough water to make 12 L of total embalming solution. There was no blood drainage during the embalming process. Once the head was perfused, cannulas were switched and the body injected with the remainder of the embalming solution. **Day Two:** 12 L of embalming solution was injected. **Day Three:** Another 12 L of embalming solution was injected. When the body was adequately perfused, the gluteal region was hypodermically injected to increase overall vascular pressure. The cadaver was placed into a heavy-duty body bag and stored.
CHAPTER 9 REFERENCES


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