# ANALYSIS OF TRANSGENIC MOUSE MODELS TO STUDY MAMMALIAN SPERMATOGONIA

by

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Spermatogenesis in the mammalian testis results in the daily production of millions of spermatozoa. This developmental process is founded upon the actions of a small population of spermatogonial stem cells (SSCs). As SSCs divide, their progeny must either remain an SSC to maintain the stem cell pool (self-renewal) or become an undifferentiated progenitor spermatogonium that proliferates prior to differentiating in response to retinoic acid (RA) and entering meiosis. Our laboratory is focused on understanding the mechanisms that regulate these fate decisions as well as defining the changes that occur downstream of RA that prepare spermatogonia for entry into meiosis. Currently, researchers have been unable to readily isolate pure populations of spermatogonia in the developing or adult testis. To address this, I have examined multiple transgenic mouse models to identify those with germ cell-specific expression of fluorescent reporter genes that will enable us to isolate spermatogonia using fluorescence-activated cell sorting (FACS). The results in this thesis describe these

efforts, and document the identification of an excellent mouse model for future studies in our laboratory to understand the mechanisms underlying spermatogonial development. This work was supported by a grant from the NIH/NICHD (HD090083) to C.B.G.

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by

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| | |
|---|---|
| Ab | Antibody |
| AZF | Azoospermia factor |
| BDAD | WIN 18,446 (bis-(dichloroacetyl)-diamine |
| DAPI | 4',6-diamidino-2-phenylindole |
| E | Embryonic Day |
| EGFP | Enhanced Green Fluorescent Protein |
| FACS | Fluorescence activated cell sorting |
| GFP | Green Fluorescent Protein |
| IF | Immunofluorescence |
| IIF | Indirect Immunofluorescence |
| IHC | Immunohistochemistry |
| MACS | Magnetic-activated cell sorting |
| NOA | Non-obstructive azoospermia |
| OA | Obstructive azoospermia |
| PGC | Primordial germ cell |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| P | Postnatal day |
| RA | Retinoic acid |
| SSC | Spermatogonial stem cell |

# CHAPTER 1: Introduction

*General introduction to spermatogenesis – mitosis, meiosis, spermiogenesis*

The survival of all living organism depends on reproduction. In diploid organisms, sexual reproduction relies on actions of the gametes, the sperm and the egg. These distinct gametes must find each other, recognize that they belong to the same species, and then fuse their haploid genomes to create a new diploid organism. Gametogenesis in females, termed oogenesis, is significantly different than spermatogenesis in males. The resulting gametes are also distinct. Eggs are generally larger, immotile, and possess abundant cytoplasmic material necessary for early embryonic development, sperm are compact, motile, carry minimal cytoplasm, and are produced in large quantities. The continuation of life on Earth for sexually-reproducing species is dependent on spermatogenesis and oogenesis.

Spermatogenesis is a complex and highly efficient process producing millions of terminally differentiated and specialized sperm every day in males. Male germ cells must pass through multiple differentiation steps in order to become sperm [1], and these steps can be used to divide the process into three main developmental phases. The first, or mitotic phase, involves spermatogonia. Male germline stem cells (also termed spermatogonial stem cells, or SSCs) proliferate to amplify their population as well as to produce committed progenitor spermatogonia that undergo multiple mitotic divisions before entering the second phase. The second phase is meiosis, and involves spermatocytes. During meiosis, DNA is replicated without division and the resulting chromosomes synapse in pairs and recombine their DNA before 2 subsequent

reduction divisions produce haploid spermatids. In the third phase of spermiogenesis, spermatids undergo a morphogenetic transformation to become sperm. Detailed aspects of these processes will be discussed in greater detail later in the introduction.

## Formation of the male germline and prenatal development

The germline is set aside early during embryonic development in mammals. This process has been best described in the mouse, although evidence in humans reveals that the processes are remarkably similar. A small number of cells become committed to the germ cell lineage and are termed primordial germ cells (PGCs). PGCs can be first identified as a cluster of approximately 40 alkaline phosphatase positive cells around embryonic day (E)7.25 [2]. Over the next few days of development, PGCs undertake a long voyage to eventually take up residence in the developing gonads. They first migrate to the developing hindgut endoderm by E7.75 [3], then into the mesentery by E9.5 [4], and then arrive at the genital ridges (future gonads) around E10.5 [3, 5]. Sex determination occurs around E10.5, and subsequently the PGCs in the testis become prospermatogonia (also known as gonocytes). Prospermatogonia proliferate until approximately E15.5 and then exit the cell cycle and enter a prolonged period of mitotic arrest in $G_0$ [6, 7]. This quiescent period persists until after birth when, in response to an unknown signal(s), prospermatogonia reenter the cell cycle to become spermatogonia at P3-P4 [8]; this event defines the beginning of spermatogenesis.

### *The initiation of spermatogenesis – postnatal development*

This initial cohort of spermatogonia in the mouse testis is heterogenous, and

contains SSCs which will undergo self-renewal as well as proliferating and differentiating spermatogonia. SSCs undergo asymmetric cell divisions and progeny either remain an SSC to maintain their population or become undifferentiated spermatogonia that will eventually become differentiating spermatogonia. The first differentiating spermatogonia then proceed through the 3 stages of spermatogenesis (mitosis, meiosis, and spermiogenesis) in the mouse without temporal interruption. Once differentiated, type A spermatogonia undergo 6 divisions before entering meiosis and becoming the first preleptotene spermatocytes at P8. At the completion of meiosis, haploid and round spermatids undergo dramatic morphological and chromatin changes during spermiogenesis prior to being released into the lumina of seminiferous tubules as testicular sperm. These first testicular sperm are produced as early as ~P35 in mice [9] (Figure 1.).

The mitotic divisions - spermatogonia

The foundation of spermatogenesis throughout the lengthy male reproductive lifespan is provided by spermatogonia, which represent a tissue-specific stem cell system conceptually similar to those in other body systems (e.g. intestine, skin, hematopoiesis). These mitotically-active spermatogonia exist as the aforementioned 3 main subtypes (stem/SSC, undifferentiated progenitor, and differentiating). SSCs undergo asymmetric cell divisions, and progeny either become another stem cell (self-renewal) or a transit-amplifying undifferentiated progenitor spermatogonium [10] that proliferates before committing to differentiate in response to retinoic acid (RA) [11]. Maintaining the delicate balance between self-renewal and differentiation is critical to

sustained production of functional spermatozoa throughout the male reproductive lifespan. Impaired SSC self-renewal or enhanced differentiation result in dramatic reduction or total loss of the germline, resulting in infertility. Improper maintenance of the spermatogonial populations has also been proposed to cause carcinoma in situ (CIS), the precursor lesion to testicular germ cell tumors (TGCTs) [12].

Spermatogonial fates are established as early as P3-4 in the mouse, with $A_{undiff}$ (SSC and progenitor) and $A_{diff}$ (differentiating) spermatogonia expressing specific mRNAs and protein markers [13]. Many of these markers have been directly linked to spermatogonial fate based on analysis of mouse models in which these genes have been deleted; the relative role of specific gene products in spermatogonial development can be assess by analyzing reproductive phenotypes and by performing heterologous transplantation assays [13, 14]. A large number of protein markers have been identified that are expressed in $A_{undiff}$ spermatogonia, including GFRA1 [15, 16], RET [17], ID4 [9], ZBTB16/PLZF [18], [19], CDH1 [20], and SOX3 [21]. However, few proteins have been identified that are upregulated in or only detectable in $A_{diff}$ spermatogonia; the few that are currently known include KIT [22-25], STRA8 [26, 27], SOHLH1, and SOHLH2 [28].

The undifferentiated spermatogonial pool is heterogeneous and provides the foundation for spermatogenesis. It is clear that the $A_{undiff}$ spermatogonia contains both the SSCs as well as the transit-amplifying spermatogonia that proliferate and are poised to differentiate (Ellen Velte, unpublished results). As isolated undifferentiated spermatogonia (termed $A_{single}$ or $A_s$) divide, their progeny can remain connected by an intercellular bridge (ICB), and these are termed $A_{paired}$ or $A_{pr}$ spermatogonia. Further divisions grow the length of these ICB-connected chains of spermatogonia, which are

now termed $A_{aligned}$ or $A_{al}$ spermatogonia [12]. The function of ICBs is not fully understood; however, they are evolutionarily well-conserved among species, and large enough for passage of macromolecules and even organelles such as mitochondria [29]. Studies from Dr. Braun show that X- and Y-linked gene products can be shared among adjacent spermatids that are connected by ICBs suggesting that the products of most genes may be equally distributed among spermatids [30].

Evidence derived from rodent studies reveals that the majority of stem cell activity resides within the $A_s$ population. The most widely accepted model depicting the dynamics of the SSC and progenitor spermatogonial pool is referred to as the "$A_{single}$ model". The first evidence supporting this model came from studying spermatogenesis in rats in 1971 [31, 32]. These and multiple reports derived from rodent studies suggested that stem cell activity resides exclusively within the isolated $A_{single}$ population, with appearance of an $A_{paired}$ spermatogonia signifying commitment to a differentiation pathway. Transplantation studies done later in mice support this model [9, 33]. However, recent studies show, that while the "$A_{single}$" hypothesis states that all SSCs exist as $A_{single}$ cells, not all $A_{single}$ cells are believed to be SSCs. Based on transplantation assays, it has been estimated that only ~10% of the $A_s$ population exhibit stem-cell capacity, or the ability to seed spermatogenesis when transplanted into a testis lacking a germline [34].

Multiple reports have described heterogeneity of a variety of genes and/or encoded protein markers among undifferentiated spermatogonia. Several proteins (GFRA1, ID4, LIN28, PAX7, ZBTB16/PLZF) show expression patterns that vary among $A_{undiff}$ spermatogonia with different clone length and between different spermatogonial

clones of the same length [9, 35-37]. Heterogeneous expression patterns have also been reported within individual spermatogonial clones for markers such as GFRA1 or NANOS2 [35, 36] suggesting $A_{undiff}$ exist as multiple dynamic subpopulation. Another functional characteristic of neonatal spermatogonia is their ability to respond to RA signaling [38]. Our laboratory reported recently that both ZBTB16/PLZF and CDH1 are expressed in nearly all spermatogonia from P1 through P7, while markers of $A_{diff}$, STRA8 and KIT, are only detectable in a subset of spermatogonia at P4, coincident with onset of RA signaling. From P10 and further, distinct populations of $A_{undiff}$ and $A_{diff}$ exhibit a unique pattern of expression of spermatogonial fate markers. This indicates either entry into differentiation phase or retention of the SSC phenotype [14]. Taken together, the evidence in the literature clearly supports the presence of heterogeneity among male germ cells in the neonatal testis.

Spermatogonial differentiation is a complex process that is initiated by RA, which is a bioactive metabolite of vitamin A (retinol) and a key regulator of spermatogenesis. Evidence in the literature reveals that the primary source of RA in the developing mouse testis is Sertoli cells. The deletion of retinol dehydrogenase (Rdh10), a key enzyme in biosynthesis of retinal from retinol [54] in Sertoli cells, resulted in loss of differentiating spermatogonia [53]. Literature suggests that RDH10 may be a key enzyme responsible for the postnatal testicular RA synthesis. RA signaling is mediated through three RA receptors (RARs) that are localized within the nucleus, and involved in both ligand-dependent activation of target genes [39].

The current understanding of critical importance of RA in spermatogenesis has been acquired from studies employing dietary, genetic, and chemical approaches. The

effects of dietary depletion of RA has been studied by many laboratories though analyzing Vitamin A-Depleted (VAD) testes. VAD mice can be supplemented with RA during different time points to reinitiate spermatogenesis, and this approach has provided us an extensive collection of gene expression data [40, 41], as well as providing an insight of SSCs ability to maintain their “stemness” in low vitamin A environment in the adult animal [42].

Unfortunately, the effects of dietary vitamin A depletion on spermatogenesis has only been collected from adult rodents, as complete vitamin A depletion requires several weeks to be achieved. In order to investigate the RA action in neonatal testis, animals carrying null mutations of the retinoid storage (LRAT) or transport (RBP4) enzymes have been developed. The *Lrat*-null model was used to demonstrate another important feature of RA, the role in meiotic initiation during the first round of spermatogenesis. Spermatogonia that failed to enter meiosis in this model were arrested in an undifferentiated state [43]. The *Rbp4*-null model also exhibited the same phenotype. Collectively, these finding suggest that vitamin A and its metabolites regulate spermatogonial differentiation and meiotic initiation. It is clear, however, that much more experimental data must be gathered to gain a complete understanding of the role of RA in the initiation of meiosis. [41, 44-47].

Chemical approaches have become common methods to suppress RA levels in mice in vivo. This method is based on oral administration of chemical compound Bis-dichloroacetyl-diamine, also known as BDAD. One specific BDAD, WIN 18,446 (WIN) was shown to reversibly block spermatogenesis. Ex vivo studies have shown that WIN acts as a blocker of RA production, while in vivo studies demonstrated reduced RA

levels, reduced levels of RA responsive genes, and an accumulation of undifferentiated spermatogonia [48-50]. This method has been shown to be faster, and safer compare to the alternatives described earlier. The manipulations of RA levels has become a commonly used procedure in our laboratory and we plan on employing this technique on transgenic mouse models described later in the introduction section of this work.

Following RA exposure, undifferentiated spermatogonia become type $A_1$, and they will undergo five additional divisions to become $A_2$, $A_3$, $A_4$, intermediate (In), and type B spermatogonia before finally entering meiosis as preleptotene spermatocytes [32, 51]. Therefore, the developmental progression that spermatogonia make towards meiosis requires a consistent, albeit perhaps not continuous supply of RA [52].

*The SSC Niche*

The maintenance of the SSC population theoretically relies on a unique niche microenvironment within the testis. This niche would be predicted to involve the adjacent somatic Sertoli cells, peritubular myoid cells and interstitial vascular cells, Leydig cells, and macrophages [55-57].

Sertoli cells are thought to be among cells that are most involved in the formation of the putative niche microenvironment. These somatic cells are positioned alongside spermatogonia within the seminiferous epithelium (Fig. 4A), and will eventually support all phase of germ cell development.

These cells are critically important for SSC niche, and this is demonstrated by their abundance and direct association with fluctuation in the SSC pool.

A study published in 2011 by the Oatley laboratory showed that increasing the number

of Sertoli cells in mouse testes by manipulating thyroid hormone levels increased the number of SSCs [58].

Another important function of Sertoli cells is formation of junctional complexes of BTB. The BTB divides the seminiferous epithelium into adluminal and basal compartments, and provides a functional barrier to prevent passage of biomolecules and toxins from the circulatory and lymphatic systems into the adluminal compartment [59-61]. This division creates an immune-privileged area for germ cell meiosis and spermatid development. The BTB is a critical component that is required for continuous spermatogenesis in the adult.

In addition to tubular Sertoli cells, cells within the interstitial tissue between seminiferous cords and tubules also would be predicted to influence the regulation of spermatogenesis, perhaps by regulating the SSC niche. The interstitial tissue consists of Leydig cells, macrophages, peritubular myoid (PTM) cells, as well as abundant cell types associated with vasculature and innervation. It has been shown that spermatogenesis is dependent upon hormonal stimuli provided by somatic cells that result in a complex pattern of intratesticular signaling pathways [62]. The two main hormones responsible for its control are pituitary follicle stimulating hormone (FSH), and testosterone, which is produced by the testicular Leydig cells under the influence of pituitary luteinizing hormone (LH). The importance of these hormones on spermatogenesis has been demonstrated in pioneering works of Greep and Smith in the 1920s and 1930s [63]. Both hormones play a role in the initiation of spermatogenesis, germ cell proliferation, development and differentiation.

Another example of critical importance of somatic cells is PTM cells. These

squamous smooth muscle cells reside in the interstitium of the fetal testis and migrate to surround the exterior of the testis cords in the neonatal testis [64, 65]. Similar to Sertoli cells, PTM cells provide structural support to developing germ cells. In recent years it has become more apparent that PTM cells are also critical regulators of spermatogenesis. For a long time Sertoli cells were considered the only source of secreted glial cell line-derived neutrorophic factor (GDNF), the ligand that binds to the GFRA1/RET co-receptors to support undifferentiated spermatogonia. Studies published in 2014 demonstrate the PTM cells as a secondary source of GDNF in neonatal testis [66, 67].

Unfortunately, we as a field have a limited understanding of all the factors involved in establishment and maintenance of SSC niche. I believe that this work will provide a number of tools that may result in acquiring better knowledge of niche microenvironment is the testis.

*Meiosis of Spermatocytes*

Meiosis in spermatogenesis is characterized by a single round of DNA replication followed by two cell division, in which one diploid cell gives rise to four haploid cells. Cells before the first division are termed primary spermatocytes, and before second division are named secondary spermatocytes. Primary spermatocytes are the largest germ cells within the germinal epithelium and can first be observed at P8 in the mouse testis. The biggest difference between mitotic and meiotic phases is the prophase of the first meiotic division. In the mouse, this stage lasts several day, during which genetic recombination takes place. The sub-stages of meiotic prophase are preleptotene,

leptotene, zygotene, pachytene, diplotene, and a brief [68] (Fig. 2). During the prophase of meiosis a number of events occur. Homologous chromosomes must find each other, align, and then synapse. At least one recombination event must occur within each homolog pair, and it must be highly regulated to ensure proper exiting of meiotic prophase [69]. After the first meiotic division, each primary spermatocyte produces two secondary spermatocytes. The secondary spermatocytes are short-lived, which explains why they are seldom observed in testicular biopsies. Each secondary spermatocyte undergoes quick division to become two haploid round cells, termed spermatids [70]. The two maturation divisions of each spermatocyte result in production of four spermatids, which will undergo a process called spermiogenesis.

*Spermiogenesis*

Spermiogenesis is the term describing the final stages of spermatogenesis that occur in the seminiferous epithelium; it is during this phase that postmeiotic haploid round spermatids undergo the morphogenetic transformations that will result in the formation of spermatozoa. During this process, nucleosomal chromatin is transformed into compacted chromatin fibers by the replacement of histones with transition protein which are in turn replaced by protamines [54]. The spermatid ceases active gene transcription as nucleosomes disappear and the chromatin is remodeled and compacted. Another significant event occurring during spermiogenesis is the assembly of sperm flagellum. Soon after the completion of meiosis, a major of component of flagellum, the axoneme, can be observed. As elongation of spermatids continues, the additional structures necessary for flagellum functions (mitochondrial sheath, fibrous

sheath) are assembled around the central axoneme [55]. At the conclusion of the maturation process of spermiogenesis, a partial extrusion of cytoplasm into the tubular lumen occurs, and this extruded cytoplasm is termed a residual body [56]. The final product of spermiogenesis is spermatozoa, a cell with a unique shape that is suitable for the transport to the female gamete.

*When spermatogenesis goes awry: Male infertility*

Infertility is a disease of the reproductive system defined in humans by the failure to achieve pregnancy after 12 months of regular unprotected sexual intercourse [57]. Infertility affects nearly 80 million couples worldwide, and the male partner is responsible for approximately 50% of these cases [58-60] (Figure 3). Male infertility is most often accompanied by deficits (either singly or in combination) in sperm concentration and motility as well as by defective morphology. These conditions have been described using terms such as azoospermia (absence of sperm in the ejaculate), idiopathic oligozoospermia (sperm numbers below 15 million/ml), asthenozoospermia (reduced sperm motility), and aspermia (ejaculation failure) [61]. A combination of two pathologies such asthenozoospermia and oligozoospermia can be often observed in infertile males [62]. Azoospermia is defined as the absence of sperm in the ejaculate and effects approximately 1% of male population, and 15% of infertile men. It can be subcategorized into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). In OA, patients have normal spermatogenesis, exocrine and endocrine function, but have a physical blockage that prevents the release of sperm. OA accounts for approximately 40% of azoospermia cases [63], but can be surgically treated by

removing the blockage. NOA is defined as no sperm in the ejaculate, which results from a failure of spermatogenesis, and is considered to be the most severe form of male infertility, and the number of treatments is very limited due to lack of understanding of specific mechanism of spermatogenesis. Oligozoospermia refers to a low sperm concentration due to failure of primary spermatocytes to progress in development. The exact cause of spermatogenic maturation arrest is not understood, but several factors such as heat stress and chromosomal failure may play a role [64].

The incidence of male infertility has risen in developed countries over the past 50 years [65]. Therefore, there is an increasing interest in defining the underlying environmental and genetic factors that are responsible, although the number of substances definitively proven to have negative effects on spermatogenesis is currently low. Exposure to various environmental agents, such as cadmium, mercury, dioxin, and bisphenol A (BPA) have all been shown to have a detrimental effect on male reproductive capacity in both humans and rodents [66, 67]. Unhealthy lifestyle, smoking, [68], obesity, and environmental toxicants are also all known factors that can negatively impact male infertility.

There is a great number of genetic causes that are thought to underlie male infertility. These include genes involved in a variety of physiological processes, such as spermatogenesis [69], and have been a major focus of research. Chromosomal anomalies are the most well-known genetic contributors leading to male infertility, and are present in an estimated 5% of infertile men [70]. Chromosomal aberrations, either structural or numerical, have profound effects on fertility and are often observed in azoospermia and severe oligospermia, at frequencies ranging from 10%-24% and from

1%-13%, respectively [71, 72]. The second most common genetic cause of male infertility is microdeletions of the azoospermia factor (AZF) regions of the Y chromosome [73]. Microdeletions in this region cause defects in spermatogenesis, that leads to development of azoospermia and oligozoospermia. AZF is a locus on the Y-chromosome that contains 16 coding genes that are required for normal spermatogenesis, code for RNA binding proteins, and may be involved in RNA splicing, and RNA metabolism [74]. These AZF microdeletions occur in 3-15% of azoospermic or oligozoospermic men [75].

Model organisms, especially rodents, have been used to define the molecular and cellular pathways regulating normal male reproduction. It is only by gaining a more complete understanding of spermatogenesis that we can identify the underlying causes of male infertility and begin to develop clinical approaches to treat this disease.

*How can we design a drug to block spermatogenesis: Male contraception*

With the Earth's population significantly rising in recent decades, there is a need for readily available, inexpensive, effective, and safe male contraceptive. Currently, the 2 most commonly used methods of male contraception are condoms and vasectomy. The condom provides a non-surgical physical method of contraception that has been widely used for a long time, and has the added benefit of preventing sexually transmitted diseases. However, condoms are not optimal choices due to breakage, incorrect use, and poor long-term use [76]. A vasectomy is a surgical method of male contraception that can be performed under local anesthesia. Briefly, the vas deferens are isolated and brought out from the scrotum through an incision followed by division

and ligation. This technique has been optimized, and currently minimally-invasive options are available. A “no scalpel technique” minimizes blood loss and reduces the possibility of infection [77]. While this method is effective and the rate of unwanted pregnancy is about 1% [78], it has a number of disadvantages. The reversibility of this procedure is not always successful [79]. With the time elapsed from the procedure increase, the reversibility rate decreases. Many patients may also develop anti sperm antibodies, which may potentially bring down the fertility rate. Other common complication of the vasectomy are significant testicular discomfort [80], hematoma formation (2%), sperm granulomas (10%-30%), chronic orchalgia (12%-52%), and infection (3.4%) [81].

As a modern alternative to external and surgical contraceptives, researchers are currently exploring ways to reversibly block spermatogenesis using pharmaceutical approaches to target genes or signaling pathways required for sperm production. The selectivity, specificity, and lesser side effects of non-hormonal contraceptives make these approaches more attractive compare to hormonal alternatives. For example, indenopyridines are compounds that have been used experimentally to alter expression of Sertoli-germ cell adherens junction proteins, which may result in germ cell loss [82]. It is also used in combination with a gonadotropin-releasing hormone (GnRH) antagonist in studies with monkeys, and was shown to have a specific antispermatogenic effect, which can be reversed under proposed conditions. This study also suggests that this compound can be potentially used as a nonhormonal oral male contraceptive [83]. Another example of nonhormonal contraceptive is gossypol, a plant extract derived from the cotton plant. In study done of Chinese men, this approach showed an adequate

suppression of sperm concentration to levels required for contraceptive [84]. However, the effect of gossypol was irreversible in 20% of the patients, and other dose-dependent side effects included hypokalemia and periodic paralysis. Literature shows, that while we are getting closer to discovering nonhormonal molecular targets that can be used to design a pharmaceutical male contraceptive with limited side-effects, there is still a large amount of work to be done.

*Techniques to study spermatogenesis*

Spermatogenesis is a technically challenging process to study, and this has dissuaded many labs from studying the process, and has hampered progress by labs who do study spermatogenesis full-time such as ours. There are several limitations that must be overcome. First, there is no currently-accepted cell line for in vitro studies. Researchers have generated several spermatogonial lines, but none of them faithfully represent in vivo spermatogonia. Second, spermatogenesis has not been recapitulated in vitro or using ex vivo organ culture systems. Third, it is difficult-to-impossible to isolate individual germ cell types because the testis contains a complex mixture of both germ and somatic cell types whose ratios change dramatically during development. At any age, biochemical studies on germ cells cannot be performed or interpreted using whole testis cell suspensions or lysates. Instead, they must be done on isolated germ cell populations. In the neonatal testis, the germline makes up a relatively small proportion of the overall cell population [42]. In the adult testis, the germline makes up ~90% of the cells within the testis; however, all germ cell types are present, from SSCs to testicular sperm. To-date, several approaches have been used to isolate distinct cell

types. These include STA-PUT, magnetic-activated cell sorting (MACS), and flow cytometry. Each of these approaches has limited utility, and will be discussed below.

*STA-PUT*

This technique was introduced in the 1970s, and is a means to isolate cells from a single cell suspension using differential velocity sedimentation [85]. Sedimentation occurs through a linear BSA gradient, and separates cells based on their size and mass; this technique can yield purities of some, but not all cell types of interest approaching 90% [86]. Researchers have taken advantage of the significant differences in relative sizes of specific germ cell types (e.g. prospermatogonia, pachytene spermatocytes, round spermatids) at specific time points during development to isolate relatively pure cell populations. In our laboratory, we routinely isolate tens of millions of pachytene spermatocytes (~85% purity) and spermatids (~90% purity) using only a few adult mice. The viable spermatogeneic isolated by STA-PUT can be maintained in culture, but not expanded (since they are post-mitotic), and they can be used for the isolation of macromolecules such as mRNAs and protein. The significant disadvantages of STA-PUT include the following: 1 – it cannot be used to isolate cells unless they are significantly larger or smaller than other cell types; 2 – it requires specialized glassware and equipment; 3 – it requires an expert eye to discriminate spermatogenic cell types using light microscopy.

*Magnetic-activated cell sorting (MACS)*

MACS is another powerful tool used for isolation of viable minority cell

populations from single cell suspensions. To isolate enriched cell populations, researchers must have a specific antibody against a unique cell surface-localized protein that can be bound in live cells. This antibody is conjugated to a magnetic bead; after incubating cells with this antibody-bead conjugate, the suspension is passed through a specialized column to which the magnetic bead will bind. Afterwards, multiple washes eliminate unbound cells, and finally the bound cells are eluted from the column [87]. MACS-enriched cells are viable, and can be used for initiating cultures; this has been done with spermatogonia [88]. Spermatogonia have been isolated using antibodies recognizing cell surface proteins including the KIT receptor tyrosine kinase (differentiating spermatogonia [89, 90]) as well as THY1 (undifferentiated spermatogonia) [91]. Although both of these antibodies capture spermatogonia, they also recover significant numbers of interstitial somatic cells, which also express both KIT and THY1. MACS is currently not useful for isolating spermatocytes and spermatids, as appropriate cell surface proteins for immunocapture have not been identified [92].

*Flow cytometry*

Flow cytometry is a well-established technique in biomedical research. While this revolutionary technology combines several concepts, the originality of this technique resides in observation of aligned cells one behind another into a fluid sheath first described in 1934 [93]. In 1980s, optical emission systems appeared, which led to creation of the first high speed cell sorter, that was used for human chromosome separation and up to three color analysis. By the 1990s, flow cytometer capacity

increased, allowing the simultaneous measurement of 7 fluorophores. Now, 84 years from the starting point, this highly sophisticated technology is capable of simultaneously detecting up to 14 parameters (size, shape, granularity, pigments, autofluorescence, DNA content, apoptosis, membrane structures, internal and external receptors, physiological activity, transfection efficiency, time, and cell/particle concentration) [94]. Another important task that can be accomplished by flow cytometer is cell sorting. A specialized flow cytometer has the ability to physically isolate cells of interest with high degrees of purity into separate collection tubes based one or more parameters listed above from a heterogeneous cell mixture.

Today, this technology has a seemingly limitless number of applications. Flow cytometry is often used to characterize disease in animal research laboratories and clinical settings [95]. Some of the common uses are phenotyping (the identification of specific observable characteristics), analysis of DNA abnormalities, identification of tumor cell surface receptors, and detection of specific immunophenotypic characteristics of different hematologic cancers. Flow cytometry has been successfully employed to sort fixed or live germ cell populations based on DNA content [53], and mitochondrial mass or activity [96, 97]. Several germ cell types such as primary spermatocytes, secondary spermatocytes, round, and elongated spermatids were isolated by flow cytometry, using Bis-benzamide Hoechst 33342 (Ho) dye to discriminate between these cell types [98]. The Ho dye allows the detection of variations in chromatin structure and DNA content [99]. What makes flow cytometry stand out from other technologies is the ability to analyze, measure, and study heterogeneous populations of cells, one cell at a time, and allows the identification of various cell types without loss of the information

[100]. The drawbacks of this technique include: 1 – expensive instrumentation; 2 – necessity for highly-trained operators; 3 –results in some cell loss when focused on purity; and 4 – it provides little information on intra-cellular distributions. Our laboratory has access to the ECU flow cytometry and cell sorting facility, and therefore this method is available to us to isolate different spermatogenic populations.

*Transgenic mice enable FACS*

Animal research was revolutionized by the development of transgenic animal technology, which arose from the pioneering work of Drs. Rudolf Jaenisch and Ralph Brinster. In independent experiments, they successfully transferred exogenous DNA into early developing embryos [101]. These early advances set the stage for the generation of a multitude of increasingly sophisticated transgenic mouse models, from those with random insertion of reporter genes to conditional and whole-body "knockouts" (KO) or "knock-ins" (KI) at specific gene loci.

The utilization of reporter transgenes has allowed for the development of mouse lines in which specific populations of cells can be identified by expression of a fluorescent marker, usually GFP, or alternatives, such as RFP (tdTomato, mCherry), YFP, and CFP. The Green fluorescent protein (GFP) is a 238 amino acid protein endogenous to the bioluminescent jellyfish Aequorea Victoria [102]. Dr. Shimomura first isolated the gene encoding GFP from these jellyfish in 1992, and now scientists have adopted it as a powerful tool for monitoring gene expression and protein localization in cells and organisms [103]. This protein is stable, and can be detected using flow cytometry and fluorescent microscopy [104]. One of the key features of GFP is its ability to generate the intrinsic chromophore without cofactors or enzymatic components [105].

Nowadays, GFP and its variants and homologs of different colors are used in a variety of application to study the organization and function of living systems. Enhanced green fluorescent protein (EGFP) is a newer GFP variant that has numerous advantages over other reporter markers [106]. It does not require additional cofactors, gene products, or substrates [107] to fluoresce 20 to 35 brighter than the wild-type GFP [108]. Tandem dimer tomato (tdTomato) is a red fluorescent protein that under right conditions shows equal or brighter photostability than green fluorescent protein (GFP) [109]. TdTomato has been successfully used for multiple applications. It was used as a promoter reporter for GEX3 promoter to study pollen [110]. Tdtomato was also employed as a fusion protein [111], and successfully used to generate transgenic mouse models [112]. The advantages of transgenic mouse lines expressing fluorescent proteins are numerous. Reporter transgenes make it possible to perform live tissue "whole mount" analyses, allowing for identification of certain cell subtypes that are expressing the factor of interest without the disruption that is caused by sectioning of tissues [9]. The development of transgenic mouse line also allows for FACS isolation of selected cell populations. Transgenic mouse models analyzed in this work are focused on employing these advantages to broaden our understanding of spermatogenesis.

*Germline-expressed fluorescent reporter strains*

Scientists studying germ cell biology have created a variety of transgenic mouse models with the intention of fluorescently labeling specific germ cell types in mammalian testis (Table 1.). Unfortunately, all current models have significant flaws; most have been discarded, and only a precious few remain in use. One of the best models for

isolating postnatal spermatogonia, the *Id4*-eGfp line, is in current use in our laboratory. These mice were generated by Dr. Oatley's laboratory (Washington State University) via pronuclear injection and "mimic" the ID4 protein expression. Briefly, a 17-kb fragment containing the promoter of the mouse Id4 gene was captured from a BAC clone. Then, an eGfp-Ura3 cassette was inserted in-frame of exon 1, deleting nucleotides 13-29. The transgene construct was then used for pronuclear injection to generate founder lines on an FVB genetic background. From five transgenic founder lines, one was chosen for expansion and backcrossed onto the C57BL/6J genetic background [9]. These Id4-eGfp mice have predominant expression of EGFP in undifferentiated spermatogonia in the neonatal testis. Additionally, the intensity of EGFP is linked to spermatogonial fate. The ID4-EGFP$^{bright}$ population is enriched for SSCs; all SSCs appear to be Id4-EGFP$^{bright}$, but not all ID4-EGFP$^{bright}$ spermatogonia are SSCs. The ID4-EGFP$^{dim}$ population also contains undifferentiated spermatogonia, but this population contains very few SSCs; these represent the proliferating progenitor population. STRA8+/KIT+ differentiating spermatogonia are ID4-EGFP- [9]. Based on this expression pattern, this model is useful for isolation of SSCs and progenitor spermatogonia via FACS, but does not allow for isolation of differentiating spermatogonia. For our laboratory, a better model would express a fluorescent reporter in all spermatogonial cell types, thus allowing the isolation of SSCs and progenitor and differentiating spermatogonia via FACS.

*Summary*

Due to the variation in abundance of somatic (Sertoli, Leydig, epithelial, and

myeloid cells) and spermatogenic (spermatogonia, spermatocytes, round spermatids, condensing spermatids, and spermatozoa) cells, it is challenging to study SSC proliferation and differentiation [10]. The ability to isolate germ cells at each point during the first wave of spermatogenesis would provide our laboratory with the ability to isolate discrete spermatogonial populations and perform a wide array of complex molecular assays such as proteomics, metabolomics, and transcriptomics.

In the work described in this thesis, I analyzed 5 different transgenic mouse models, each with putative expression of recombinant fluorescent protein in the male germline. The impetus for these efforts was to identify a powerful tool for the isolation of live spermatogonia to advance our studies on mouse spermatogenesis. The ideal model will have discrete expression of fluorescent protein confined to spermatogonia and exhibit bright epifluorescence in both live and fixed cells. These characteristics will enable our laboratory to perform advanced biochemical assays and make advances that have been previously impossible.

**Table 1**. Germline-expressed fluorescent reporter strains

| Promoter | Fluorophore | Expressing Cells | References |
|---|---|---|---|
| *Dazl* | GFP | spermatocyte, spermatids | [113] |
| *Sohlh1* | mCherry | spermatogonia | [114] |
| *Dppa3/Stella* | GFP | PGCs | |
| *Pou5f1/Oct4* | GFP | PGCs | [115] |
| *Ifitm3* | | PGCs | [116] |
| *Id4* | EGFP | spermatogonia, spermatocytes, spermatids | [9] |
| *Sycp1-Cre* | N/A | spermatocytes | [117] |
| *DDX4-Cre* | N/A | spermatogonia, spermatocytes, spermatids | [118] |

**Figure 1. Overview of Mammalian Spermatogenesis.** The developmental progression of germ cells in the testis, that begin as spermatogonia and can either remain undifferentiated as SSC or progenitors or differentiate in response to RA and enter meiosis as spermatocytes. After meiosis, spermatids undergo morphogenetic transformations during spermiogenesis to form testicular spermatozoa [119].

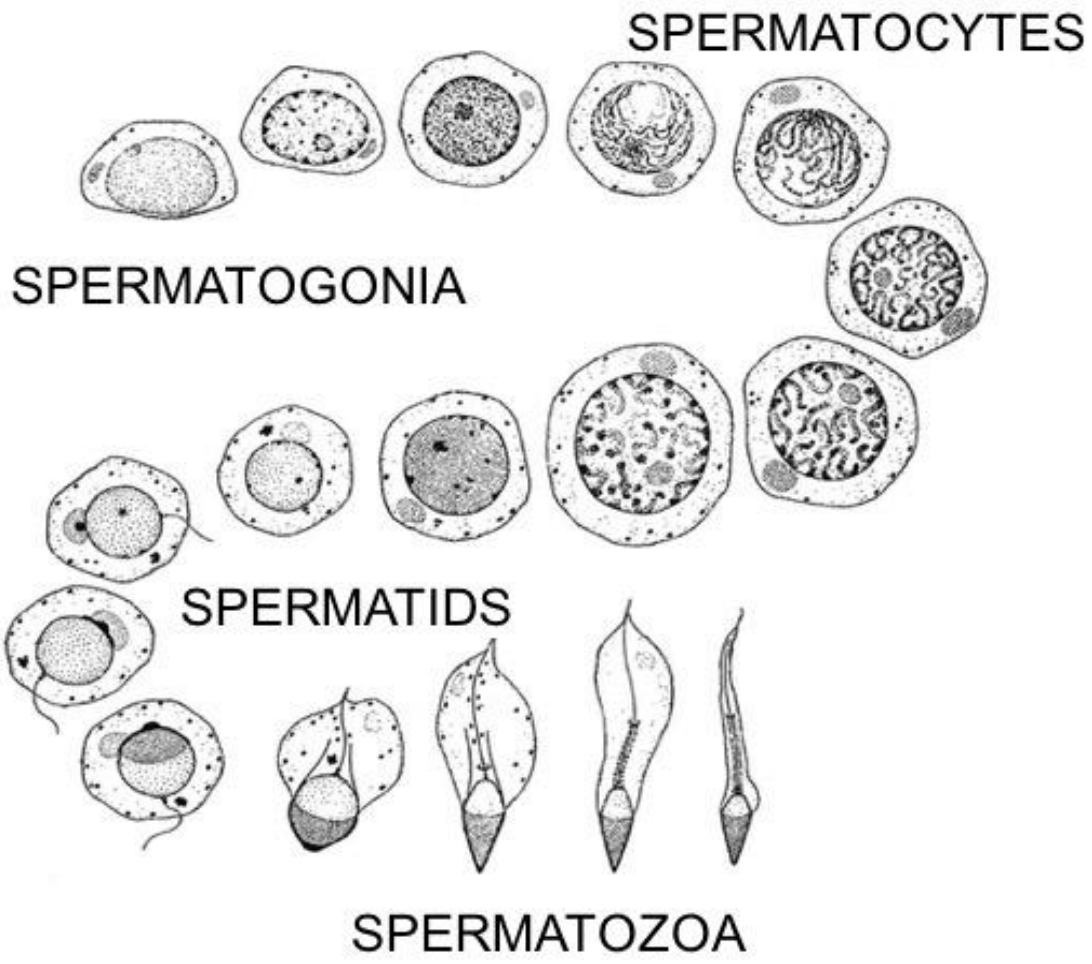

SPERMATOCYTES
SPERMATOGONIA
SPERMATIDS
SPERMATOZOA

**Figure 2. Stages of spermatogenesis and steps of spermiogenesis in the mouse.** The specific phases of cell development designated by the symbols are: type A spermatogonia in mitosis (mIn), intermediate spermatogonia in mitosis (Inm), type B spermatogonia (B), type B spermatogonia in mitosis (Bm), preleptotene spermatocytes (Pl), leptotene spermatocytes (L), zygotene spermatocytes (Z), pachytene spermatocytes (P), diplotene spermatocytes (D) and secondary spermatocytes in meiosis (m2°m) [120, 121]. Adapted from Russell et al. (2008).

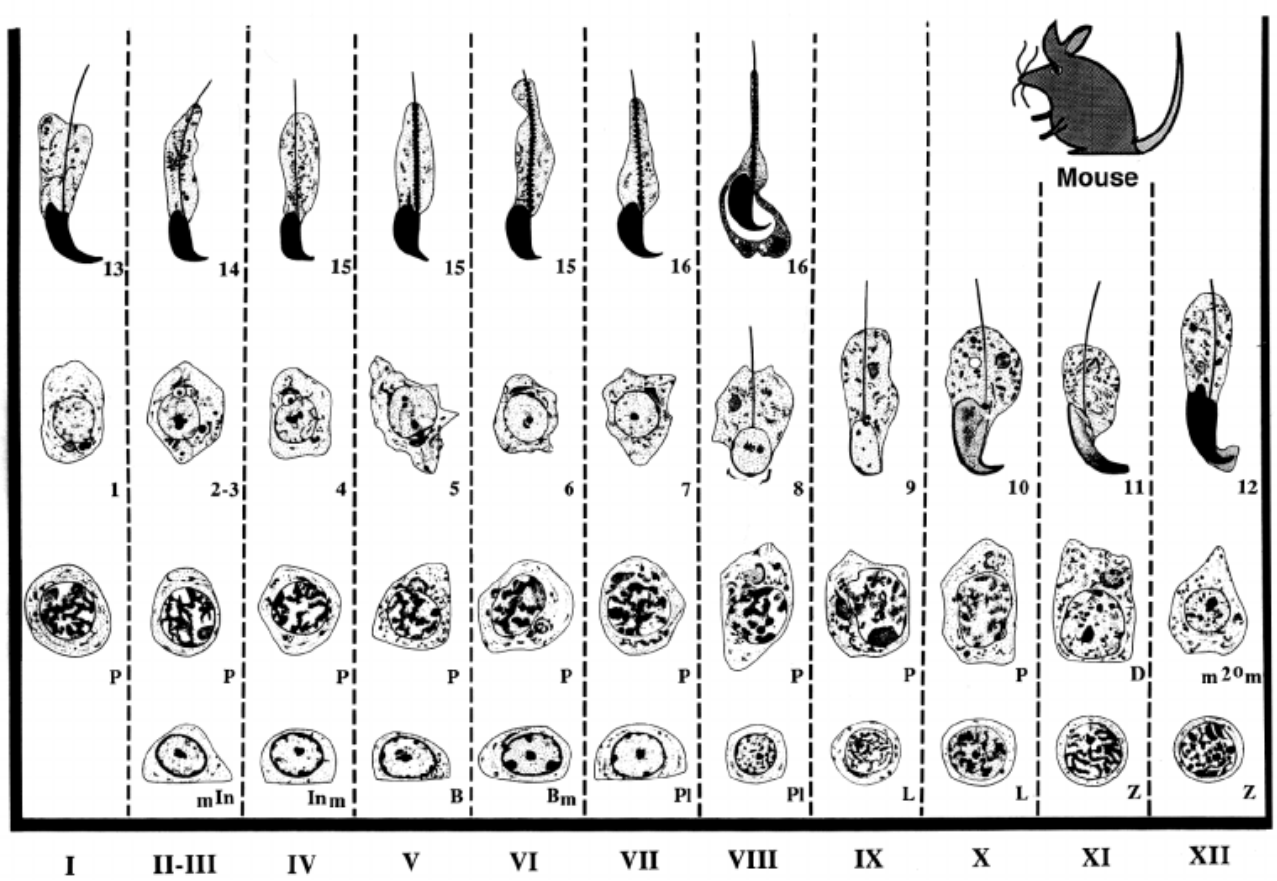
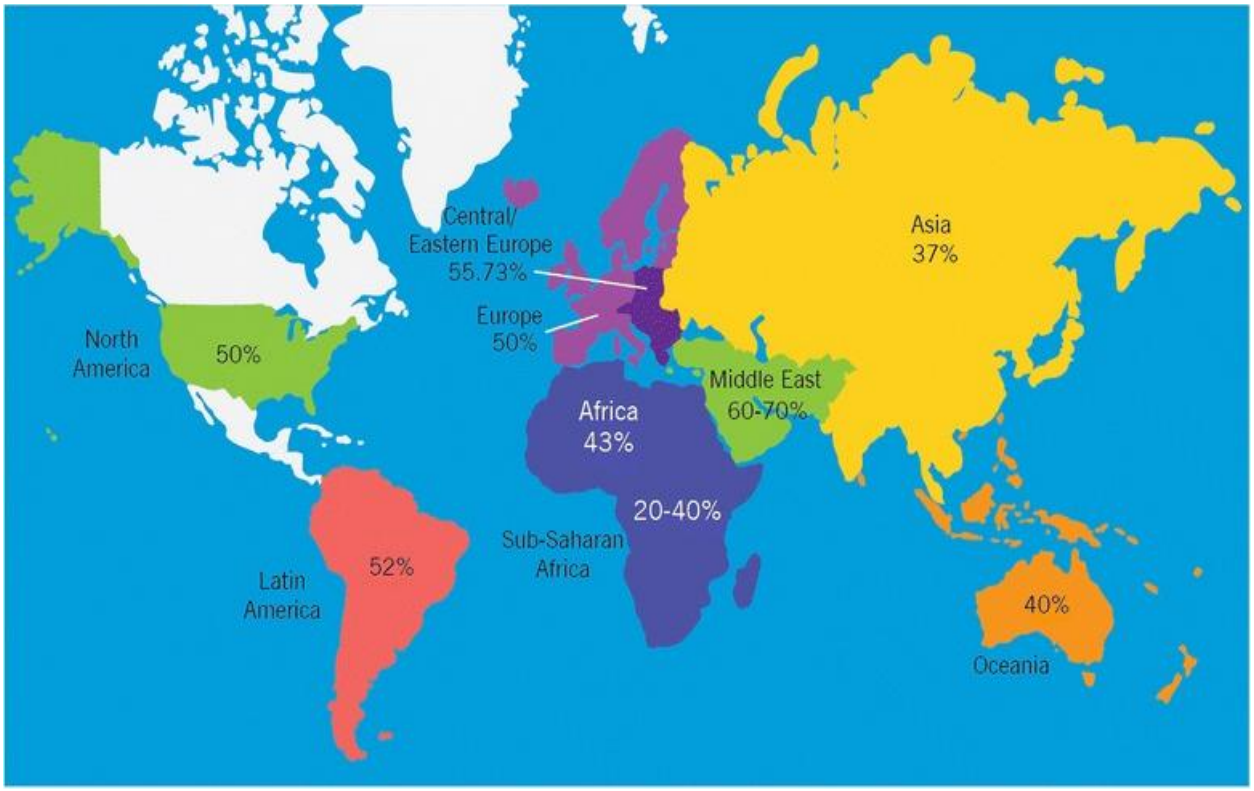

Mouse
13
14
15
15
15
16
16
1
2-3
4
5
6
7
8
9
10
11
12
P
P
P
P
P
P
P
P
P
D
m 2o m
mIn
In m
B
Bm
Pl
Pl
L
L
Z
Z
I
II-III
IV
V
VI
VII
VIII
IX
X
XI
XII

**Figure 3. World map displaying percentages of infertility cases per region that are due to male factor** [122].


Central/
Eastern Europe
55.73%
Asia
37%
Europe
50%
North
America
50%
Middle East
60-70%
Africa
43%
20-40%
Sub-Saharan
Africa
52%
Latin
America
40%
Oceania

# CHAPTER 2: Materials and Methods

## Animal Handling and Genotyping

All animal procedures were carried out using protocols prepared in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of East Carolina University (AUP #A198). Euthanasia of neonatal and juvenile mice up to P7 was performed by decapitation, and euthanasia of mice older than P7 was done by $CO_2$ asphyxiation followed by cervical dislocation or decapitation.

### *Ddx4*-tdTomato

Ddx4-tdTomato transgenic mice carrying one copy of the construct were created by Cyagen Biosciences, Inc. The 8419 bp regular plasmid gene expression vector was inserted downstream of *Ddx4/Vasa* promoter. Three founder lines were generated by pronuclear microinjection. Line #3 was used to propagate the strain. TdTomato alleles were identified by PCR-based genotyping using the following primers: tdTomato forward 5′- CTACACCATCGTGGAACAGTACGA, tdTomato reverse 5′-ACACCTCCCCCTGAACCTGAAAC. PCR conditions: Denaturation Temp: 94°C for 30 sec, annealing Temp: 60°C for 30 sec, extension: 72°C for 30 sec, number of cycles: 35. These mice were maintained on the C57Bl/6 genetic background.

### *Rosa26*-EGFP

*Rosa26*-EGFP expressing EGFP were created by crossing homozygous

female mice carrying a floxed EGFP allele (#004077, The Jackson Laboratory) with young (<P60) male mice carrying the *Ddx4*-Cre transgene (#006954, The Jackson Laboratory.) PCR conditions: Denaturation Temp: 94°C for 30 sec, annealing Temp: 60°C for 30 sec, extension: 72°C for 30 sec, number of cycles: 35. The resulting transgenic pups were on a C57Bl/6 background

**DNA isolation**

Approximately 0.5 mm of tail tissue was carefully snipped and placed into 1.5 ml microfuge tubes with tight-fitting caps. 0.5 ml of DNA digestion buffer (1M Tris pH 8, 5M NaCl, 0.5M EDTA, SDS) with proteinase K was added to 1 mg/ml final concentration and incubated at 55°C overnight while shaking at 900 rpm. Samples were spun at 13,000 rpm, and 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added. Samples were spun 13,000 rpm for 10 min to separate aqueous (top) from organic (bottom) layers. The top aqueous layer was removed and transferred into 1.5 ml microfuge tubes. 500 µl of chloroform was added and tubes were spun at 13,000 rpm for 10 min. Two layers were separated again, and the top aqueous layer was transferred to a 1.5 ml microfuge tube. 750 µl of 100% ethanol was added to each tube and mixed by inverting multiple times (~20) to precipitate DNA. Tubes were spun at 13,000 rpm for 10 min and liquid was carefully removed without disturbing the pellet on the bottom of a tube. Pellets were washed twice with 750 µl of 80% ethanol to remove salts. Tubes were spun at 13,000 rpm for 1 min, and remaining ethanol was removed. Pellets were dried for 15 min at room temperature until all residual ethanol had evaporated. 500 µl of Tris pH 7.5 was added to each

pellet and vortexed. Tubes were incubated at 37°C with shaking at 900 rpm overnight. The quantity of DNA was assessed using a NanoDrop™ 2000 spectrophotometer.

**Indirect immunofluorescence (IIF) staining**

Testes were immersion-fixed in fresh 4% paraformaldehyde, washed in 1X PBS, incubated overnight in 30% sucrose at 4°C, and frozen in O.C.T. Five micrometer sections were placed on slides, and each section was incubated in blocking reagent (1X PBS containing 3% BSA+ 0.1% Triton X-100) for 30 min at room temperature. Primary antibodies were diluted with blocking reagent and incubated on tissue sections for 1 hr at room temperature. The primary antibodies used are listed in Table 2. Primary antibody was omitted from one section in each technical replicate to serve as a negative control. Following three stringency washes in the wash buffer (1X PBS + 0.1% Triton X-100), sections were incubated in blocking reagent containing secondary antibody (Alexa Fluor-405, -488, -555, or -559, each at a 1:500 dilution, Invitrogen) and/ or phalloidin-635 (1:500, Invitrogen) for 1 hr at room temperature. Stringency washes were performed as above. Coverslips were mounted using Vectastain containing DAPI (Vector Laboratories). IIF on isolated cells was performed using the same protocol.

Whole mount indirect immunofluorescence was performed using the same steps as above, but with different time points. All washes and incubations were performed on the tube rocker. Tissues were permeabilized in wash buffer for 30 min, incubated in block buffer for 1 hr, followed by incubation in block buffer containing

primary antibodies overnight at 4°C. Tissues were washed in wash buffer 3 times for 30 min each time, and then incubated in block buffer containing secondary antibodies for 3 hr. Tissues were again washed in wash buffer 3 times for 30 min each time, then placed on a slide, Fluoroshield Medium with DAPI (Abcam #ab104139) diluted 1:1 in 1X PBS was added to cover the tissue, and a coverslip was placed on top and sealed with nail polish.

**Testicular single cell suspension**

Pups or adult mice were euthanized and testes were carefully removed and placed in a warmed dish containing Enriched Krebs-Ringer Bicarbonate Medium (EKRB). Testes were detunicated beneath a dissecting stereomicroscope, and placed into a 15 ml conical tube containing 4.5 ml Trypsin + 0.5 ml of Deoxyribonuclease I (DNase I). The solution was repeatedly drawn and expelled to begin breaking up the tissue. The tube with the solution was placed into 37°C water bath for 3 min. 1 ml of DNase I was added to the solution and pipetted repeatedly to break up the tissue as above. This procedure was done twice. 1 ml of FBS was added to stop the digestion of tissues. The mixture was filtered through a 40 µm sieve (Falcon #352340) into a 50 ml conical tube. The initial 15 ml conical tube was rinsed with 2 ml of Hank's Balanced Salt Solution (HBSS). The solution was transferred into a new sterile 15 ml conical tube. The cell suspension was centrifuged at 500 x g for 7 min. Cells were resuspended in sorting buffer (FBS/EDTA/HEPES/PBS) or DMEM with penicillin-streptomycin (pen-strep). Cells were counted using the hemocytometer and either plated or used for fluorescent

activated cell sorting (FACS, described below).

**Fluorescent activated cell sorting (FACS)**

FACS was performed by East Carolina University Flow Cytometry/Confocal Microscopy Core Facility using a Becton Dickinson AriaFusion Cell Sorter. This equipment is supported by NIH 1S10OD021615-01.

**Cell seeding**

Isolated cells were seeded on fibronectin-coated 6-, 12-, and 96-well plates at the density of $2 \times 10^6$, $1 \times 10^6$, and $0.05 \times 10^6$ respectively. Cells were also plated on fibronectin-coated coverslips at the density of $2 \times 10^6$, and incubated in 37ºC overnight. Cells were then washed with PBS, and fixed with 4% PFA for 15 minutes.

**Imaging**

Images of live tissue were obtained using Zeiss fluorescent microscope. Images of stained tissues were obtained using a Fluoview FV1000 laser scanning confocal microscope (Olympus America).

**Table 2.** Immunofluorescence Staining Antibodies.

| Antibody | Vendor (Catalog Number) | Dilution |
|---|---|---|
| α-TRA98 | Abcam (ab82427) | 1:1000 |
| α-DDX4 | R&D Systems (AF2030) | 1:800 |
| α-GATA4 | R&D Systems (AF2606) | 1:500 |
| α-PLZF/ZBTB16 | Abcam (ab18949) | 1:800 |
| α-UCHL1 | Cell Signaling (13179) | 1:1000 |
| α-KIT | Cell Signaling (3074) | 1:1000 |
| α-GFRA1 | R&D Systems (AF560) | 1:800 |

# CHAPTER 3: Results

## *Npy*-GFP

The first transgenic mouse model I investigated was 'neuropeptide Y' (*Npy)*-GFP. These mice were housed in Dr. Hu Huang's laboratory (Department of Kinesiology, East Carolina University) by inserting a humanized Renilla GFP (hrGFP, Stratagene) sequence into the translational start site of the mouse Npy gene. Following isolation of the transgenic construct NPY-GFP, the transgene was used for pronuclear injection into fertilized one-cell stage FVB embryos. One line was chosen to propagation and these mice were bred on C57BL/6 genetic background [123].

Neuropeptide Y is a 36 amino acid peptide containing several tyrosine residues synthesized primarily in neurons in the brain [124]. In the hippocampus, NPY has been suggested to function as an endogenous anti-epileptic peptide [125]. In the hypothalamus, neurons containing NPY are thought to be critical for energy homeostasis [126]. Curiously, *Npy* mRNAs were also detected in the testis, potentially in Leydig cells and Sertoli cells [127, 128]. In addition, microarray results revealed that *Npy* mRNA levels decreased from P0-P6, a pattern often observed for genes expressed in SSCs; the pool of germ cells with SSC capacity is thought to diminish over this interval [129]. We considered the possibility that NPY was expressed in a subset of male germ cells during testis development, and decided to assess whether NPY-GFP is expressed in a subset of germ cells.

We analyzed testicular tissues from 2 adult (P>60) *Npy*-GFP hemizygous mice. These tissues were fixed, sectioned, and stained with DAPI in accordance with our

protocols. Upon examination, I observed that GFP was detectable in peritubular myoid cells and, to a lesser degree, in interstitial Leydig cells (Fig. 4B-D). Unfortunately, GFP was not detectable in any germ cells. While this mouse line is not suitable for our area of research, it may be successfully employed by investigators interested in isolating somatic Leydig and peritubular myoid cells.

*Ddx4*-tdTomato

The second transgenic mouse model I investigated was one produced recently by the Geyer laboratory. A 1.4 kb fragment of the proximal 'DEAD-Box Helicase 4' (*Ddx4*) promoter, which was previously shown to successfully direct male germline expression [130], was placed upstream of the coding sequence for tdTomato. The *Ddx4* gene is conserved across multiple species and is specifically expressed in the germline of Drosophila [131], zebrafish [132], monkeys [133], mice [134], and humans [130]. The endogenous Ddx4 gene is expressed as early as E15.5 in the mouse male germline in prospermatogonia, and is expressed to varying levels throughout the remainder of spermatogenesis [135]. We hypothesized that tdTomato would be detectable in the same germ cells expressing endogenous DDX4, and that expression would be sustained throughout development.

Three transgenic *Ddx4*-tdTomato founder mice were generated by pronuclear microinjection by Cyagen Biosciences. These founders were bred to WT C57Bl/6 mice to expand 3 lines with unique transgene integration sites. Not unexpectedly, we discovered considerable differences in the expression of tdTomato in the different founder lines. Adult testes from line 1 had no detectable tdTomato expression, while

those from mice in lines 2 and 3 had bright tdTomato epifluorescence (Fig. 5). Line 2 failed to produce a sufficient number of transgenic male offspring and was discarded from the study. Offspring from line 3 were used to expand a colony for further evaluation.

Surprisingly, *Ddx4*-tdTomato+ testes had no detectable tdTomato protein at P6, P8, and P10. However, expression became detectable at P15, and increased as the mice aged (Fig. 6A-O). I used adult mice to generate single cell suspensions for FACS to separate tdTomato+ and tdTomato- cells. Adult testes appeared to have the highest tdTomato expression levels (Fig. 6M-O). Sorting and plating adult tdTomato testicular cells revealed an abundance of tdTomato+ germ cells and residual bodies. Indirect immunofluorescence (IIF) on isolated cells showed these observed residual bodies lacked nuclei (Fig. 7D). Also, not all cells were tdTomato+ post sort (Fig. 7B-D). We conclude that this model did not recapitulate our expected expression pattern for tdTomato. This model did not produce desired results, and therefore will not be used in future studies in our laboratory to isolate germ cells.

*Rosa26*-EGFP;*Ddx4*-Cre

The third transgenic mouse model I investigated was one that I generated by crossing 2 strains of mice available from the Jackson Laboratory: *Ddx4*-Cre (stock #006954) and *Rosa26*-EGFP (stock #004077). The latter has a transgene within the Rosa26 locus, which is on chromosome 6 and is commonly used as a site for integration of transgene constructs to achieve ubiquitous or conditional gene expression in mice [136]. Fluorescence in these mice is predicted to occur following Cre-mediated

excision of a lox-STOP-lox sequence; this brings the promoter in juxtaposition with the EGFP coding sequence (Fig. 8).

We predicted that *Ddx4*-Cre expression early in the male germline would drive germline expression of EGFP beginning in fetal prospermatogonia and then in all subsequent germ cells. I first examined freshly dissected live seminiferous cords of P6 mice under the fluorescent microscope to detect EGFP a high expression of fluorescent protein in these tissues. I next stained PFA-fixed frozen sections from P6 mice with the pan germ cell marker TRA98 and observed that EGFP was detectable only in spermatogonia (Figure 9A-D). As expected, EGFP was not detected in testes of WT littermate controls.

One significant disadvantage of the *Ddx4*-Cre line is that Cre-donating males must be young; after ~P80, Cre expression is not tightly restricted to the germline. In analyzing multiple litters from the same crosses, we observed that EGFP became detectable in both germ cells and somatic cells as the Cre-donating sire aged. In agreement with expected Mendelian ratios, the number of transgenic male pups was limited per litter ($n \leq 3$). This number is insufficient to provide enough cells after FACS. Altogether, we conclude that the *Rosa26*-EGFP;*Ddx4*-Cre model is suitable for isolation of male germ cells, but it is not an optimal model because few usable litters are produced per breeder cage setup, and few pups will be produced for isolating cells.

*Dnd1*-EGFP

The fourth transgenic mouse model I investigated was one generated in Blanche Capel's laboratory at Duke University. Her laboratory is interested in understanding early steps in testis development by defining the molecular mechanisms underlying the

events in somatic cells that commit bipotential gonad to testis or ovarian fate. In one recent study, Dr. Capel's laboratory created mice carrying a transgene with the Dnd1 promoter directly upstream of EGFP. These mice were created by CRISPR-mediated knockin of EGFP into the *Dnd1* locus. The insertion of EGFP did not affect DND1 function, and homozygous mice were viable and fertile (Capel, personal communication). Having a homozygous mouse model would be ideal for our laboratory's studies, because it would save time and resources on genotyping procedures, as well as provide us with number of animals required to perform cell sorting.

The 'Dead-end gene' (*Dnd1*) gene encodes two RNA binding protein isoforms, α and β, due to alternative splicing of transcripts. It is expressed in germ cells as early as E7.25 and was shown to be required for survival of PGCs [137]. The inactivation of this gene leads to Ter mutant mouse strain, which results in germ cell deficiency, sterility, and development of TGCTs and results in PGC deficiency [138, 139].

We expected *Dnd1*-EGFP mice to exhibit germ cell-specific expression of EGFP. To examine this, we obtained testes form mice at the following ages: P1, P3, P6, and P10. I fixed, sectioned, and stained these tissue in accordance with lab protocols. Upon examination I found that all ages express EGFP in both germ cells and Leydig cells (Fig. 10A-O). The EGFP signal was not as bright as we expected. P1 showed the brightest expression of EGFP in germ cells, and it appears that the intensity of the endogenous EGFP decreases with age. In Leydig cells, however, the expression remained unchanged.

Based on low EGFP expression in germ cells and expression in Leydig cells, these mice do not appear to be suitable for FACS until a separation of tubular (germ cells and Sertoli cells) and extratubular (Leydig cells and peritubular myoid cells) components is successfully done. A number of protocols have been developed over the years that allow this separation using collagenase treatments. Briefly, after removing the testes' tunica albuginea, seminiferous tubules are dissociated and transferred into the collagenase solution. This allows the initial separation of seminiferous tubules from the interstitium, as the tubules rapidly sink to form a pellet at the bottom of the tube, while the interstitial components remain in the supernatant.

This method does not work as well on neonatal testes. Since our laboratory is mostly interested in this phase of germline development, we often use P6 mouse pups as a source of testicular tissue. While the digestion protocol has been optimized for adult testis, the preferred concentration of the enzyme that can be used on neonatal testes is yet to be identified. We conclude that this model is most useful for isolation of germ cell in prentatal stages of development, but significant challenges currently prevent this line from being useful for postnatal isolations.

*Uchl1*-eGfp

The fifth transgenic mouse model I investigated was one generated in Dr. Pembe Ozdinler's laboratory at Northwestern University. Her laboratory is interested in understanding the cellular and molecular basis of selective neuronal vulnerability, and primarily focuses on the corticospinal motor neurons (CSMN), the neurons that are important for the initiation and modulation of voluntary movement. Dr. Ozdinler's laboratory created a fluorescent reporter mouse model with eGFP expression under

UCHL1 promoter that genetically labels CSMNs and a subpopulation of degeneration-resistant spinal motor neurons in an ALS [140]. The 'Ubiquitin carboxyl-terminal hydrolase L1' (*Uchl1*, also known as protein gene product (PGP) 9.5) gene encodes a deubiquitinating enzyme with ligase and hydrolase activities [141]. It is highly expressed in sensory and sympathetic ganglia [142, 143], retina [144], and has been used as a marker of neural elements. UCHL1 has also been localized to germ cells in multiple species, including dogs [145], pigs [146], cattle [147], and mice [148]. Studies done by Kon et al. in 1999 demonstrate the expression of UCHL1 in mouse testis specifically in spermatogonia in neonates, and in Sertoli cells in adult. [149]. In 2015, Dr. Ozdinler reported expression of UCHL1-EGFP in P30 testis from Uchl1-eGfp mice [150]. Taking these discoveries together, we expected a germ-cell specific expression of EGFP in neonatal testes of *Uchl1*-eGfp mice.

This model was created by pronuclear injections. Briefly, a BAC clone encoding the UCHL1-eGFP was directly inserted into approximately 200 pro-nuclei of fertilized oocytes of C57BL/6 mice. Out of nine line expressing EGFP, three showed strong EGFP expression that was evident with consistent pattern and intensity of expression from litter to litter for >8 generations. We obtained testes from these mice to investigate the expression of EGFP in neonatal testis. Upon the examination of fixed and sectioned P6 testis, I observed that EGFP expression overlapped with UCHL1+ cells identified by IIF (Fig. 11 M-O). To extend these findings and determine whether EGFP was only expressed in germ cells, I stained P6 testis sections for the pan germ-cell marker TRA98 (Fig. 11D-F), and the Sertoli cell marker GATA4 (Fig. 11A-C). The expression of EGFP was strictly confined to TRA98+ cells, with no expression detectable in GATA4+

Sertoli cells. This confirmed that UCHL1-EGFP+ cells were indeed germ cells and not somatic cells. I also observed a difference in EGFP brightness among germ cells. To determine whether brightness was associated with spermatogonial fate (stem, progenitor, or differentiating), I performed IIF using antibodies against markers of differentiating (KIT) and undifferentiated (GFRA1) spermatogonia. These analyses revealed that KIT+ spermatogonia were both UCHL1-EGFP$^{bright}$ and UCHL1-EGFP$^{dim}$ (Fig. 11G-I). However, only UCHL1-EGFP$^{dim}$ spermatogonia were GFRA1+ (Fig. 11J-L), suggesting that these cells are a subset of undifferentiated spermatogonia. We conclude that *Uchl1*-eGfp transgenic mouse model is an ideal candidate for isolating enriched populations of spermatogonia from developing mouse testes, and will serve as our primary model for future studies.

**Figure 4. NPY-GFP is expressed in Leydig cells of adult *Npy*-Gfp mouse testis.** (A) Structure of mammalian testis. (B-D) Representative images of immunostaining for pan germ cell marker TRA98, and cell nuclei marker in cross-sections of seminiferous tubules from testes of adult Npy-Gfp mice. Solid lines identify Leydig cells. Dashed lines identify peritubular myoid cells. Scale bar (in B) = 20 μm.

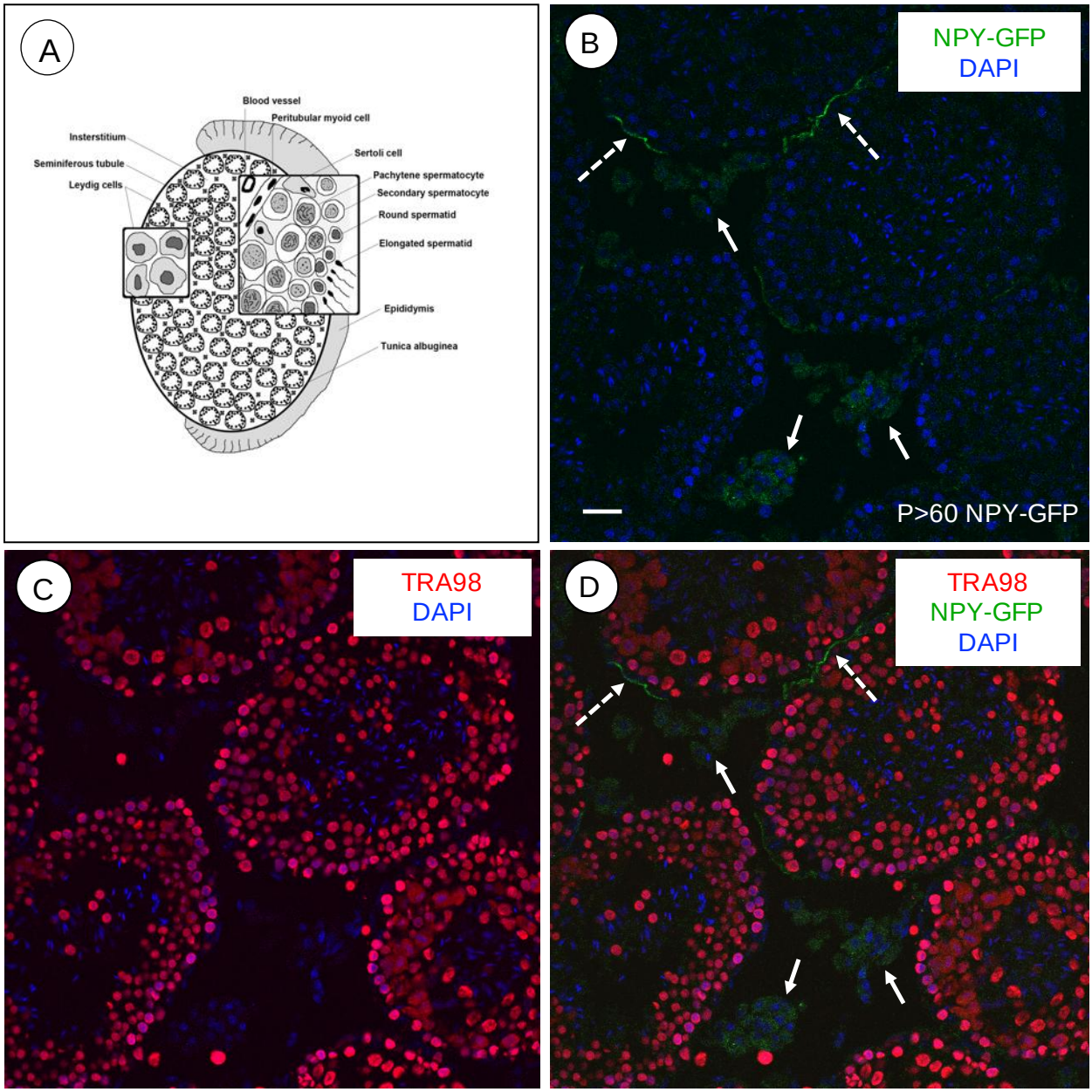

A
Blood vessel
Peritubular myoid cell
Insterstitium
Seminiferous tubule
Leydig cells
Sertoli cell
Pachytene spermatocyte
Secondary spermatocyte
Round spermatid
Elongated spermatid
Epididymis
Tunica albuginea
B
NPY-GFP
DAPI
P>60 NPY-GFP
C
TRA98
DAPI
D
TRA98
NPY-GFP
DAPI

**Figure 5. DDX4-tdtomato is expressed in 2 out of 3 *Ddx4*-tdTomato founder lines.**

Red is endogenous tdTomato protein. Scale bar = 150 μm.

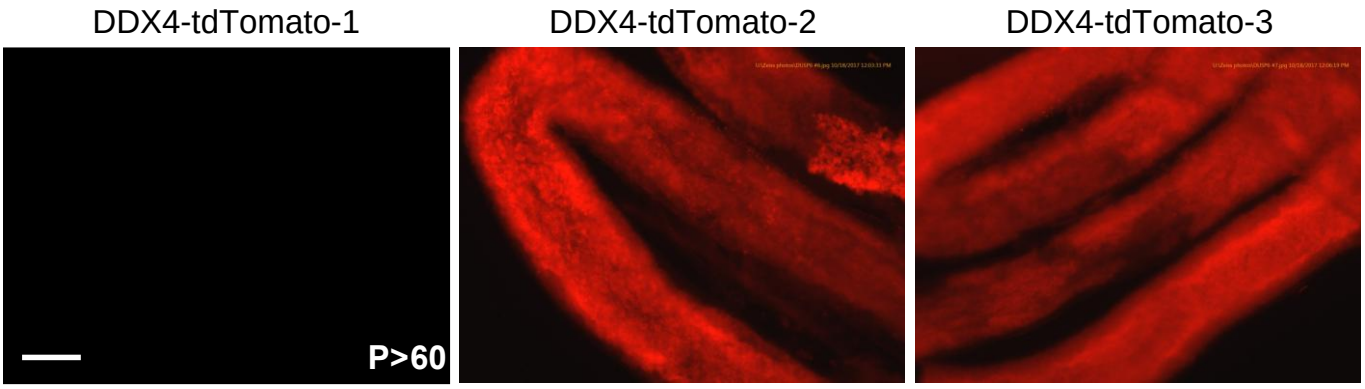

DDX4-tdTomato-1
DDX4-tdTomato-2
DDX4-tdTomato-3
P>60

**Figure 6. DDX4-tdTomato expression in germ cells increases with age (P8, P15, P21, P>60) in testes of *Ddx4*-tdtomato mice.** (A,D,G,J,M) Representative images of fixed frozen sections stained with cell nuclei marker DAPI (blue) and endogenous tdTomato (red), (B,E,H,K,N) germ cell marker TRA98 (green) and DAPI (blue), (C,F,I,L,O) TRA98 (green), DAPI (blue) and endogenous tdTomato (red). Scale bar (in A) = 20 μm.

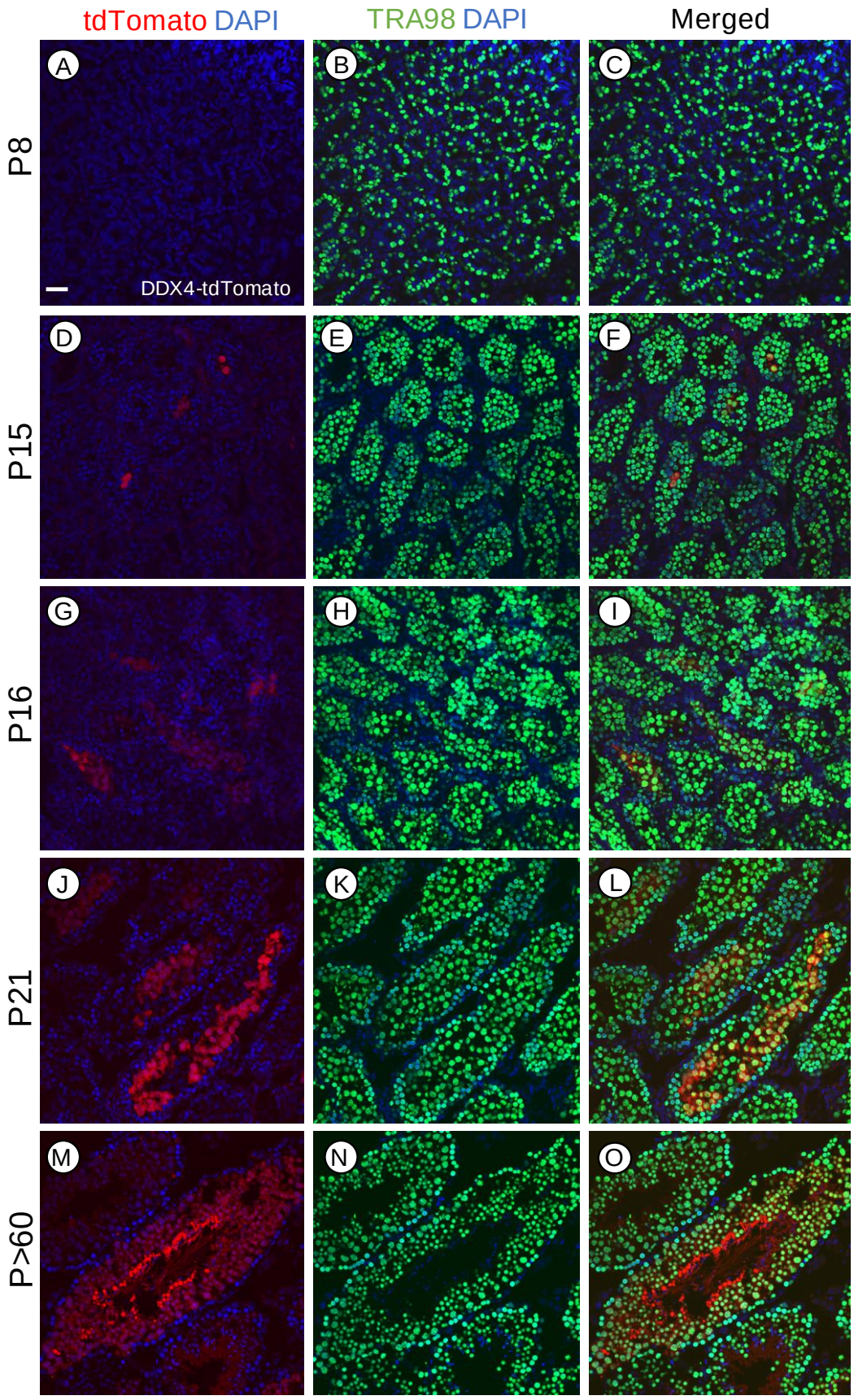

tdTomato DAPI
TRA98 DAPI
Merged
P8
P15
P16
P21
P>60
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
DDX4-tdTomato

**Figure 7. DDX4-tdTomato is expressed in germ cells and residual bodies after FACS of adult *Ddx4*-tdtomato testes.** (A) Representative image of testicular single cell suspension. (B-D) Representative image of post-sort plating and fixation of DDX4-tdtomato+ cells stained with cell nuclei marker DAPI (blue). Red is endogenous tdTomato protein. Scale bar = 10 μm.

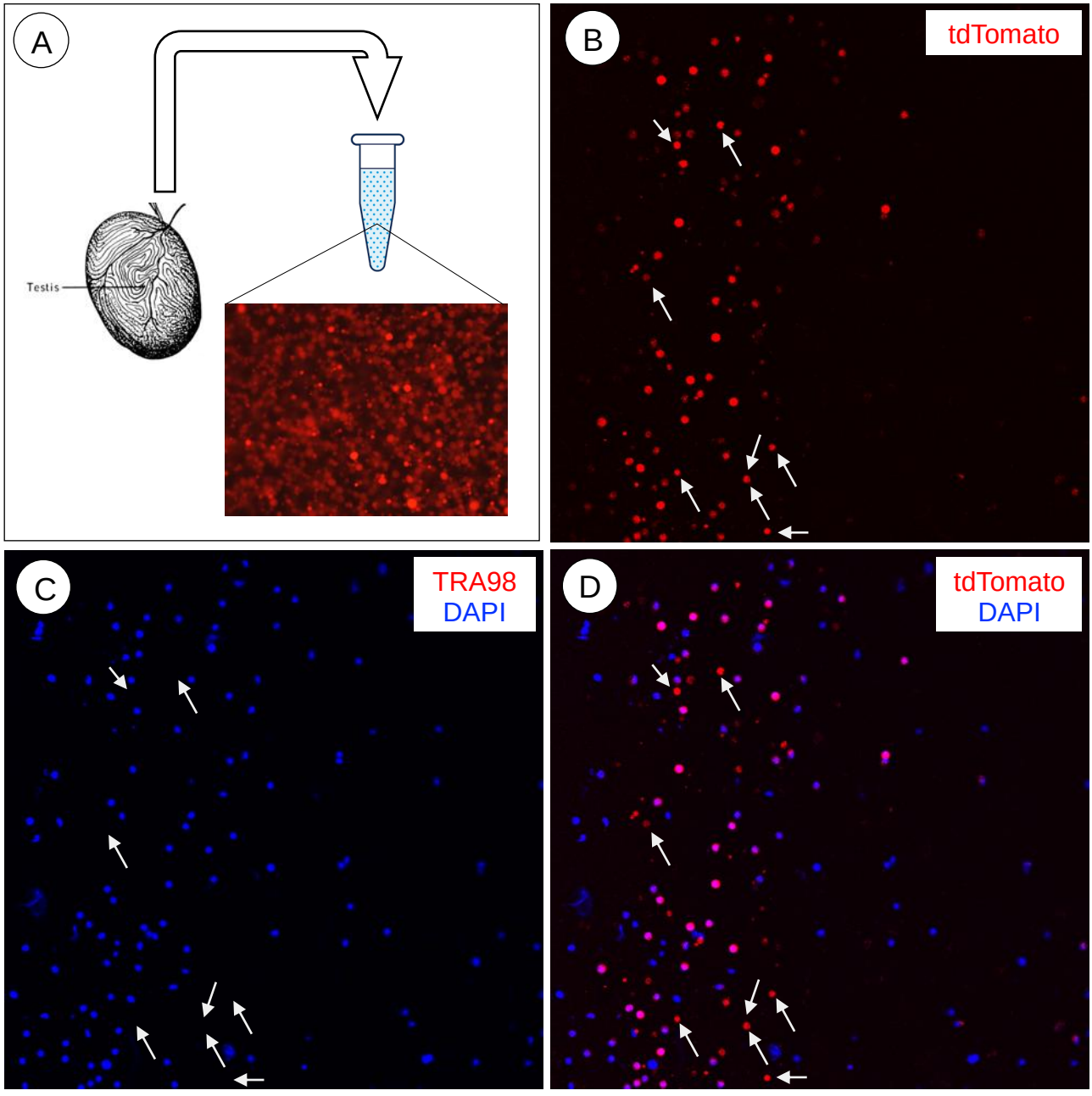

A
Testis
B
tdTomato
C
TRA98
DAPI
D
tdTomato
DAPI

**Figure 8. Cre recombination and expression of GFP (The Jackson Laboratory).**

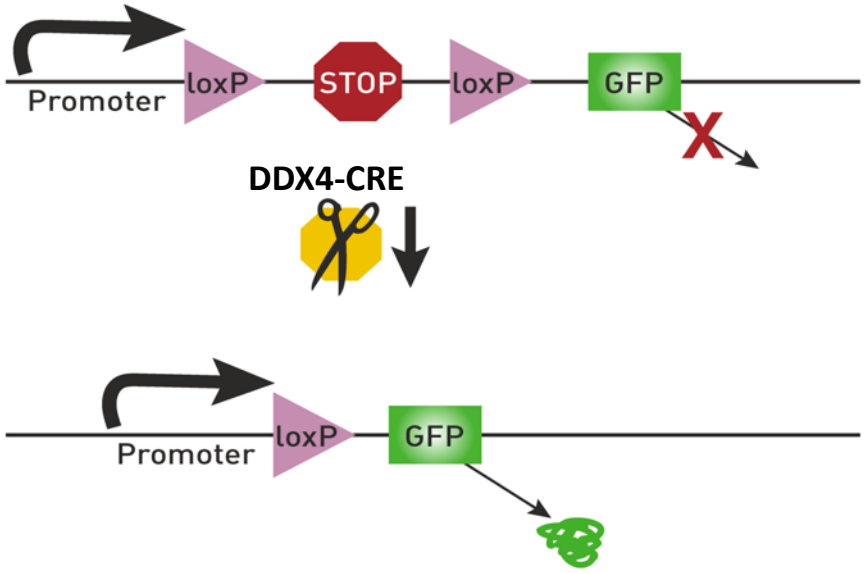

Promoter
loxP
STOP
loxP
GFP
DDX4-CRE
Promoter
loxP
GFP

**Figure 9. Rosa26-EGFP is specifically expressed in spermatogonia of P6 *Rosa26*-EGFPx*Ddx4*-Cre mice.** (A) Representative images of immunofluorescence staining on frozen sections with pan germ cell marker TRA98 (red), GFP (green), and 4',6-diamidino-2-phenylindole (DAPI) (blue), (B) GFP (green) and DAPI (blue), (C) TRA98 (red) and GFP (green), (D) TRA98 (red) and DAPI (blue). Scale bar = 20 μm.

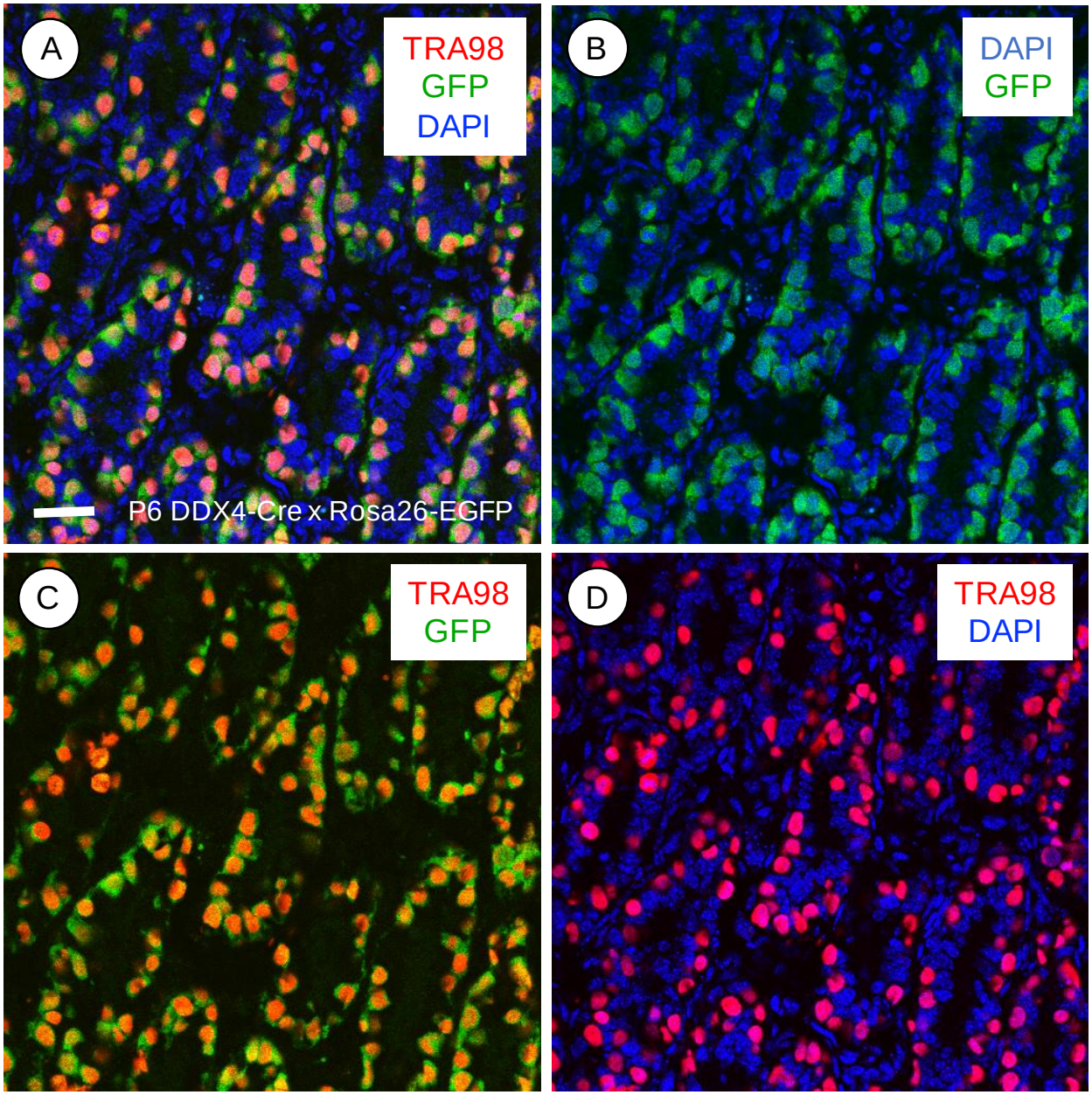

A
TRA98
GFP
DAPI
P6 DDX4-Cre x Rosa26-EGFP
B
DAPI
GFP
C
TRA98
GFP
D
TRA98
DAPI

**Figure 10. DND1-EGFP is expressed in both germ cells and Leydig cells of *Dnd1*-EGFP mice at multiple stages of postnatal development.** (A,D,G,J,M) Representative images of immunofluorescence staining on frozen section fixed with PFA using anti-GFP antibody (red) and DAPI (blue), (B,E,H,K,N) endogenous EGFP (green) and DAPI (blue), (C,F,I,L,O) anti-GFP (red), endogenous EGFP (green), and DAPI (blue). Scale bar = 20 μm.

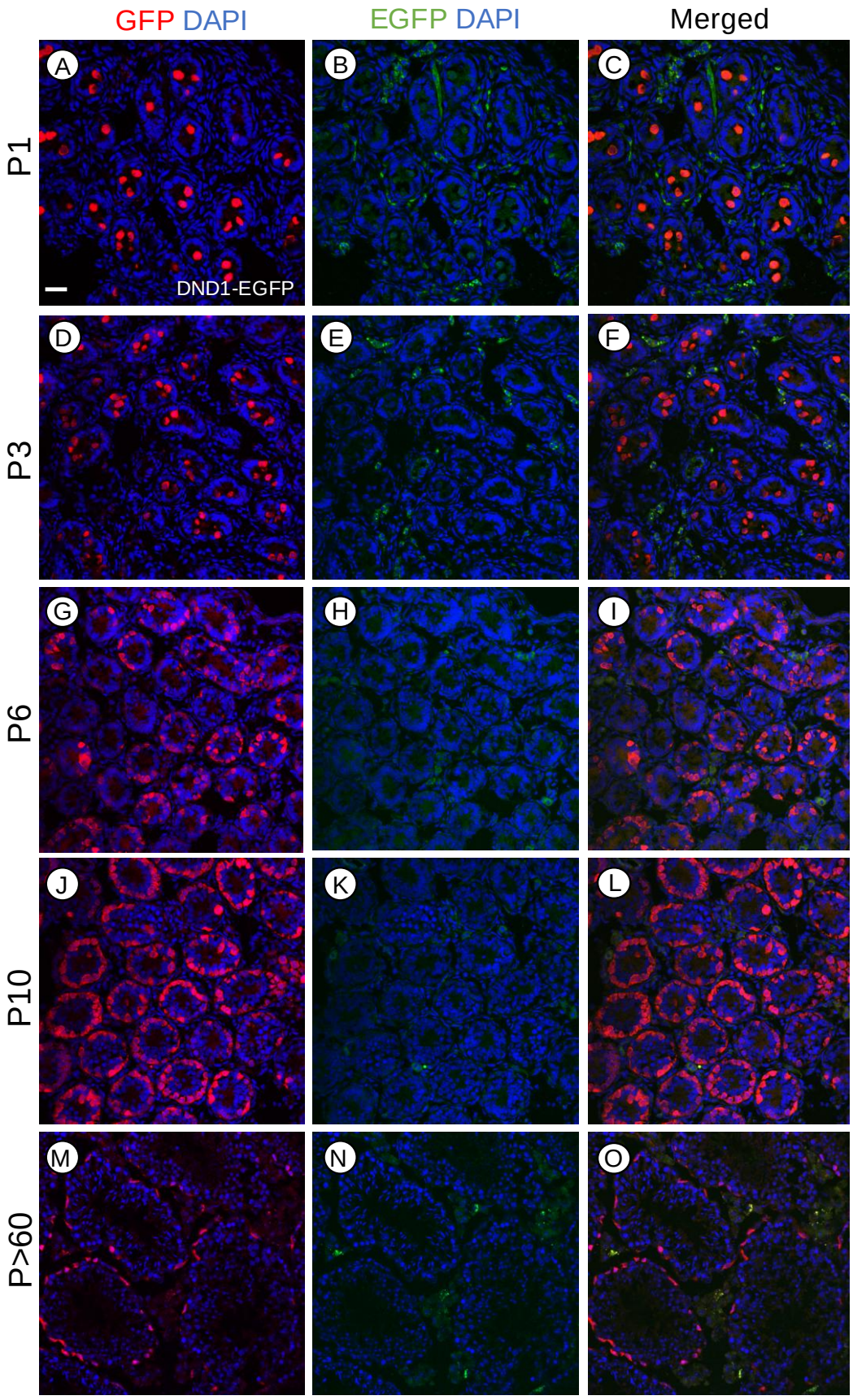
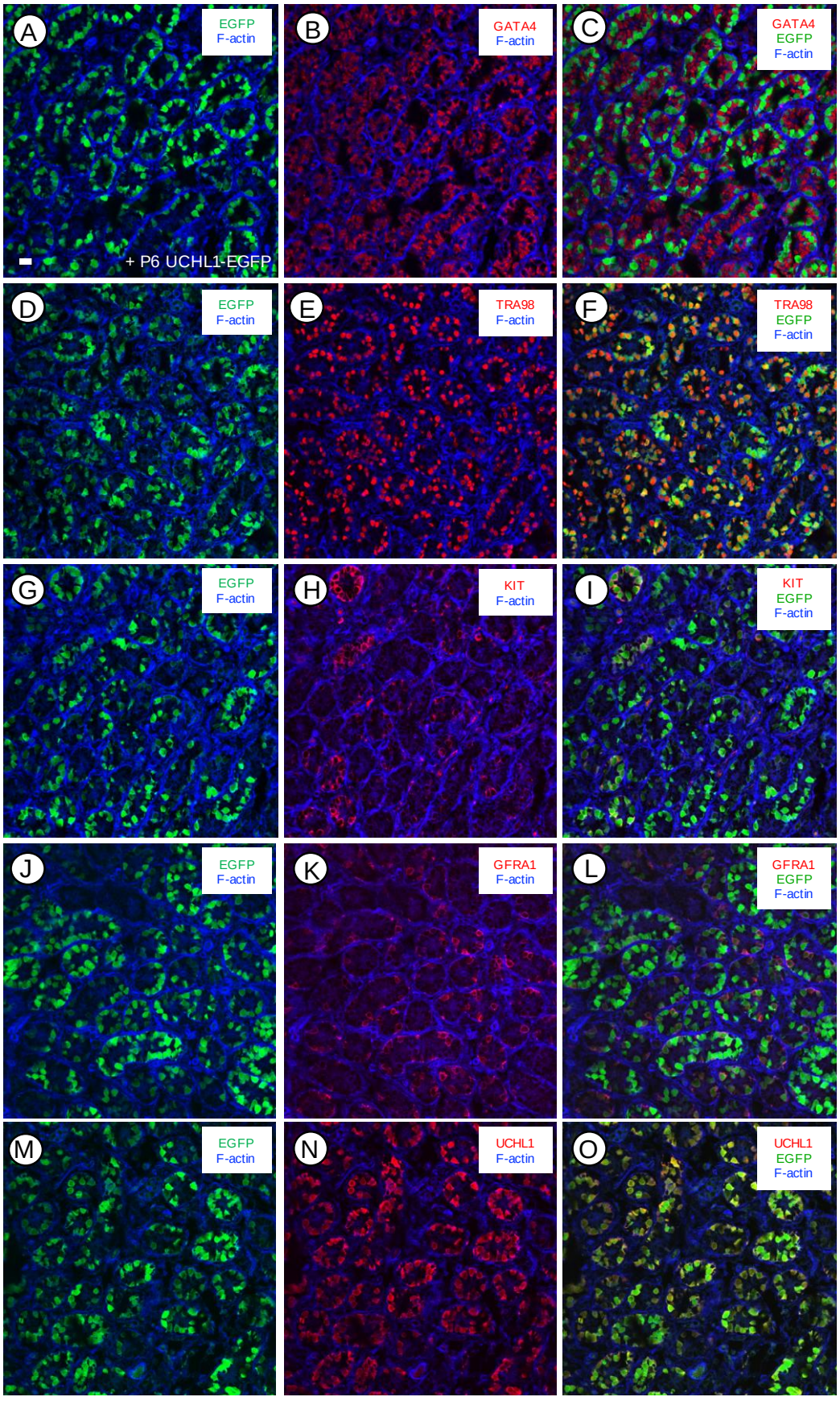

GFP DAPI
EGFP DAPI
Merged
P1
P3
P6
P10
P>60
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
DND1-EGFP

**Figure 11. UCHL1-EGFP is specifically expressed in spermatogonia in testes of P6 *Uchl1*-eGfp transgenic mice.** Representative images of immunofluorescence staining on frozen sections fixed in PFA using antibodies against (A-C) marker of Sertoli cells (GATA4), (D-F) pan germ cell marker (TRA98), (G-I) marker of spermatogonial differentiation (KIT), (J-L) marker of undifferentiated spermatogonia (GFRA1), and (M-O) UCHL1. Scale bar = 20 μm.


A
EGFP
F-actin
+ P6 UCHL1-EGFP
B
GATA4
F-actin
C
GATA4
EGFP
F-actin
D
EGFP
F-actin
E
TRA98
F-actin
F
TRA98
EGFP
F-actin
G
EGFP
F-actin
H
KIT
F-actin
I
KIT
EGFP
F-actin
J
EGFP
F-actin
K
GFRA1
F-actin
L
GFRA1
EGFP
F-actin
M
EGFP
F-actin
N
UCHL1
F-actin
O
UCHL1
EGFP
F-actin

# CHAPTER 4: Discussion

The primary goal of this study was to evaluate five transgenic fluorescent reporter mouse models for their usefulness in isolating pure populations of germ cells by flow cytometry. Our field is currently lacking a reliable model for fluorescence-based isolation of germ cell populations, and this has impeded progress in addressing a number of outstanding questions. While a sizeable number of mouse models have been developed over the years, most of them do not work well. We found that, although 4/5 of the models we investigated were either not suitable or not ideal for isolation of spermatogenic cells, all could be used to study some aspect of mammalian testis biology.

The *Npy*-GFP mouse model lacks expression of GFP in germ cells. However, expression was observed in Leydig cells, suggesting a role for NPY in regulation of testis hormonal activity. Studies in rats have shown that NPY inhibits testosterone secretion in the rat testis [151]. In 2006, Terado et al. demonstrated the expression of endogenous NPY exclusively in the interstitial cells of the testes in mice, and our findings support that study. While this model is not suitable for studies in our laboratory, it may be of interest to a laboratory studying the postnatal development of Leydig cells.

The next transgenic mouse model (*Ddx4*-tdTomato) was expected to provide us with desirable expression of fluorescent protein in germ cells. Recently, a rat model was developed using a similar construct (*Ddx4*-GFP), and was successfully utilized for isolation of germ cells [152]. This rat model was also created via pronuclear injection, and one of the caveats of this approach is the transgene inserts randomly into the

genome and expression is therefore subject to position effect variegation (PEV). It is possible that the tdTomato expression variability we observed in our mice was due to construct insertion into the genome in a spot that was unfavorable for expression. This is not unexpected, that only approximately 10% or less of the founders generate offspring that contain the transgene [153]. This resulting mouse model is not useful in our laboratory to isolate germ cells. This method can be tested again, but we recommend obtaining more than three founders for each expression construct to increase chances of desirable expression.

To continue our search for an ideal mouse model, we analyzed the *Ddx4*-Cre;*Rosa26*-EGFP transgenic mouse model. A study done in rats in 2007 was focused on generation of rat line with genomic integration of a ROSA26-EGFP transgene with specfiic expression of EGFP in the germ cells [154]. Unlike our model that uses Cre-mediated recombination of transgenic construct to direct EGFP expression, those rats were produced by microinjection of the transgene into pronuclei of rat embroys. Although our mice demonstrated desired germ cell-specific expression of EGFP and may be successfully used for isolation of spermatogonia, the efficient production of usable pups per litter (~2-3) is low, and would require a large number of breeder pairs. This makes this approach less than ideal for our laboratory.

The *Dnd1*-eGfp transgenic mouse model, created at Duke University, is currently used by Dr. Capel's laboratory to investigate fetal male germ cell development, and to study teratomas. Since these mice express EGFP in PGCs and prospermatogonia, and Dnd1 mRNAs are upregulated in neonatal testis, we expected to observe strong EGFP expression in neonatal spermatogonia. Although DND1-EGFP was detectable in germ

cells in testes of various ages (P1, P3, P6, P10, and P>60), it was also highly expressed in Leydig cells. These results suggest, that while it is possible to use this model for isolation of germ cells via FACS, additional experimental steps would be required to first remove EGFP+ Leydig cells. If we decided to pursue this model, we would need to optimize an enzymatic digestion protocol to separate tubular and interstitial components of mouse neonatal testis. This approach would likely reduce germ cell recovery.

The last transgenic mouse model we examined is *Uchl1*-eGfp. It was first reported in 1988 that the neuronal protein UCHL1 was localized specifically to spermatogonia in neonatal mouse testes [148]. The results presented in this work confirm this observation. EGFP was expressed specifically in spermatogonia, but not in more developmentally advanced germ cells and somatic cells. Additionally, our findings suggest that the brightness of EGFP may be associated with fate of spermatogonia; UCHL1-EGFP$^{dim}$ spermatogonia may be a subset of undifferentiated spermatogonia. Our future work will optimize the FACS-based isolation of UCHL1-EGFP+ spermatogonia. These spermatogonia will be used in a variety of biochemical assays, such as metabolomics, proteomics, transcriptomics, and results will provide a clearer understanding of spermatogonial biology – particularly differentiation – and may result in discovery of new regulators of spermatogenesis.

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# APPENDIX: IACUC approval

East Carolina University
Tomorrow starts here.

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

November 14, 2017

Chris Geyer, Ph.D.
Department of ECDOI
ECHI Building, MS#743
East Carolina University

Dear Dr. Geyer:

The Amendment to your Animal Use Protocol entitled, "Using the Mouse to Study Neonatal Testis Development" (AUP #A198) was reviewed by this institution's Animal Care and Use Committee on November 14, 2017. The following action was taken by the Committee:

"Approved as submitted"

***Please contact Aaron Hinkle at 744-2997 prior to hazard use***

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

SB McRae

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure