Oct4 Lineage Tracing of Spermatogonial Stem Cells in the Adult Mouse Testes

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Dedication

This thesis is dedicated to my family and friends for being supportive and encouraging throughout my academic career. I also want to thank my father for sparking my interest in stem cell biology and giving me all the great scientific learning opportunities he has.

Abstract

Spermatogenesis in mammals occurs in the testes to produce mature sperm for reproductive purposes. Spermatogonia are derived from primordial germ cells. Undifferentiated spermatogonia are thought to arise from a small population of cells arranged around the basal membrane of the seminiferous tubule, called spermatogonial stem cells (SSCs). SSCs divide either symmetrically or asymmetrically to maintain the stem cell pool and give rise to daughter cells. The identity of the spermatogenic stem cell remains unclear. Numerous researchers have attempted to isolate the SSC population but in most cases they also labeled progeny of the SSCs, so a definitive population of SSCs has yet to be isolated. Oct4 is a protein that is expressed in pluripotent cells and has been described as being expressed in undifferentiated SSCs. In adult male Oct4:Cre^{ER} mTmG transgenic mice GFP expression indicating Oct4 driven tamoxifen inducible Cre^{ER} recombination is observed only in the testes. Here we describe using this transgenic mouse to identify and lineage trace the progeny of Oct4:Cre^{ER} expressing cells in the adult mouse testes using multiple tamoxifen pulse and chase experiments. In 4-day chase experiments single GFP expressing cells along the edge of the basal lamina are observed. Longer 10-day chase experiments show aligned spermatogonia expressing GFP in chains of 2, 4 and 8 cells linearly placed on the basal lamina of the tubule. Fallowing extended chase periods after tamoxifen administration GFP expressing cells are seen in all stages of spermatogenesis. Our results are consistent with the cells labeled immediately after tamoxifen addition being the spermatogenic stem cell.

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Introduction

Testes are one organ in the mammalian body that possesses their own stem cell population. These stem cells are called spermatogonial stem cells (SSCs) and are responsible for proliferation of spermatogonia throughout adult life. Even though research on spermatogonial stem cells dates back to 1971, the spermatogonial stem cell population alone has not yet been identified by a protein for study of the population. Spermatogonial stem cells line the inside of the seminiferous tubule along the basal lamina and are derived from gonocytes present in newborn testes. Differentiation begins when a population of spermatogonial stem cells (called A_{single} (A_s)), which give rise to progeny called spermatogonia for proliferation and differentiation to mature sperm (de Rooij and Grootegoed 1998). As spermatogonia divide to give two more proliferating cells they are called A_{paired} (A_{pr}) spermatogonia. $A_{aligned}$ (A_{al}) cells are then generated by a series of mitotic divisions of the spermatogonia (Olive and Cuzin 2004). A_{al} cells divide horizontally for symmetric division around the outside of the basal membrane for expansion and also divide in groups toward the center of the tubules asymmetrically for differentiation. As the A_{al} cells divide toward the center of the tubule they break off in clones to create spermatocytes through mitosis. Spermatocytes differentiate further toward the center of the tubule to spermatids during the second phase of meiosis, which will contain half of the genetic material of each spermatocyte. Finally the spermatids will terminally differentiate to mature sperm, which will be released from the center of the tubule to the epididymis for storage (Fig.1). Defining the SSC's and their niche within the seminiferous tubule is important to further the developmental understanding of spermatogenesis. Research has been conducted to find a protein that labeled specifically the SSC population but most proteins are also expressed in the progeny of the SSC population.

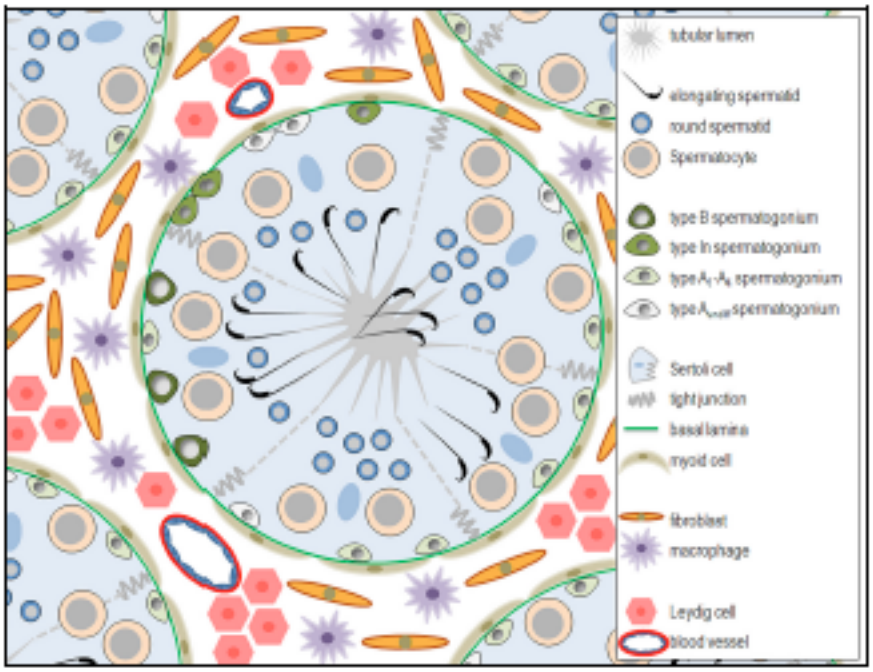


Figure 1: Cross section of testicular tissue (Makela; 2012)

Origin of Spermatogonia

Primordial germ cells (PGCs) during embryonic development migrate from the epiblast to the genital ridge where the PGCs will associate with somatic cells to form the gonad. In males the gonad somatic cells inhibit PGCs from entering meiosis and direct them to a spermatogenic fate (prospermatogonia), remaining proliferative until birth. Prospermatogonia develop into SSCs postnatally during a 6-day period in rodents (Oatly *et al;* 2012). During the first three days after birth SSCs migrate from the center of the seminiferous tubules to the to the basement membrane. Once this process is complete three days after birth that the SSC population becomes functional.

Spermatogenesis

Spermatogenesis is a cyclic process with two main stages. The first stage includes undifferentiated spermatogonia that repopulate the testes through spermatogenesis (Fig.2). Spermatogonia undergo proliferative mitosis in the first stage to give rise to clones of spermatocytes. During mitosis two daughter cells are formed with identical

DNA from a parent cell. The steps of mitosis are as fallows: G1 is the first phase where parent cells grow, S phase where replication of chromosomes occurs, and the G2 phase where cells prepare for mitosis and replication of organelles and synthesis of microtubules occurs. Most of the cells in this category will differentiate to maturity and include single SSCs (A_s), pairs (A_{pr}) and chains (A_{al}) of spermatogonia. During mitosis in spermatogenesis A_s cells divide either asymmetrically or symmetrically giving rise to daughter cells that can migrate away from each other and become two new single cells. The daughter cells go through self-renewing divisions maintaining the SSC population or stay together and form pairs (called A_{pairs}, $A_{pr)}$ with intracellular cytoplasmic bridges connecting them. Although A_s and A_{pr} spermatogonia are distinguishable by their topographical arrangement there are no other morphological differences between the cell types. The pairs of cells then divide linearly to form A_{al} chains of 4, 8 or 16 cells along the basal lamina of the tubules. The A_{al} spermatogonia then differentiate into A_1 spermatogonia, which are the first generation of differentiating spermatogonia (de Rooij *et al;* 1998).

The A_1 spermatogonia differentiate to form A_2, A_3, A_4, intermediate and finally B spermatogonia. The B spermatogonia will then give rise to more mature spermatocytes through the last mitotic division. Spermatocytes further differentiate into spermatids through meiosis, which will reach full maturity as sperm. A primary spermatocyte is transformed into two secondary, more mature, spermatocytes during the first phase of meiosis. As the spermatocytes continue through the second stage of meiosis they differentiate to become spermatids, which develop in 12 stages in mice. A_{al} spermatogonia then differentiate to A1 spermatogonia in stages VII and VIII. This indicates the transition from random cycling of the undifferentiated cells to a six division process that is more controlled for differentiation of the spermatogonia (Fig.3, de Rooij *et al;* 1998). The entire spermatogenic process takes 28 days in rodents. The second stage of spermatogenesis is the population of cells that will transition directly into spermatogonia that contribute to the first round of spermatogenesis but do not renew (Oatley *et al;* 2012).

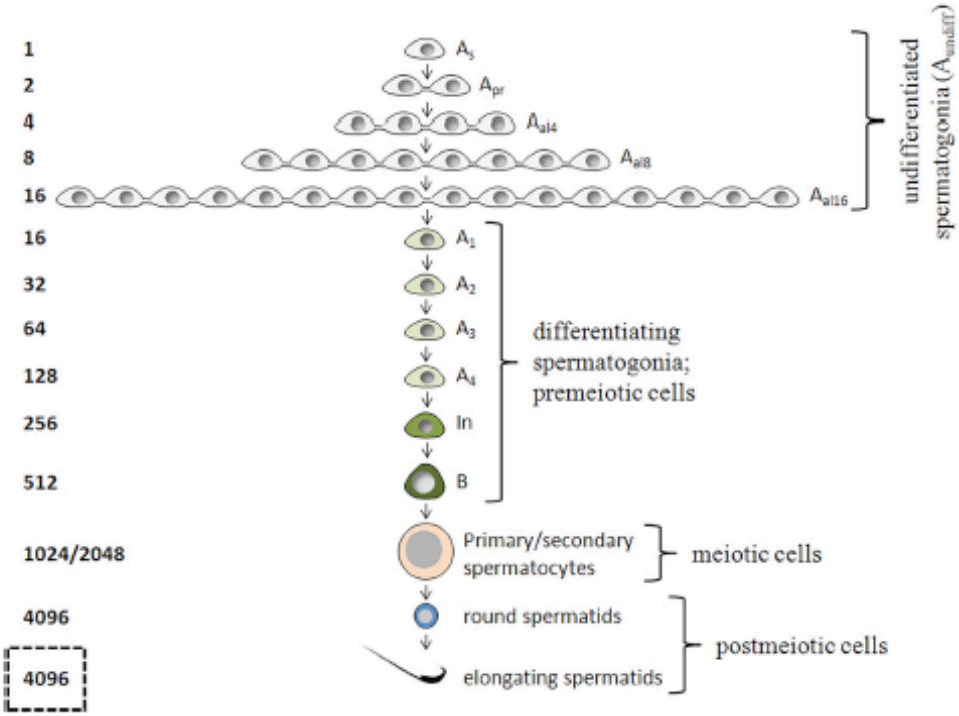


Figure 2: Scheme of stem cell renewal and expansion (De Rooij *et al;* 2012).

Seminiferous Tubule Stages

The stages of spermatogenesis take place at different places throughout the seminiferous tubule (Fig.3). The beginning epithelial stages of spermatogenesis follow the length of the tubules and the beginning of the tubule is seen to be stage VII where all A_1 spermatogonia go through the S phase of mitosis at the same time as stage IX (De Rooij *et al;* 2006). Following A_1 mitosis during stage XI, A_2 spermatogonia are formed and go through the G_1, S and G_2 phases of mitosis synchronously to become A_3 spermatogonia. Each stage of spermatogonia is present in only a few stages of the seminiferous epithelium cycle and the cycle is maintained synchronously (De Rooij *et al;* 2006). During stages X and III the undifferentiated spermatogonia (A_s, A_{pr}, and A_{al} spermatogonia) will proliferate at random times and are not coordinated with the differentiating spermatogonia. A_{al} spermatogonia are rare from stages VIII to XI. During stages VII and VIII the A_{al} spermatogonia differentiate into A_1 spermatogonia and during

stage VIII the preleptotene (the earliest stage of prophase in meiosis) spermatocytes enter leptotene stage (meiotic prophase) and move towards a more adluminal position in the tubules (Fig.3, De Rooij *et al;* 2006).

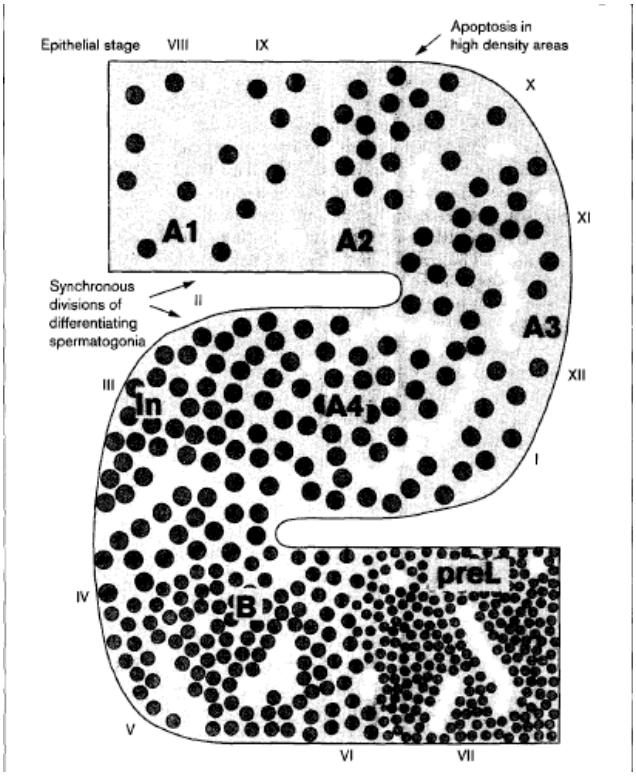


Figure 3: Seminiferous Tubule Stages (De Rooij *et al;* 2006).

Spermatogonial Stem Cell Niche

The fate decisions made by the SSC population are highly influenced by a specific niche within the seminiferous tubules. Specific somatic support cells are present in the seminiferous tubules to aid in decision of the SSCs. Niche support cells secrete cytokines that regulate SSC function. There are also adhesion molecules in the SSC niche that help direct the SSCs where to go within the tubules. According to Oatley and Binster in 2012 Sertoli cells have been shown to be an important somatic cell type that is closely oriented to SSCs (Oatley and Brinster, 2012. Sertoli cells influence the function and formation of the SSC niche by secreting soluble factors and orchestrating contributors of other support cells within the niche. Niche support cells are important in the development

and differentiation of SSCs to keep the tubules populated. As stated above SSCs are theorized to have the ability to differentiate symmetrically (creating two new SSCs or two new differentiated cells), asymmetrically (creating one stem cell and one progenitor cell), or simultaneous symmetric and asymmetric divisions. Fate decisions are tightly regulated and the SSC niche plays an important role in regulation by providing cues and factors that influence symmetric versus asymmetric division (Oatley and Brinster; 2012).

To sustain the adult SSC population the niche provides cues during steady state conditions that promote self-renewal and continued differentiation. There are many other cell types that contribute to the niche including blood vessels (vasculature), Lydig cells, and peritubular myoid cells (Fig.4a). Sertoli cells are the only cells shown to secrete factors influencing the SSC population but other cells contribute to the niche as well. In mature mouse testes vasculature is an important contributor to the SSC niche. Spermatogonia are closely associated anatomically with branch points in blood vessels and follow the vasculature pattern in their basal membrane position (Yoshida *et al*; 2007). Undifferentiated spermatogonia are recruited by blood vessels, which play an important role in the SSC niche formation. Lydig cells are closely related to blood vessels and possibly originate in blood vessels. Lydig cells secrete androgens (hormones that control male characteristics by binding to androgen receptors) and other factors to produce niche microenvironments for SSC maintenance (Yoshida *et al;* 2007). They showed that Lydig cells surround blood vessels and are therefore in close proximity to undifferentiated spermatogonia and the association of branch point vessels reflects the importance of Lydig cells since they are plentiful in these areas. Lydig cells and peritubular myoid cells may secrete the cytokine colony-stimulating factor-1 (CSF-1), which influences the self-renewal of SSCs in mice (Fig.4a, Oatley and Binster; 2012). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) regulate Sertoli and Lydig cell functions. These hormones are released by the anterior pituitary gland in response to stimulation of gonadotropin-releasing hormone (GnRH) (Oatley and Brinster 2008).

The seminiferous tubule is surrounded by seminiferous epithelium and interstitial fluid (Fig.4). Within the seminiferous epithelium Sertoli cells and undifferentiated germ cells are closely associated (Fig.4a). Tight junctions are formed between Sertoli cells to

compartmentalize the seminiferous epithelium, which then constitute the blood-testes barrier, a physical barrier between blood vessels and seminiferous tubules (Fig.4b). The regulation of stem cell proliferation and survival is controlled by Sertoli cell secretion, glial cell derived nuerotrophic factor (GDNF) and fibroblast growth factor two (FGF2) (Fig.4). According to Oatley and Brinster in 2008 bone morphogenic protein 4 (BMP4) and neuroregulin1 influence differentiation of SSCs but the source of these secretions is unknown.

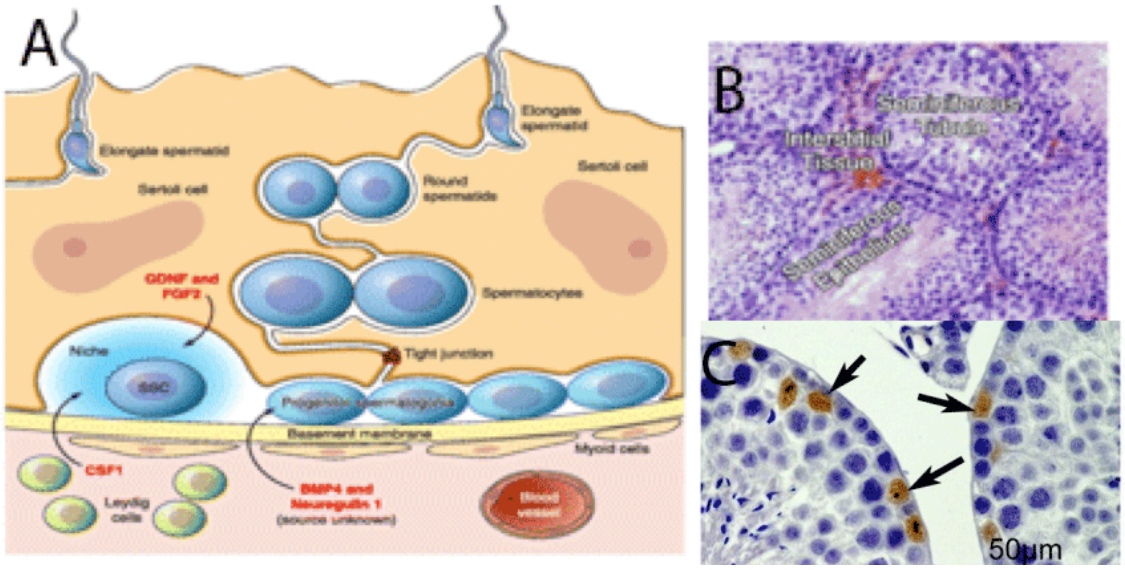


Figure 4: Spermatogonial stem cell niche and testes environment A) schematic overview of the SSC niche, B) tight junctions are seen between Sertoli cells and visualized through cross sections of adult mouse testes (Oatley and Brinster 2012), C) WT1 staining of Sertoli cells in adult mouse testes (Wang *et al;* 2013).

Biological markers have been identified that are expressed in the SSC niche. Wilm's tumor protein (WT1) has been shown to be critical in Sertoli cell regulation and can be used as a marker to identify Sertoli cells (Wang *et al;* 2013). In figure 4c the placement of Sertoli cells labeled by WT1 near the basement membrane of the seminiferous tubule enforces the close association of these cells with the SSC population.

Spermatogonial Stem Cell Markers

Many researchers have investigated marking and isolating the spermatogonial stem cell population with various endogenous factors. CD9 was a proposed surface marker for mouse and rat germ line stem cells (Kanatsu-Shinohara *et al;* 2003). CD9 was thought to be a specific SSC marker because of its expression in embryonic stem cells, hematopoietic stem cells, and neural stem cells. The group used FACS sorting of ROSA mouse testes stained for CD9. 4.7% of testes cells expressed CD9 and staining showed that cells positive for CD9 were located near the basal lamina of the seminiferous tubules in the A_{al} pattern (Fig.5a). This data suggests that a population of undifferentiated spermatogonia are being labeled and not individual A_s undifferentiated spermatogonia. Without being able to identify the definitive SSC population characterization of this cell type is not possible. The A_s spermatogonia population of cells expressing CD9 is located within the basal lamina of the seminiferous epithelium but other undifferentiated spermatogonia in the A_{al} position also expressed CD9 (Kanatsue-Shinohara *et al;* 2003*).*

In 2004 Yoshida *et al* proposed that neurogenin3 (Ngn3) marks the earliest stages of mouse spermatogenesis. The mice used in this study carry the EGFP gene flanking a 230kb fragment of the Ngn3 locus to visualize cells expressing Ngn3. Whole mount florescent images show that chains of undifferentiated spermatogonia express Ngn3 in the adult mouse testes (Fig.5b). Ngn3 is not expressed solely in the A_s spermatogonia population, but is also expressed in the A_{al} spermatogonia. The cells expressing GFP represents the A_s, A_{pr}, and A_{al} spermatogonial population (Yoshida *et al;* 2004). Clones of spermatogonia with multiple intracellular bridges between one another are also positive for Ngn3 (Fig.5c). The expression of Ngn3 in the adult mouse testes can be seen in A_s, A_{al}, and more mature clone-like spermatogonia in the adult mouse testes.

CHD1(previously known as E-Cadherin) was investigated as a marker for undifferentiated spermatogonia in adult mouse testes (Tokuda *et al; 2*006). CDH1 are cell adhesion proteins and specific surface markers for spermatogonia. This group showed that CDH1-positive cells made clusters of one, two, four or eight cells as seen through whole mount immunohistochemistry in figure5d and 5e. Their testes repopulation experiment using CDH1 expressing cells demonstrate that the SSC population resides

among the CDH1-positive cells but the true SSC population cannot be distinguished from the other undifferentiated progenitor spermatogonia expressing CDH1. In 2008 Tulkunova *et al* also looked at E-cadherin (another name for CDH1) expression in the adult mouse testes. E-cadherin-positive cells were seen as A_s or A_{al} spermatogonia in staining of adult mouse testes cross sections (Fig.5e). These results shows that E-cadherin protein expression does label the SSC population but also marks other spermatogonial progenitor cells.

In 2012 EPCAM and Pou5f1 have been examined for their expression in spermatogonia. Although EPCAM has been considered a homophilic adhesion molecule, EPCAM transmits proliferation signals upon cleavage. EPCAM expression in adult mouse testes is highly expressed in progenitor spermatogonia although, not solely labeling the A_s population (Kanatsu-Shinohara *et al;* 2011). EPCAM is present and highly regulated in undifferentiated A_s spermatogonia and their progeny. Another recent study showed that Type A spermatogonia express Pou5f1 (Garcia and Hofmann; 2012). This group used the Pou5f1-GFP mouse model to visualize Pou5f1-positive cells in adult mouse testes. Immunofluorescence conducted on whole mount tissues showed that GFP expression was seen in undifferentiated A_{al} chains of spermatogonia in the adult mouse testes. Again, Pou5f1 labeled the undifferentiated A_s spermatogonia population along with the undifferentiated A_{al} population of spermatogonia.

These experiments indicate that there is has not been a protein in the adult mouse testes that the definitive spermatogonial stem cell population is expressing. All of these factors have been shown to be present in the undifferentiated A_s spermatogonia population along with other spermatogonial populations within the testes. Isolation of the true SSC population is very important in characterizing the population and its niche to further developmental and medical research in the field of spermatogenesis. There has been one transcription factor that was thought to label only the undifferentiated A_s spermatogonia, but at a high rate per tubule.

Recently, in 2011 inhibitor of DNA binding 4 (ID4) protein transcription factor has been suggested to selectively mark the SSC population in adult mouse testes (Oatley *et al;* 2011). ID4 is also expressed in Sertoli cells but the group found a specific antibody

that was expressed only in spermatogonia. ID4 labels only undifferentiated A_s cells that are rare and seen on the basil lamina of the tubules. This group investigated the expression of ID4 using a transgenic mouse model where GFP is driven by recombination of the ID4 promoter. Testes repopulation assays indicate that undifferentiated A_s spermatogonia are highly enriched by ID4 expression (Fig.5g). ID4 staining in normal adult mouse testes showed that cells expressing ID4 were single cells near the basal lamina of the tubules (Fig.5h, Ferguson *et al;* 2013*).* This group also quantified the number of A_s cells expressing ID4 per tubule cross section. Slightly less than one cell expressing ID4 through immunostaining per tubule cross section was observed in the adult control mouse testes (Fig.5i). This supports Oatley *et al*'s finding that ID4 is expressed in a rare population of single undifferentiated spermatogonial cells near the basal lamina of the seminiferous tubule, which may be the true spermatogonial stem cell population (Oatley *et al,* 2012). However, no transplant experiments were conducted with the ID4-GFP mouse model, so ID4 cannot be said to mark a stem cell population.

ID4 expression in the adult mouse testes has given researchers a possible tool for the identification and study of the rare spermatogonial stem cell population within the adult mouse testes. The high frequency of ID4 expression in spermatogonia may indicate the group is labeling progenitor cells as well as the SSC population. This question remains unclear and further study would be needed to indicate that the ID4 expressing cell population within the adult mouse testes really is the definitive SSC population.

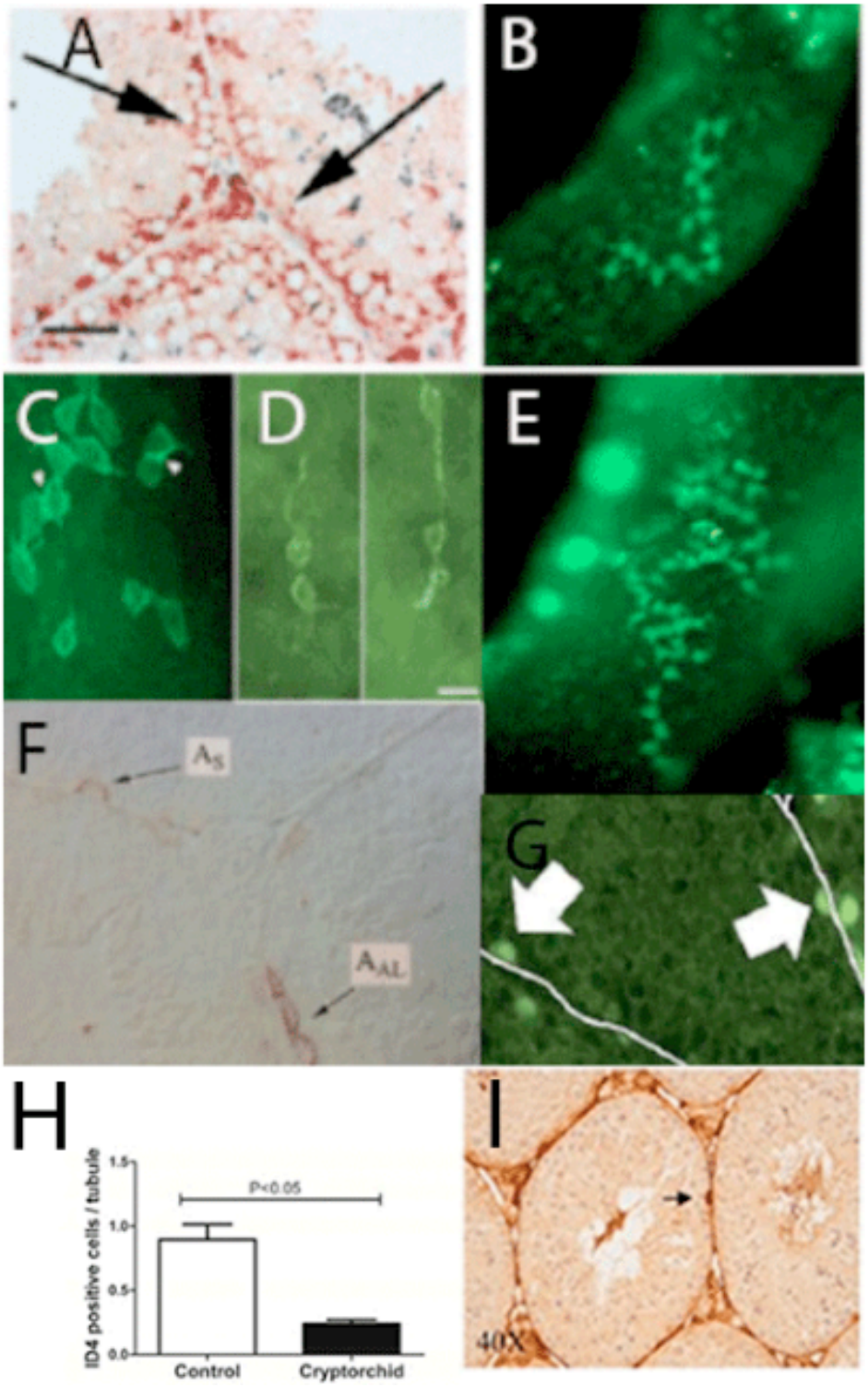

A
B
C
D
E
F
A_S
A_{AL}
G
H
ID4 positive cells / tubule
1.5
1.0
0.5
0.0
P<0.05
Control
Cryptorchid
I
40X

Figure 5: Spermatogonial markers A) CD9+ cells at the basement membrane of the testicles (Kanatsu-Shinohara *et al* 2003), B and C) mouse seminiferous tubule whole mounts showing NGN3+ cells (Yoshida *et al* 2004), D and E) mouse seminiferous tubule whole mounts showing CDH1+ cells (Tokuda *et al* 2006), F) mouse seminiferous tubule whole mounts showing E-cadherin+ cells (Tolkunova *et al* 2008), G) ID4 staining of spermatogonia in adult male testes (Oatley *et al* 2011), H) cross section of ID4 staining 3 month old mouse testes (Ferguson *et al* 2013), I) quantitative comparison of ID4 positive cells of 3 month old control mice (Ferguson *et al* 2013).

Molecular Biology of Oct4

Oct4 transcription factor is a promising candidate for marking and being able to isolate the SSC population in adult mouse testes. Oct4 is an octomer binding protein of the POU domain. It is an 8bp element found in the promoter or enhancer regions of ubiquitously expressed and tissue specific genes (Ovitt and Scholer; 1998). Oct4 is present in ESCs and was first identified by its binding protein activity in ESCs. It is a protein consisting of 352 amino acids, the POU domain being about 150 amino acids in length. The Oct4 gene is located on chromosome 17 in mice and consists of 5 exons and transcription can be initiated at multiple steps because the gene is missing a TATA box in the promoter region. The Oct4 gene sequence is 84% similar between mice and humans and the genomic organization is the same (Ovitt and Scholer; 1998). In the early embryo Oct4 expression is ubiquitous in the inner cell mass cells and is expressed in the embryo until embryonic day 6.5 after which is it rapidly down regulated. After day e8.5 Oct4 expression is confined to primordial germ cells (PGCs) (Fig.6a). PGCs are precursors for SSCs. Oct4 expression is essential for pluripotent cells (SSCs in the testes) and the entirety of the germline in the mouse embryo (Boiani *et al;* 2004).

In adult mice Oct4 expression is restricted to oocytes and spermatogonia. An 18-kb genomic Oct4 fragment containing the Oct4 coding and its 5' and 3' flanking sequences was large enough to drive gene expression comparable to endogenous Oct4 expression (Yeom *et al;* 1996). Oct4 expression in the male germ line is confined to the most undifferentiated spermatogonia (Pesce *et al;* 1998). This group looked at Oct4

expression in newborn mice and saw that the entire population of spermatogonia was expressing Oct4 (Fig.6b). Adult mouse seminiferous tubules were also looked at and Oct4 expression was still confined to the undifferentiated spermatogonia (Fig.6c). This promising information provoked more investigation into the role of Oct4 in the adult mouse testes.

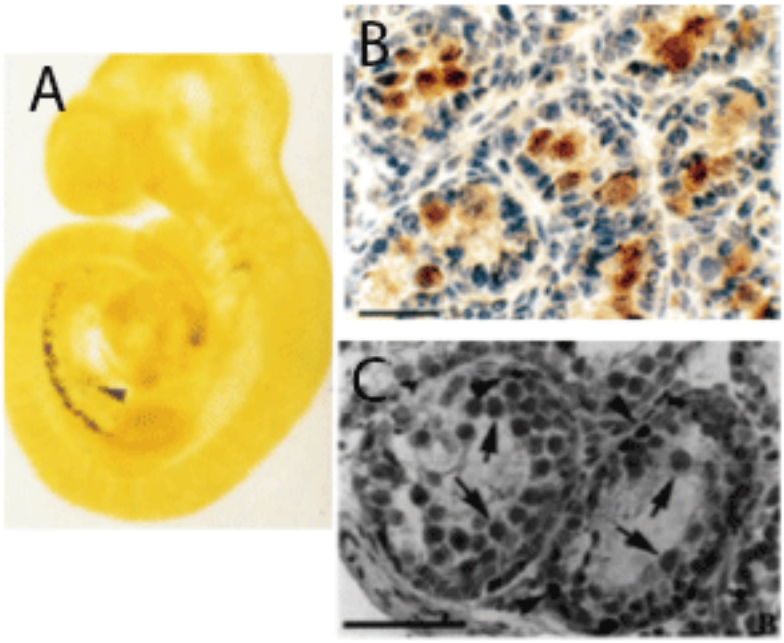


Figure 6: Oct4 expression during embryonic development A) Primordial germ cells are the only cells that express Oct4 after gastrulating, B) Spermatogonia expressing Oct4 (scale bar=50microns), C) Oct4 is confined to undifferentiating spermatogonia (scale bar=100microns), (Pesce *et al* in 1998).

Oct4:CreER mTmG Mouse Model

To enable lineage tracing of endogenous Oct4 in the testes of adult mice we used the Oct4:Cre^{ER} mTmG transgenic mouse model described by Greder *et al* in 2012. In this system a MerCreMer (an internal ribosomal entry site (IRES)-linked, tamoxifen-inducible Cre recombinase) cassette was inserted into the 3'untranslated region (UTR) of the endogenous Oct4 locus. When Oct4 is expressed Cre^{ER} is present in the cell. In the presence of tamoxifen the Cre^{ER} translocates to the nucleus and recombines the mTmG

reporter gene. In this reporter strain Cre recombinase activity removes transmembrane targeted TdTomato, which is replaced by membrane bound eGFP. As the recombination is irreversible this allows lineage tracing of cells that have expressed Oct4. The use of Cre^{ER} with tamoxifen administration enables lineage tracing of Oct4 expressing cells in post embryonic tissue (Fig.7). This transgenic mouse model allow lineage-tracing of Oct4 expression in the adult mouse testes with high temporal-spatial resolution.

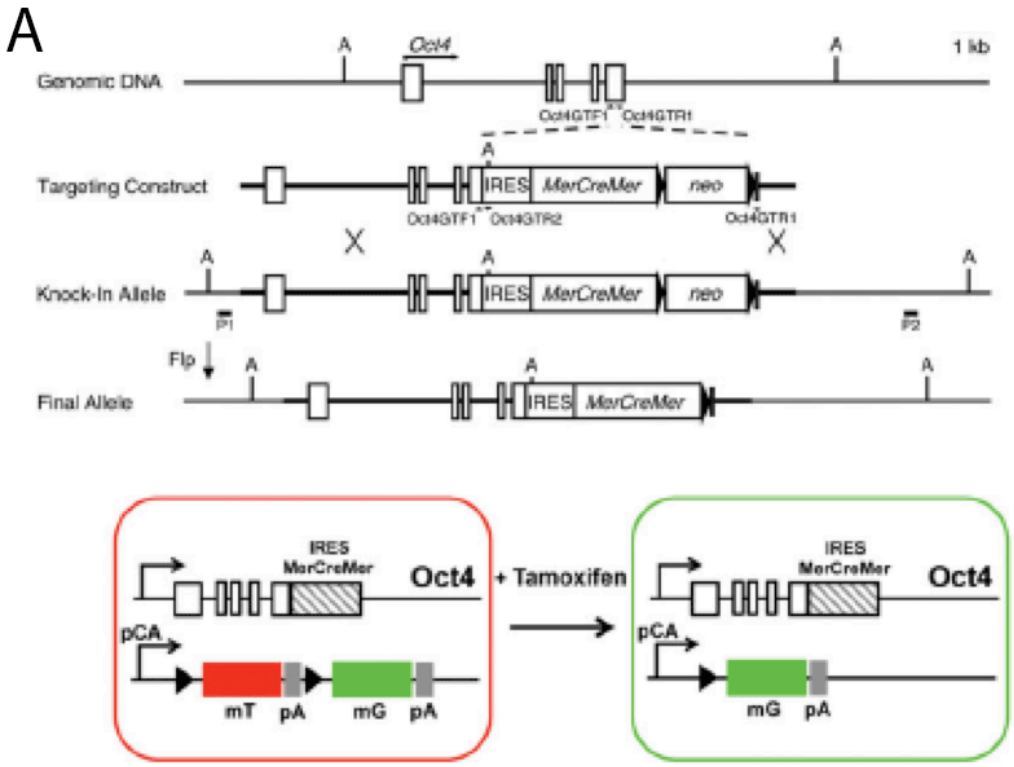


Figure 7: Oct4 Mer CreMer mouse system A) Oct4 MerCreMer mouse model (Greder *et al* 2012)

Summary

The identification of the spermatogonial stem cell is important for further developmental and medical study. There have been many factors investigated for expression in the SSC population. Most of these factors have labeled the A_s population of spermatogonia along with other undifferentiated progenitor cells. ID4 protein has been shown to be expressed in only A_s spermatogonia, but at a high rate per tubule. Here we investigate a protein known to be expressed in primordial germ cells in adult mice. The Oct4:Cre^{ER} mTmG transgenic mouse model will allow us to identify and lineage trace spermatogonia that express Oct4 in the adult mouse testes.

Methods and Materials

Generation and Characterization of Oct4:CreER Mouse Line

A MerCreMer (an internal ribosomal entry site (IRES)-linked, tamoxifen-induced Cre recombinase) cassette was inserted into the 3'untranslated region (UTR) of the endogenous Oct4 locus. Oct4-MerCreMer transgenic mice expressing EGFP in Oct4 positive cells (green florescent protein, Greder *et al* 2012, Jackson Laboratory Stock No. 016829, West Grove, PA, http://www.jacksonimmuno.com) were crossed with homozygous double-florescent Cre reporter mice strain mTmG (The Jackson Laboratory, Stock No. 007576) for the replacement of TdTomato with eGFP expression through Cre recombinase (Greder *et al* 2012) for lineage tracing of Oct4 expression in mouse testes.

Experimental Animal Model and Treatments

Oct4-MerCreMer mice (Greder *et al* 20120) were crossed with reporter mice from Dutton laboratory. For the short-term experiments 0.2mL of tamoxifen at 10mg/mL was injected intraperitonealy for 3 consecutive days and testicles were harvested on the fourth day. For the mid-term experiments 0.2mL of tamoxifen at 10mg/mL was injected intraperitonealy for 3 consecutive days and testicles were harvested 7 days after the last tamoxifen injection. For the long-term experiment 0.2mL of tamoxifen at 10mg/mL was injected intraperitonealy for 3 consecutive days and testicles were harvest 27 days after the last dose of tamoxifen.

Wizard Genomic DNA Purification

The Wizard Genomic Purification Kit was purchased from Promega.

Tissue Preparation

Genomic DNA was isolated from mouse tails and put in 1.5mL tubes where 500µl of Nucleic Lysis Solution, 120µl of EDTA, and 17.5µl of protinase K were added to the tissue and incubated overnight at 55° C in a shaker.

Lysis and Protein Precipitation

For lysis and protein precipitation 1.5ml tubes were removed from the shaker and 200µl of protein precipitation solution was added to each tube. The tubes were then vortexed and chilled on ice for 5 minutes, and then centrifuged on high for 4 minutes.

DNA Precipitation and Rehydration

The supernatant was then transferred to new tubes and 600µl of isopropanol was added to each tube. The tubes were then mixed gently by inversion and centrifuged on high for 10 minutes. Then the supernatant was removed and 600µl of 70% ethanol was added to each tube. The tubes were then vortexed and centrifuged on high for 10 minutes. The supernatant was then dumped out of each tube and the tubes were set upside down on paper towels and allowed to dry for 30 minutes. After 30 minutes 100µl of DNA rehydration solution (10mM TE buffer, 1mM EDTA, at pH 7.5) was added to each tube.

Genotyping

One microliter of DNA created from the method above was added to 25 µl of ReddyMix PCR Master Mix from Thermo Scientific to amplify DNA, which contains ThermoPrime *Taq* DNA polymerase, dNTPs, reaction buffer, magnesium chloride, dye, and precipitant to facilitate gel loading. 1µl of each of three Oct4 primers ordered from Integrated DNA Technologies (IDT) of 25nmole DNA of 100µM Oligo in 282 µl of IDTE Buffer of pH 8; Oct4GTF1: 5'CCA AGG CAA AGG TAG ACA AG3', Oct4GTR1: 5'GCT TTC TCC AAC CGC AGG CTC TC3', and Oct4GTR2: 5'GCC CTC ACA TTG CCA AAA GAC GG3') were added to ReddyMix. Thermal cycling protocol was as follows: Initial DNA denature at 94° Celsius for 2 minutes, followed by 94° Celsius for 20 seconds, 59.5° *C* for 30 seconds, and 72° Celsius for 30 seconds for 35cycles. PCR products were analyzed by agarose 2% gel electrophoresis and the fragments were visualized with ethidium bromide and UV light exposure. UltraPure Agarose from Invitrogen was used at 2% in 1X TAE buffer (1X tris, acetic acid, and EDTA).

Tamoxifen Injections

Stock Tamoxifen 10mM (Sigma) was prepared by: 100mg of tamoixfen powder put in 500mL of 100% EtOH (ethanol). After the powder is dissolved add 9.5mL of sunflower oil and mix. Sonicate the mixture 10-15 minutes or until the solution is clear. Mice are injected at a concentration of 10mg/ml so 200 micro liters per mouse by intraperitoneal(IP) injection for 3 consecutive days. Mice are harvested at either 1day, 7 days, or 27 days post tamoxifen injections.

Immunohistochemistry for Tissue Sections

Slides were fixed with 4%PFA for 10-20 minutes at room temperature. Slides were then washed two times with PBS-T (PBS +0.1% Tween20). Slides were then permeabilized for 10 minutes with PBS-T + 1% BSA + 1% Tween20. Slides were next blocked with PBS-T + 1% BSA for 30 minutes. The primary antibody was diluted (Table 1) to 1:250 in blocking solution (PBS-T + 1% BSA) and slides were left over night at 4°C. Slides were washed three times with PBS-T. Next, slides were incubated with secondary antibody diluted to 1:500 (Table 2) in blocking solution for one hour at room temperature in the dark. Slides were then washed three times with PBS-T. DAPI was added to each slide at a 1:1000 dilution in blocking solution. Cover slips were added and slides were imaged. Slides were kept for storage in 4° Celsius in the dark.

Immunohistochemistry for Whole Mount Tubules

The mouse testicles were harvested, cut in half (so that formalin can get to all areas of the tissue) and put in formalin at 4° Celsius for 24 hours. The testicles were then put into PBS for keeping in the dark at 4° Celsius. Testicles were placed in petri dishes with two milliliters of PBS and gently pulled apart with tweezers. Once individual tubules were present the tubules were collected in 1000ml pipette tips. Tubules were placed in chamber slides. Excess PBS was sucked off of the slides and Vectashield with DAPI was added to each slide before placement of the coverslips. For staining tubules were washed in 10ml specimen vials three times for five minutes each with PBS. Tubules were permiablilzed in PBS-T (PBS + 0.1% Tween20) for ten minutes at room temperature.

Tubules were then blocked in PBS-T with 5% serum at room temperature for one hour (note: blocking serum of the animal that the secondary antibody was raised in was used). Next the primary antibody was added to each vial, diluted in PBS-T + 0.1%BSA + 5% serum and incubate overnight on a shaker. Tubules were washed three times for five minutes with PBS-T. Next, tubules were blocked with PBS-T + 5% serum for 30 minutes. The secondary antibody was diluted in PBS-t + 0.1% BSA + 5% serum and added to the vials for two hours at room temperature in the dark. Tubules were washed with PBS-T three times for five minutes. Individual tubules were then placed on chamber slides and Vectashield with DAPI was added before the cover slips were put on top of the specimens. Slides were kept for storage in 4° Celsius in the dark.

Immunohistochemistry of SYCP3 and DMRT1

10millimolar (32 grams of citric acid dissolved in 2 liters of de-ionized and distilled water are autoclaved to equal a pH of 6) citric acid buffer was boiled for 3 minutes in a microwave at 100% power. Cross section slides were then added to the citric acid buffer and boiled for 15 minutes in a microwave at 30% power. The slides were then permeabilized in PBS with 0.1% TritonX-100 added for 15 minutes and put on a shaker. Slides were washed in PBS for 1 minute. Tissue samples were circled with a Daco pen and then block solution (1% goat serum in BTT (50micromolar tris HCl at pH 7.9 + 150micromolar NaCl + 0.1% BSA + 0.1% TritonX-11)) was added. The primary antibody was then added to the slides and diluted in block solution and left at room temperature over night. The slides were washed three times with PBS + 0.1% Tween for five minutes each on a shaker. Secondary antibody was diluted in the block solution and placed on appropriate slides for 2 hours in the dark. Slides were washed three times with PBS + 0.1% Tween for 10 minutes each on the shaker. DAPI was diluted in block solution and put on slides for 10 minutes. Slides were washed once with PBS and mounted with Immunomount for viewing.

Antigen	Antibody Code	Origin, Clonality	Dilution	Buffer	Manufacturer
WT1	Sc-192	Rabbit, polyclonal	1:200	PBS(pH 7.4)	Santa Cruz Biotechnology
DMRT1	_	Rabbit, polyclonal	1:100	PBS(pH 7.4)	From Dr.David Zarkower's Lab
SYCP3	_	Mouse, polyclonal	1:300	PBS(pH 7.4)	From Dr.David Zarkower's Lab

Table1: List of primary antibodies used in immunohistochemistry cross section and whole mount staining.

Conjugated to	Antibody Code	Origin	Target	Dilution	Manufacturer
AlexaFlour488	A11008	Goat	Rabbit igGs	1:500	Invitrogen
AlexaFlour557	A31572	Donkey	Rabbit igGs	1:500	Invitrogen
AlexaFlour558	A11054	Rabbit	Mouse igGs	1:500	Invitrogen

Table2: List of florescent antibodies used in immunohistochemistry cross section and whole mount staining

Tissue Preparation

Both testicles were harvested at various time points (short term, mid term, and long term). The tips of the testicles were cut off to allow for penetration of the formalin. The freshly harvested testicles were put into formalin in vials for fixing for 24 hours at 4° Celsius. The testes were then put in a 30% sucrose and 70% PBS solution to protect the testes from lysing when frozen down for 24 hours at 4° Celsius. Testes were then frozen

in O.C.T at -20° Celsius until cryosectioning. Each testis was Cryosectioned at 8-10 microns per section.

Counting and Pattern Identification

Serial sections of long-term *testis*s were taken at 10micron slices of tissue on the cryostat in a consecutive row. The number of labeled versus unlabeled tubules were counted per section. Each pattern type from 107 tubules was quantified.

ImageJ Quantifications

The desired images was opened in ImageJ software and the split color channels was chosen. Then the image was made binary and watershed. Then particle analysis was completed and the program counted each cell by the DAPI stained nuclei.

Whole Mount Quantifications

Each Z-stacked whole mount tubule image was taken at 20X magnification and un-stacked and each individual layer was quantified. A measure of tubule length was taken for each and added together for total tubule length per time point. Each pattern type (A#-immature single layer labeled cell count, B#-immature grouped labeled cell count, O, D1-spermatocyte, D2-spermatid, M-mature sperm) was quantified in each layer of the z-stacked tubules. Number of patterns per area of each tubule was also quantified. For the 4-day time point 2,390 micrometers of tubule length were quantified. For the 10-day time point 4,870 micrometers of tubule length were quantified. For the 30-day time point 4,750 micrometers of tubule length were quantified.

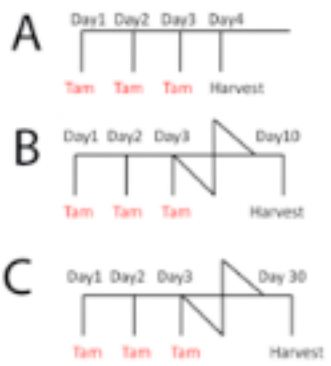
Confocal Z-stack Imaging

For each section of tubule 28 Z-stacked images were taken at 2 microns apart of whole mounted tubules. DAPI for blue florescence, GFP for green florescence, and FITC for red florescence were the lasers used for confocal microscopy.

Results

Lineage tracing Oct4 expressing cells in the adult mouse *testes*

Figure 8 illustrates the experimental design for lineage tracing the Oct4 expressing cells in the adult mouse testes.



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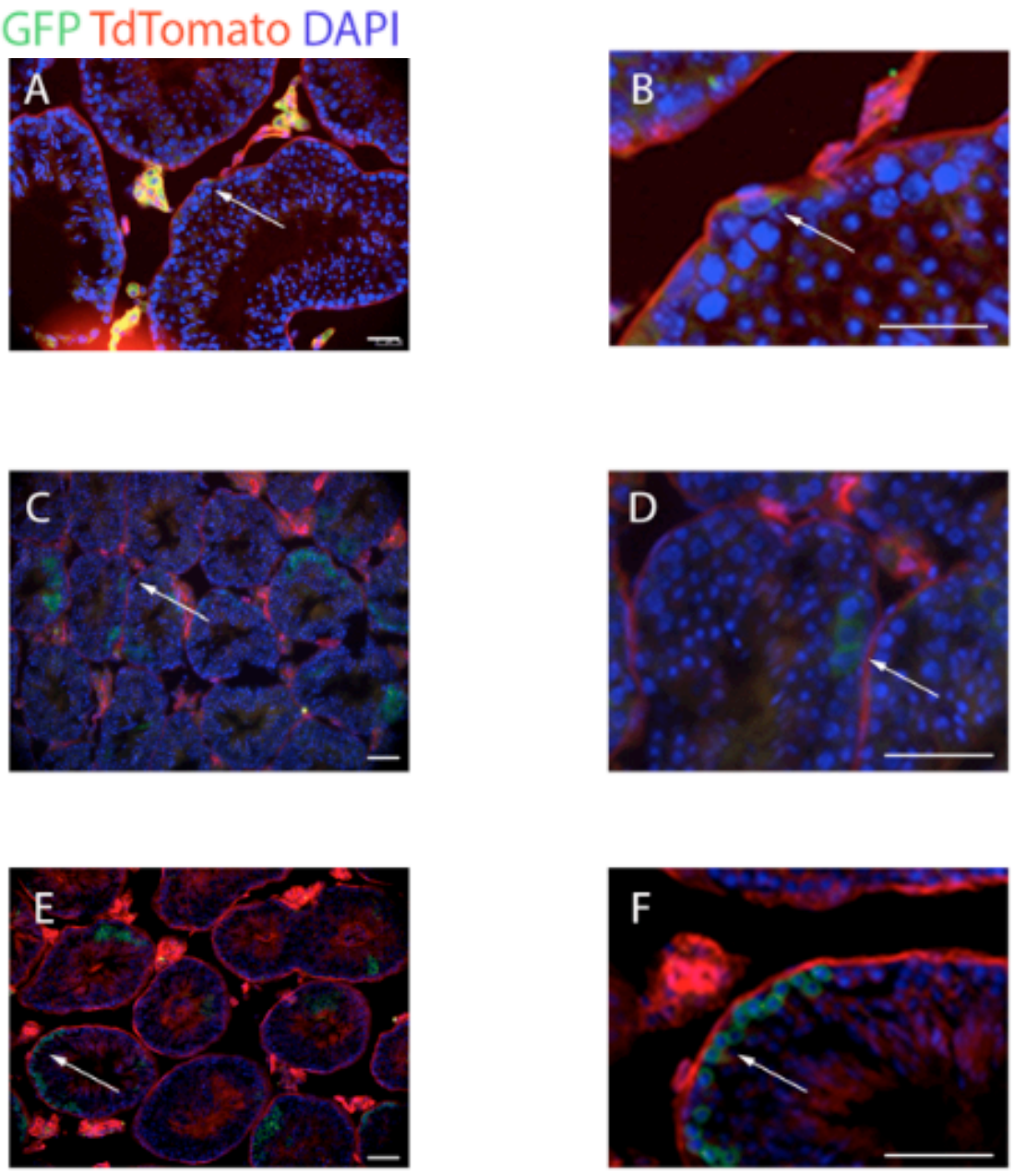
Mouse #	Mouse DOB	Time Point	Age at harvest
1	4/2/13	4-day	4 weeks
2	7/3/13	4-day	4 weeks
3	8/15/13	4-day	4 weeks
4	5/30/13	10-day	5 weeks
5	7/3/13	10-day	4 weeks
6	8/15/13	10-day	4 weeks
7	unkown	30-day	4 weeks
8	5/30/13	30-day	5 weeks
9	8/15/13	30-day	4 weeks

Figure 8: Experimental design for lineage tracing of Oct4 expressing cells in the Oct4:Cre^{ER} mTmG testes A) 4-day time point, B)10-day time point, C)30-day time point, D) Number of mice used for each time point.

Examples of patterns seen after Tamoxifen administration to Oct4CreER transgenic mice

In figure 9 examples of the patterns seen throughout the three experiments are depicted. A tubule cross section from a 4 day time point shows GFP expression in a single cell at the basal lamina of the tubule (Fig.9A). In figure 9b A_{pr} spermatogonia can be seen to express GFP. GFP expression in A_{al} patterns shown in figure9c, d, and e. Spermatocytes labeled with GFP and are seen with a large circular morphology (Makela in 2012, Fig.9f). The spermatocytes are located more interiorly in the tubule than the linear progenitor cells. GFP expression in smaller round spermatids is seen in figure9g. GFP labeling of mature sperm is seen in Fig.9h-l.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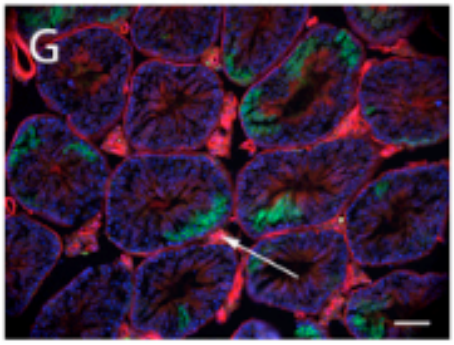
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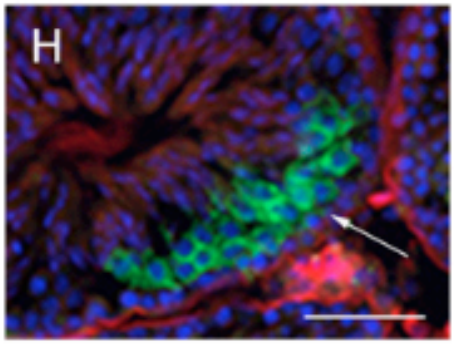
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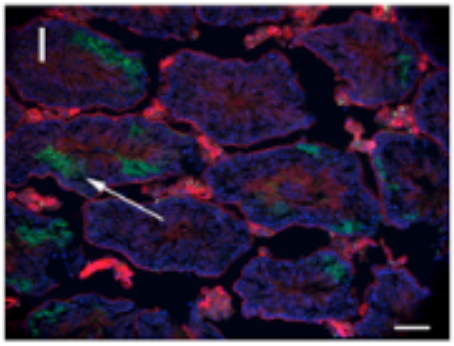
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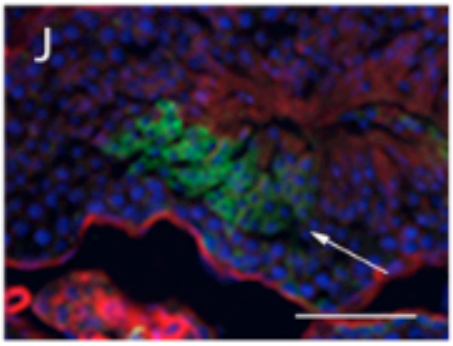
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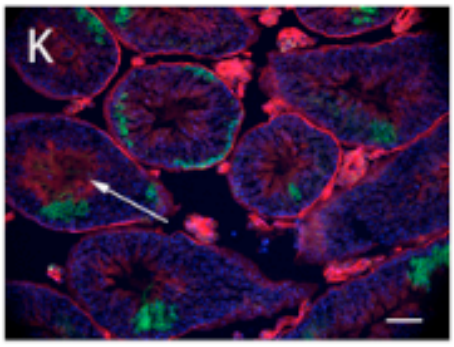
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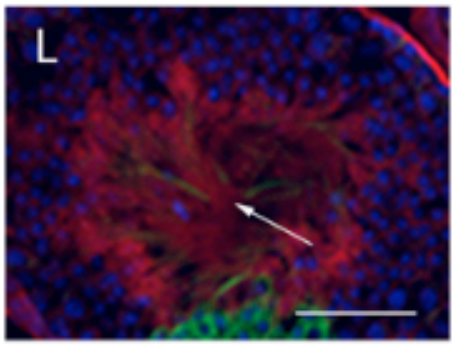
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Figure 9: Examples of GFP expression in the adult mouse testes cross sections of Oct4CreER mouse system after Tamoxifen injection at different time points A and B) Single cell, A_s, GFP labeling of spermatogonia, C and D) A_{pr} GFP labeling of spermatogonia, E and F) A_{al} pattern, G and H) Mitotic spermatogonia labeled with GFP, I and J) small round spermatid morphology seen through GFP labeling of spermatogonia in the meiotic stage of reproduction, K and L) Mature sperm labeled with GFP. Scale bar 50 microns.

Patterns in the seminiferous tubules of adult mice 1 day post Tamoxifen injection

In testes examined one day after the third tamoxifen injection (4-day time point) GFP expression is only seen in single cells adjacent to the basal lamina (Fig.10). This is consistent with the postulated position of the SSC population. The cross section image of the A_s cell in figure10a and 10b shows a flattened cell morphology on the edge of the lamina membrane, again consistent with the hypothesized characterization of the SSC.

From this we further investigated the GFP expressing A_s cells seen in cross sections and imaged whole mount tubules. Only single cells were seen to express GFP (Fig.10c-e). In figure10e confocal z-stack imaging is unstacked and it is seen that the Oct4:Cre^{ER} expressing cell is a single cell and not a chain of cells (Fig.10e).

GFP expression patterns in each tubule was determined for the 4-day time point in 3 separate mice. Whole mount images were used for pattern quantification so that the tubule could be viewed 3-dimensionally and any multiple GFP expressing cell chains could be accounted for. As seen in figure 11 100% of cells at the 4-day time point are single A_s cells.

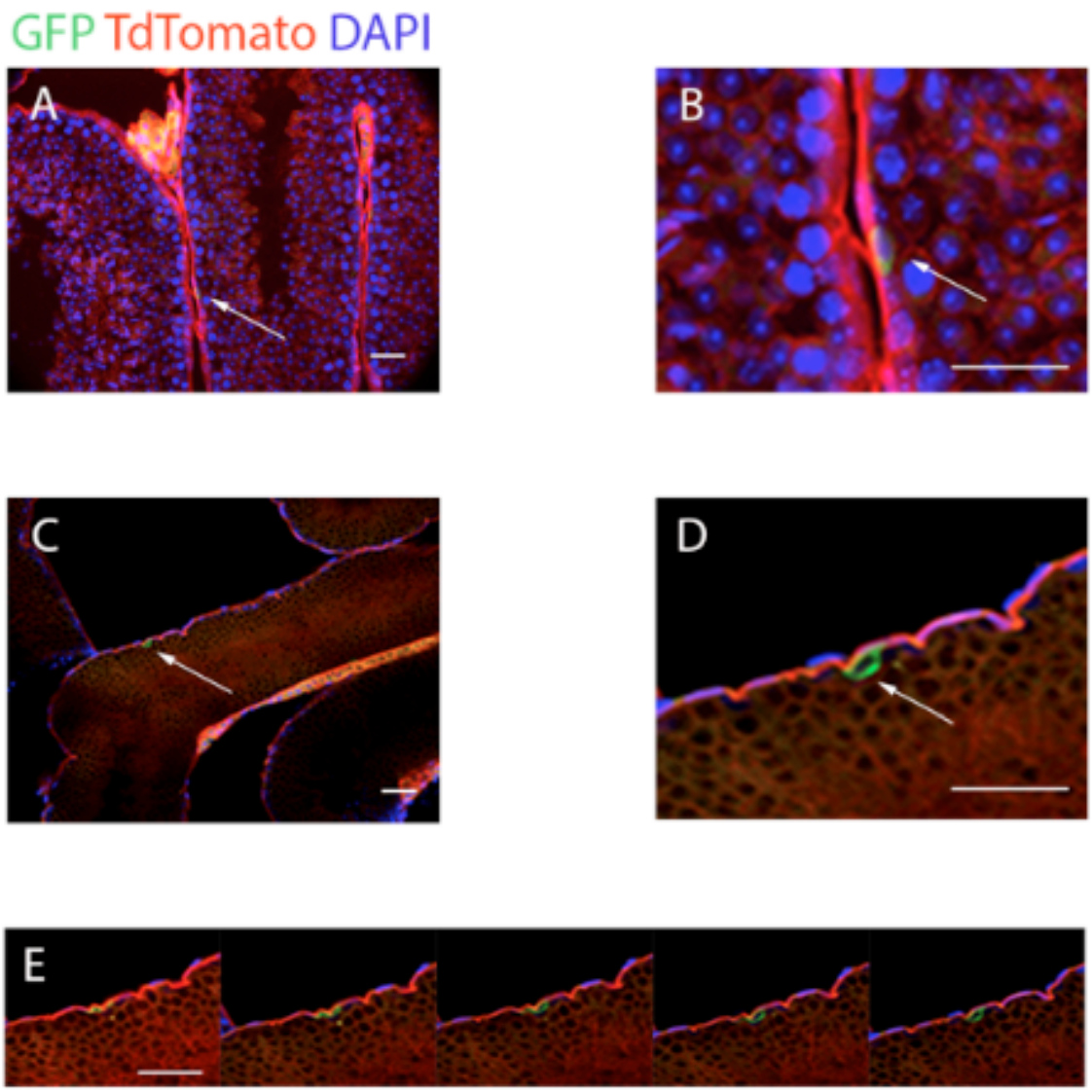


Figure 10: GFP expressing cells at the 4-day time point in adult Oct4CreER mTmG mouse testes, A and B) cross section images of single cell spermatogonia, A_s, labeled with GFP, C and D) whole mount confocal imaging (same tubule as C and D) of 20micron Z-stacked images showing single spermatogonia GFP labeling, E) Whole mount single Z-stacked images un-stacked at 4 microns each in order from top of tubule down. Scale bars 50 microns.

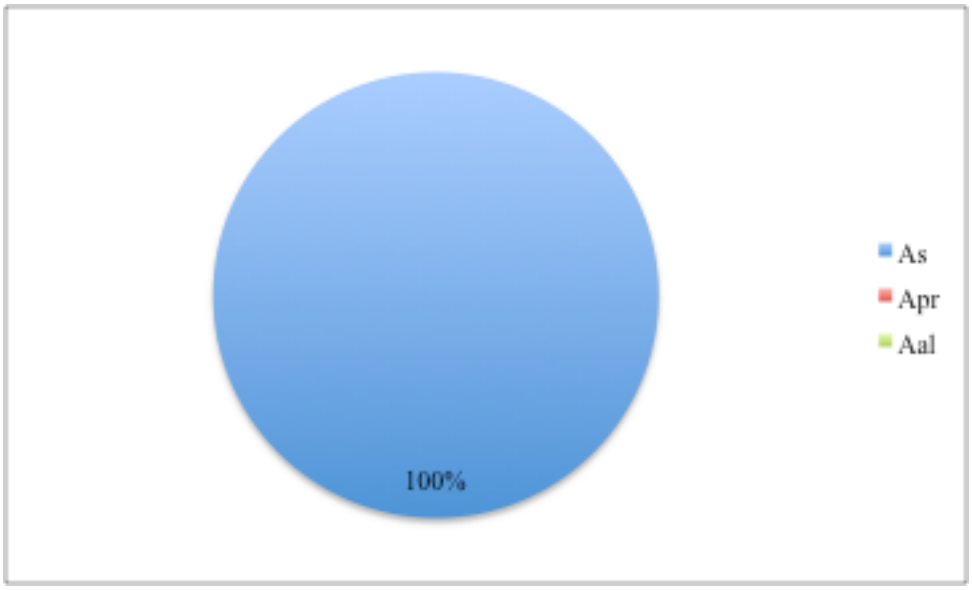


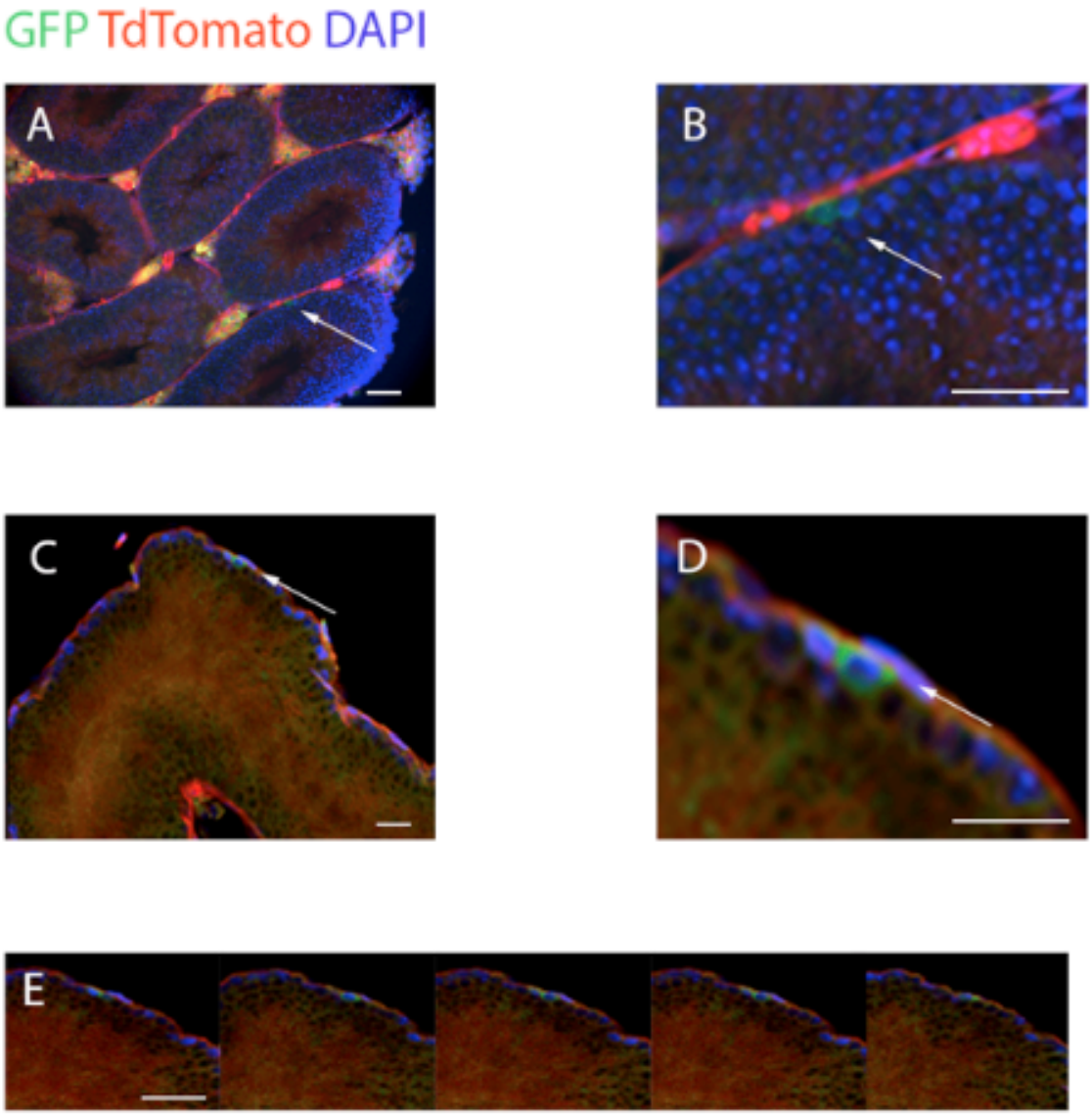
Figure 11: 4-day whole mount patterns quantified from 2390 microns of tubule from three different Oct4:Cre^{ER} transgenic mice. Confocal images of 2390 microns of seminiferous tubule whole mounted from the 4-day time point testes was imaged for GFP expression. The number of each pattern seen in 2390 microns of tubule were evaluated and quantified as a percent for each pattern type.

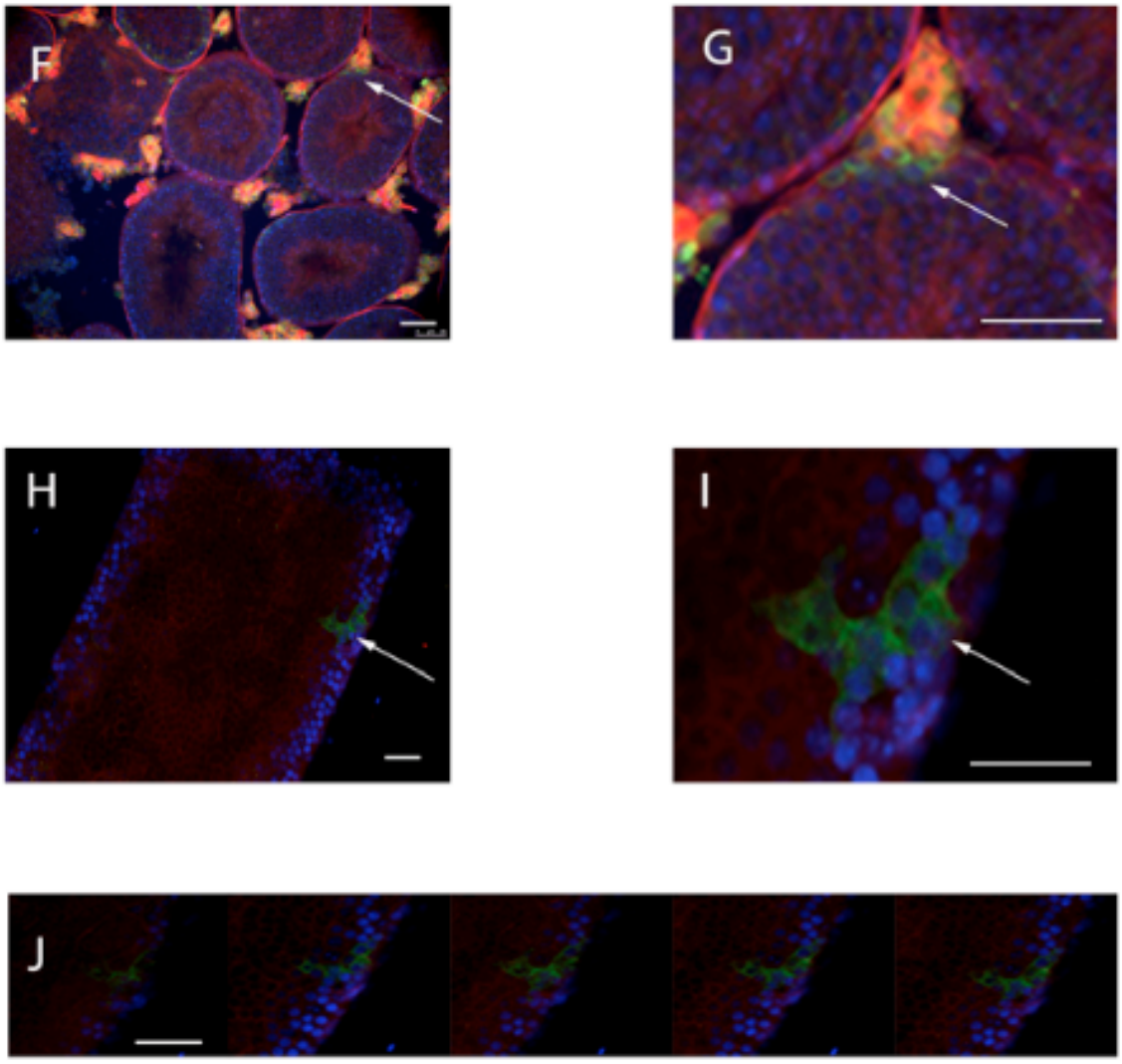
Patterns in the seminiferous tubules of adult mice 7 days post Tamoxifen injection

Examination of the tubules 7 days after the third tamoxifen injection showed A_{pr} and A_{al} cells expressing GFP within the seminiferous tubule. The cells expressing GFP 7 days post tamoxifen are in the position of cells in the mitotic stage of spermatogonial development. In figure12a and 12b, the A_{pr} pattern can be seen. In figure12c, d, and e whole mount images show the same A_{pr} pattern as seen in the cross sections. The chain of two cells can be visualized through the unstacked confocal image in figure12e. The cells are a linked chain with intercellular bridges between the two cells.

In figure12f and 12g the GFP expression is in an A_{al} spermatogonial pattern. The orientation of the A_{al} cells close to the basal lamina of the tubule is consistent with what previous researchers have seen with other protein labeling methods. The 4 cell linear spermatogonial pattern can also be seen in whole mount imaging through GFP expression, however orientation cannot be determined in whole mount images (Fig.12h,i,

and j). Unstacked whole mount images show that the GFP expressing cells are a chain of 4 cells with intercellular bridge connections (Fig.12j). The A_{al} pattern is seen with 8 cells expressing GPF (Fig.12k and l). The pattern is seen in the A_{al} position at the edge of the tubule membrane. The intracellular bridges in-between the chain of cells is visualized through whole mount confocal imaging (Fig.12m, n, and o). In figure12p and 12q there are 16 cells in the A_{al} position expressing GFP. In figure12p and 12q the cells expressing GFP are in the A_{al} position at the edge of the tubule membrane. However, when looking at whole mount images of the 10-day time point a16 cell arrangement of GFP labeled cells showed cells connecting at more than one point (Fig.12r, s, and t).

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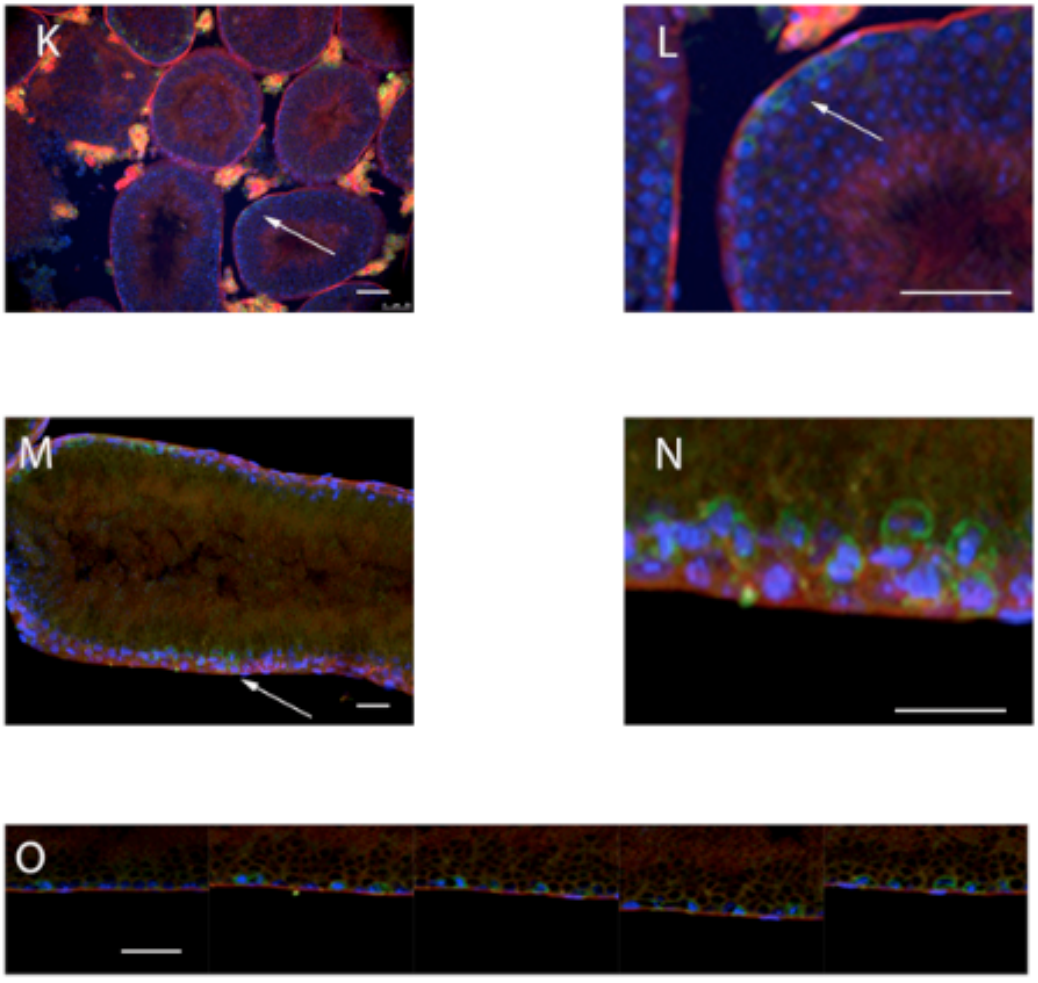
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Figure 12: GFP expressing cells at the 10-day time point in adult Oct4CreER mTmG mouse testes, A and B) Cross section showing A_{pr} spermatogonia labeled with GFP, C and D) whole mount confocal imaging showing A_{pr} spermatogonia labeled with GFP, E) whole mount single Z-stacked images (same tubule as C and D) un-stacked at 4 microns each in order from top of tubule down, F and G) Cross section images of A_{al} spermatogonia that have gone through 2 divisions to give rise to 4 GFP labeled spermatogonia, H and I) whole mount confocal images of A_{al} spermatogonia in a chain of 4 cells, J) Whole mount single Z-stacked images (same tubule as H and I) un-stacked at 4 microns each in order from top of tubule down, K and L) cross section images of A_{al} spermatogonia in a clone of 8 cells labeled by GFP, M and N) whole mount confocal images of A_{al} spermatogonia in a chain of 8 cells labeled by GFP, O) whole mount single Z-stacked images (same tubule as M and N) un-stacked at 4 microns each in order from top of tubule down. Scale bar 50 microns.

GFP expression is quantified in 7 days after the last tamoxifen injection (Fig.13). The A_{al} position of 8 and 16 cells expressing GFP were the most prominent pattern types seen in the 10-day experiment tubules. The same number of A_{pr} and A_{al} 16 cells labeled patterns were seen at 18%, slightly lower than the A_{al} 4 and 8 cells. All of the cells expressing GFP in the seminiferous tubules at the 10-day time point are still in the mitotic stage of development.

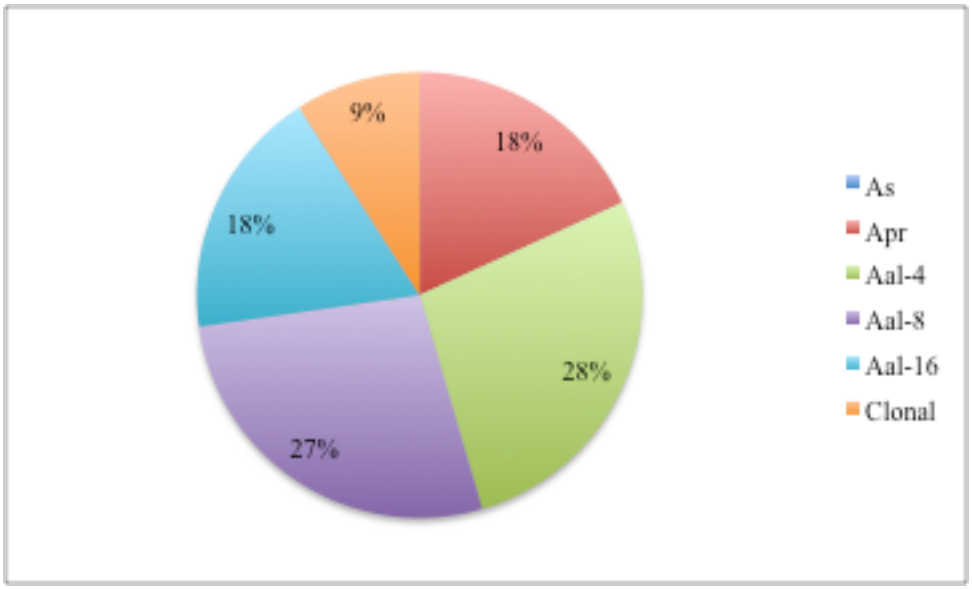


Figure 13: 10-Day whole mount patterns quantified from 4870 microns of tubule from three different Oct4:Cre^{ER} transgenic mice. Confocal images of 4870 microns of seminiferous tubule whole mounted from the 10-day time point testes was imaged for GFP staining. The number of each pattern seen in 4870 microns of tubule were evaluated and quantified as a percent for each pattern type.

Patterns in the seminiferous tubules of adult mice 27 days post Tamoxifen injection

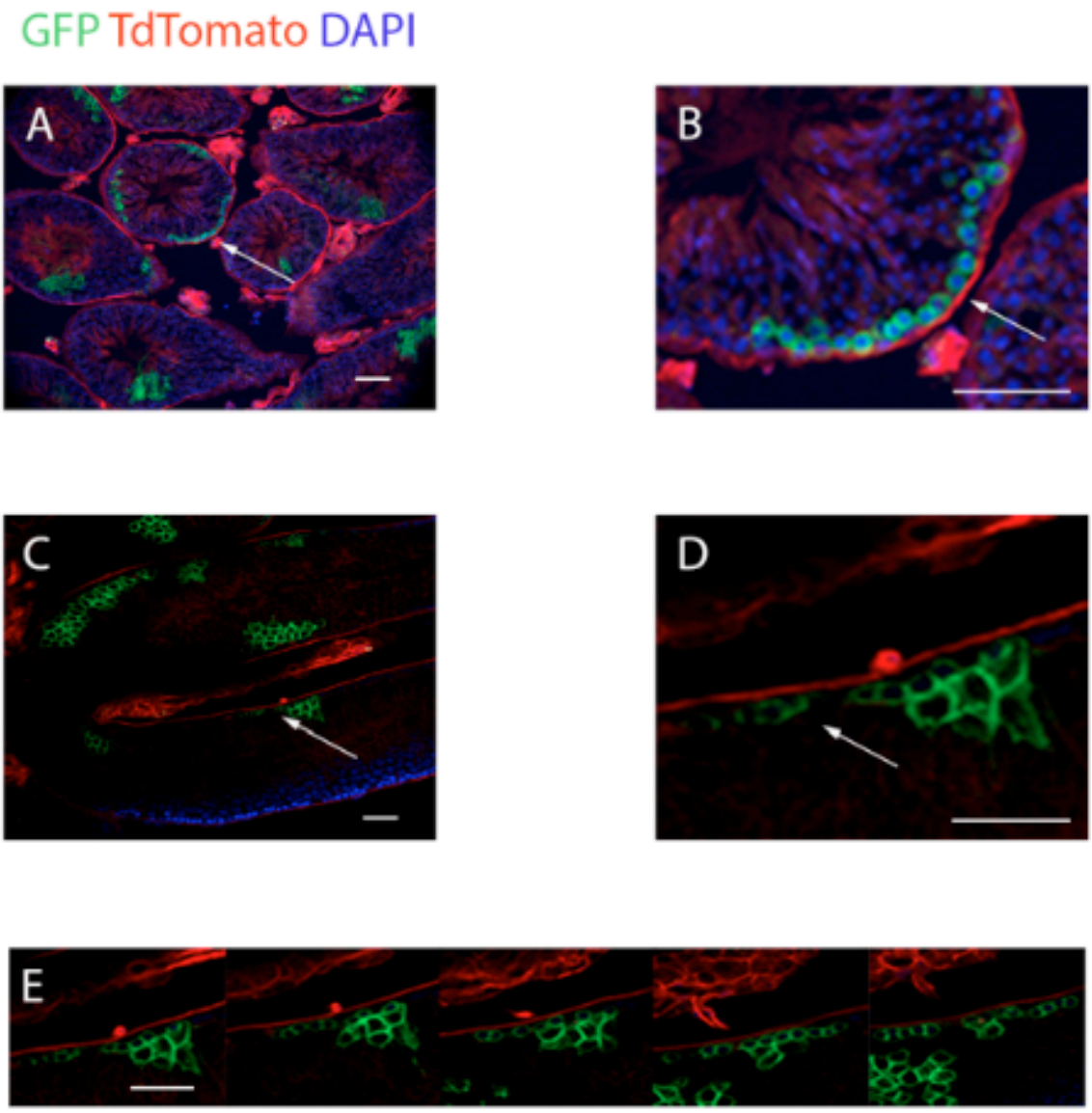
Multiple patterns of GFP expression were observed in the testes of adult mice 27 days after the third tamoxifen administration. GFP expression was seen in A_{al}, spermatocytes, spermatid and mature sperm. Figure14a and 14b show GFP expression in the A_{al} pattern of spermatogonia. A_{al} cells of 8 and 16 clones were seen in 30-day time point tubules. In figure14c-e the A_{al} pattern of cells can be seen expressing GFP in whole mount confocal microscopy. The GFP expressing A_{al} cells were seen with a slightly larger morphology, characteristic of spermatocytes (Fig.14c and d). In figure14e z-stacked confocal images of whole mount 30-day time point tubules were unstacked in 4micron increments. It is seen that the GFP expressing linear A_{al} clone expands throughout 20 microns of tubule.

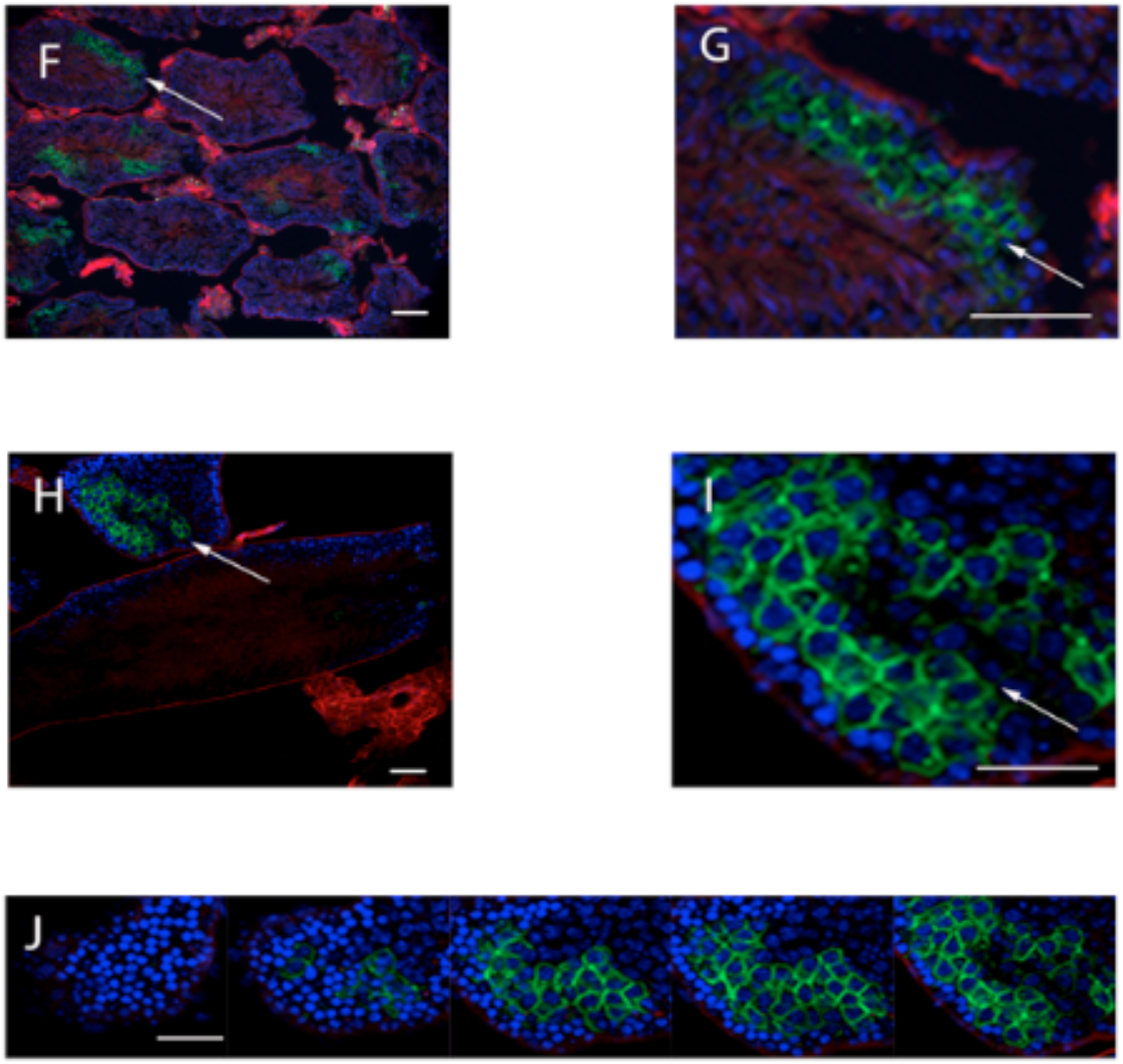
Spermatocyte-like clones are also seen to express GFP in the 30-day tubules. The spermatocyte-like GFP expressing cells seem to either be connected to A_{al} linear cells also expressing GFP or have dissociated from these cells to migrate towards the center of the tubule. In figure14f and 14g cross sections of 30-day tubules show a clone of spermatocyte-like cells most likely in the meiotic stage of development and are not in the outermost layer of cells adjacent to the basal lamina. The spermatocyte-like cells have a larger and more irregularly shaped border compared to the A_{al} cells. The spermatocyte-like cells are not associated with the membrane of the tubules since they have started differentiation toward the adluminal of the tubule (Fig.14f and g). Whole mount images show the same pattern of spermatocyte-like cells in the meiotic stage of development expressing GFP at the 30-day time point (Fig.14h-j). The morphology of the GFP expressing spermatocyte-like cells is larger and less circular then the A_{al} cells in the whole mount images as well. In figure14j 4micron increments of z-stacked confocal images were unstacked and are displayed from the top of the tubule inward. The GFP labeled cells don't appear until 8 microns of depth from the top of the tubule. The GFP expressing spermatocyte-like cells are not around the basement membrane but positioned towards the center of the tubule.

Spermatid-like spermatogonia are expressing GFP in the 30-day tubules as well. Spermatids have a very round and small appearance compared to spermatocytes and are much smaller than A_{al} spermatogonia. The morphology of the spermatid-like GFP expressing cells is round and small, characteristic of spermatids. In figure14k and 14l spermatid-like cells express GFP and are positioned towards the center of the tubule in the second meiotic stage of spermatogenesis. These cells have a different morphology than the spermatocyte-like cells seen in figure 14f-j. Interestingly the spermatid-like cells are not seen closely associated with the spermatocyte-like cells. The process of meiosis two, which spermatocytes undergo to become spermatids, is very rapid and could account for not seeing association between the two cell types in histological sections. In figure14m-o whole mount confocal images show the small round morphology of spermatids expressing GFP. In figure14o the whole mount image z-stacks were unstacked in 4 micron increments from the inward of the tubule outward. The spermatid-

like cells are farther toward the center of the tubule since the spermatid-like cells are not seen until 16 microns deep into the tubule.

Mature sperm expressing GFP are seen in the 30-day time point tubules. Mature sperm can be identified by their long and spindly tail morphology. In figure14q and 14r mature sperm with tails are seen as long linear organelles that branch directly from the spermatid-like cells.

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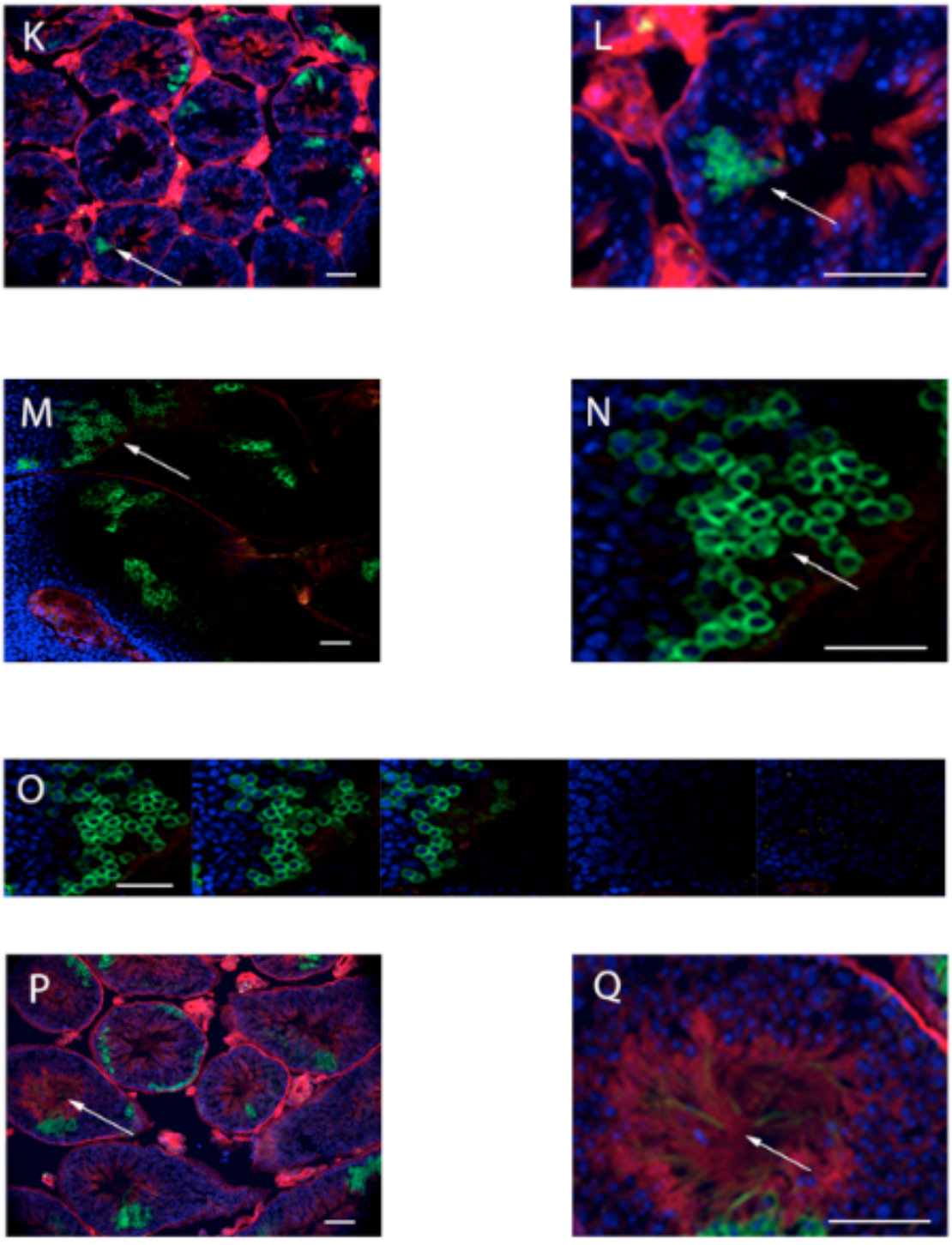
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Figure 14: GFP expressing cells at the 30-day time point in adult Oct4CreER mTmG mouse testes, A and B) Cross section showing A_{al} spermatogonia in a chain of 16 labeled with GFP, C and D) whole mount confocal images showing A_{al} spermatogonia in a chain of 8 cells labeled with GFP, E) whole mount single Z-stacked images (same tubule as C and D) unstacked at 4 microns each in order from top of tubule down, F and G) Cross section images of spermatocyte-like spermatogonia in the mitotic stage of division, H and I) whole mount confocal images of spermatocyte-like spermatogonia labeled by GFP, J) whole mount single Z-stacked images (same tubule as H and I) un-stacked at 4 microns each in order from top of tubule down, K and L) cross section images of spermatid-like spermatogonia in the meiotic stage of development labeled by GFP, M and N) whole mount confocal images of spermatid-like spermatogonia labeled by GFP, O) whole mount single Z-stacked images (same tubule as M and N) unstacked at 4 microns each in order from top of tubule down. Scale bars 50 microns.

GFP expression patterns from cross sections of 30-day testes were quantified. In figure15 most GFP expression patterns seen in the 30-day tubules are spermatocyte-like clones (65%) followed by spermatid-like clones (25%). Only 9% of tubules showed the A_{al} position of GFP expressing cells.

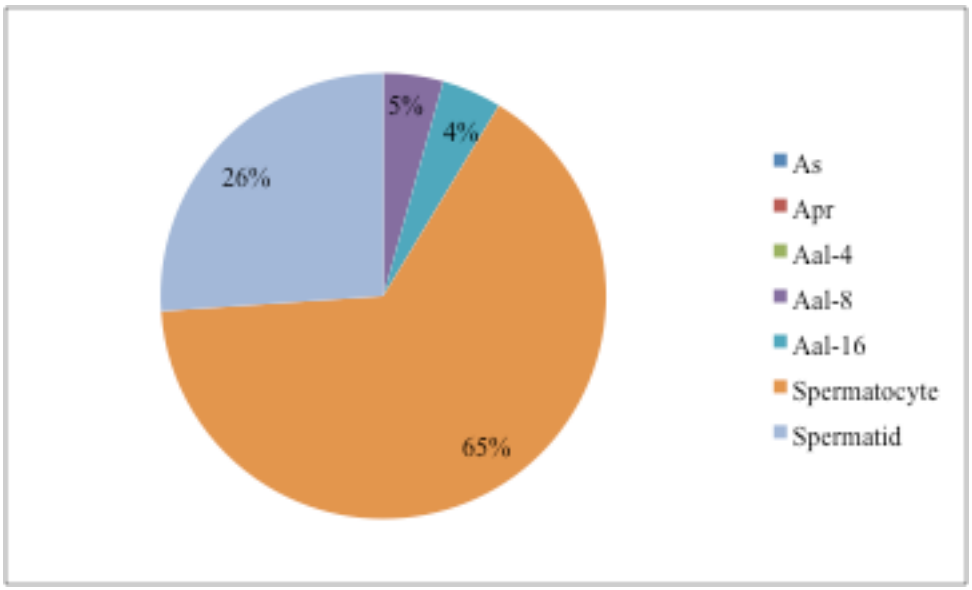
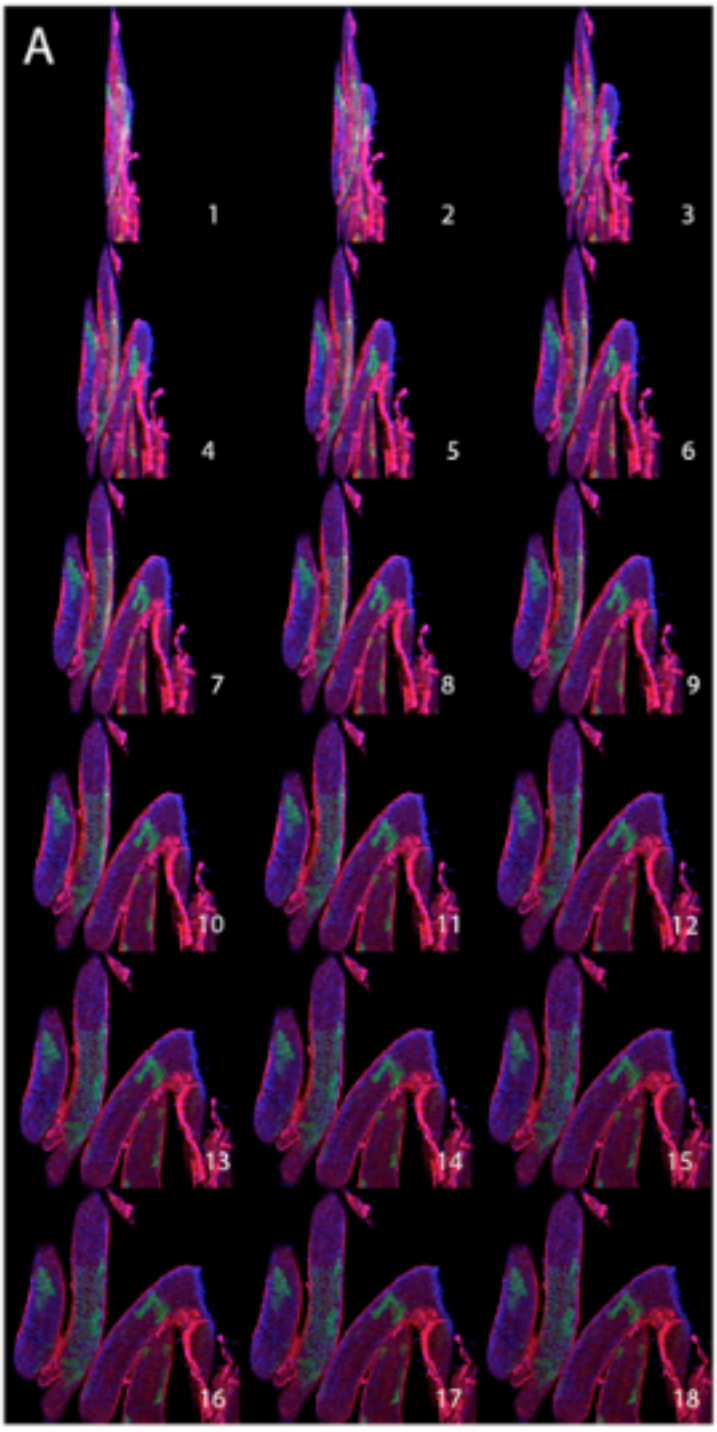


Figure 15: 30-day whole mount patterns quantified from 4750 microns of tubule from three different CreER transgenic mice. Confocal images of 4750 microns of seminiferous tubule whole mounted from the 30-day time point testes was imaged for GFP staining. The number of each pattern seen in 4750 microns of tubule were evaluated and quantified as a percent for each pattern type.

One whole mount tubule was chosen for further analysis. In figure 16a-c this tubule shows GFP labeled A_{al}, spermatocyte-like clones, and spermatid-like clone sheets. In a 3-dimensional rotation of the tubule placement of the GFP cell patterns can be seen (Fig.16a and c). The spermatid-like GFP expressing cells have formed a sheet and are present throughout the tubule whereas the spermatocyte-like cells clustered near the spermatid sheet are seen closer to the surface of the tubule. The 3-dimensional rotation in figure 16 shows that the spermatocyte-like cells are only a few cell layers deep in the tubule, whereas the spermatid-like cells are positioned throughout the center of the tubule creating a sheet. This sheet appearance could be attributed to multiple spermatocyte clones differentiating at the same time to create a sheet-like appearance of spermatid-like cells.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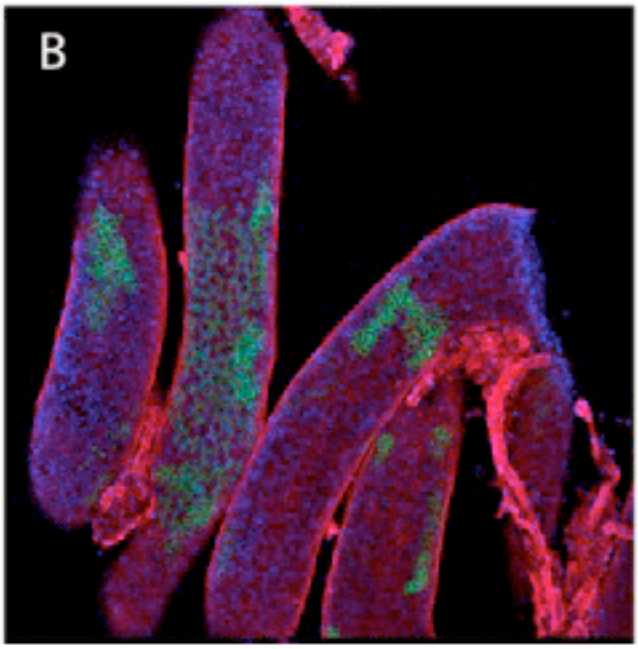
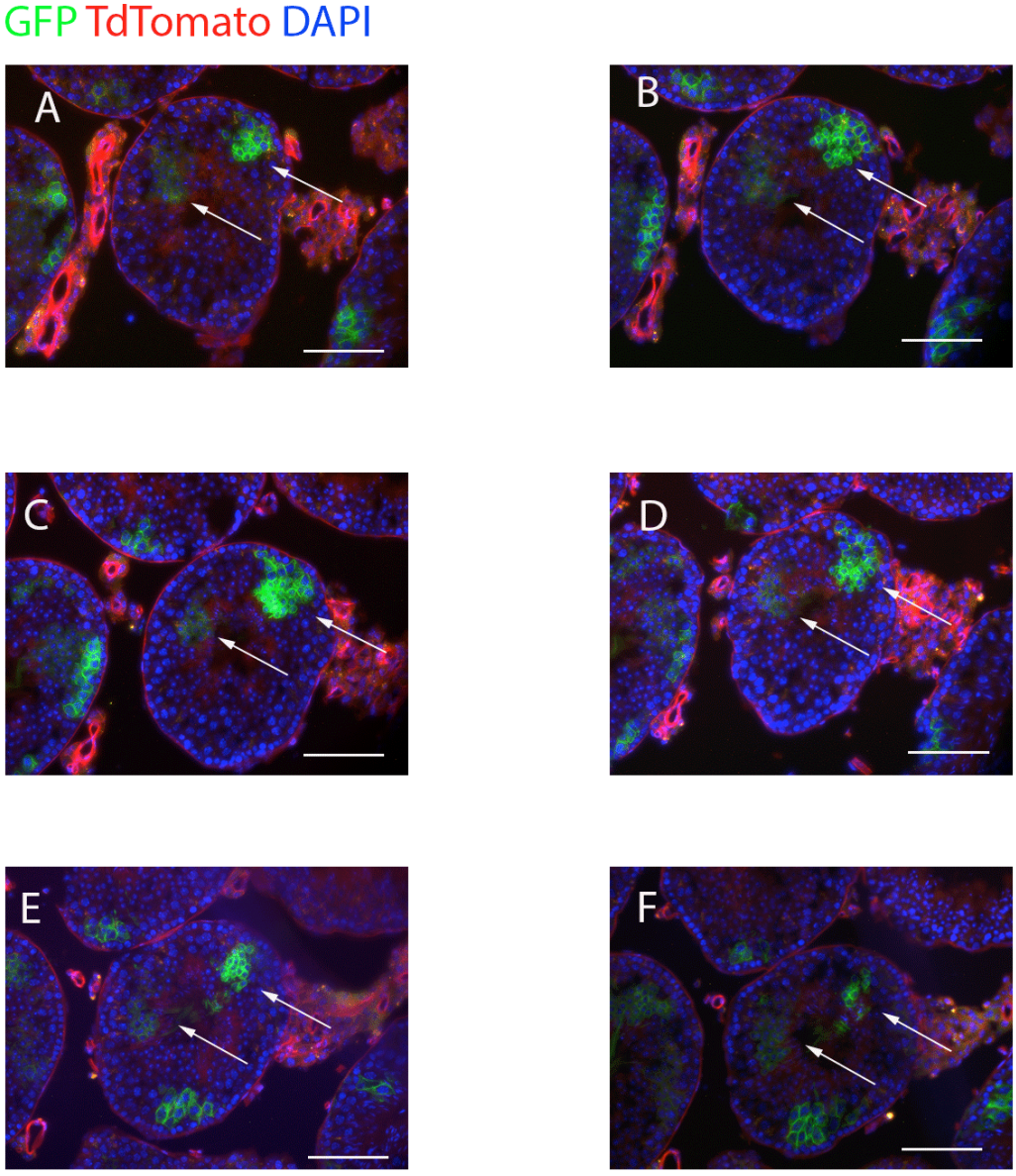


Figure 16: Confocal Z-stacked whole mount image through 20 microns of tubule of representative 30-day tubules A) 3D animation turning of tubule, B) representative confocal image.

To further investigate the depth of GFP expressing spermatogonial patterns serial sections of the 30 day time point tubules were analyzed (Fig.17a-l). Looking through 120 microns of tubule a spermatid-like clone near the center of the tubule arises (Fig.17a) and gives rise to mature sperm at 100 microns of depth (Fig.17k-l). In figure17a-e the spermatocyte-like clone has many cells that cover from the tubule membrane to toward the center of the tubule. In figure17f the spermatocyte-like clone is seen as 3 smaller clusters.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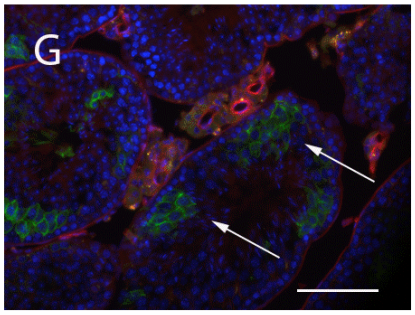
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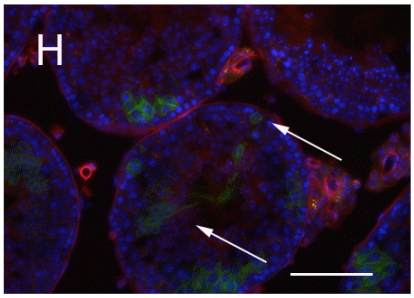
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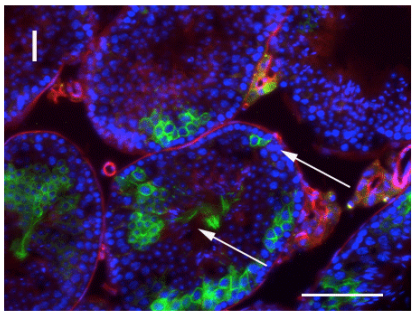
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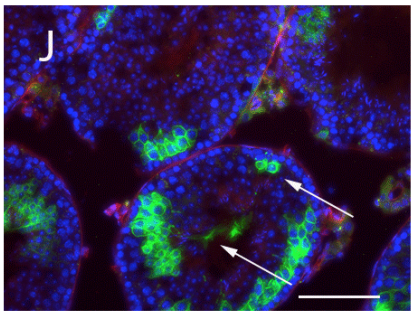
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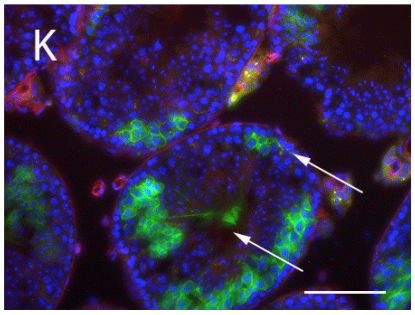
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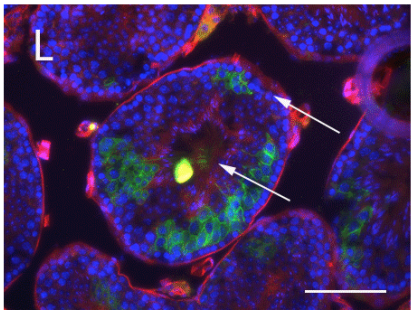
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Figure 17: GFP expression in a 30-day tubule analyzed in serial cross sections at 10microns each covering 120 microns of tubule, A) section 1, 10 microns B) section 2, 10 microns , C) section 3, 10 microns, D) section 4, 10 microns, E) section 5, 10 microns, F) section 6, 10 microns, G) section 7, 10 microns, H) section 8, 10 microns, I) section 9, 10 microns, J) section 10, 10 microns, K) section 11, 10 microns, L) section 12,10 microns . Arrows point to patterns of interest, mature sperm and a spermatocyte-like clone. Scale bars 50microns.

Comparison of GFP expression patterns seen at different time points following Tamoxifen administration

The number of GFP expressing cells was analyzed at for each time point following tamoxifen administration (Fig.18).

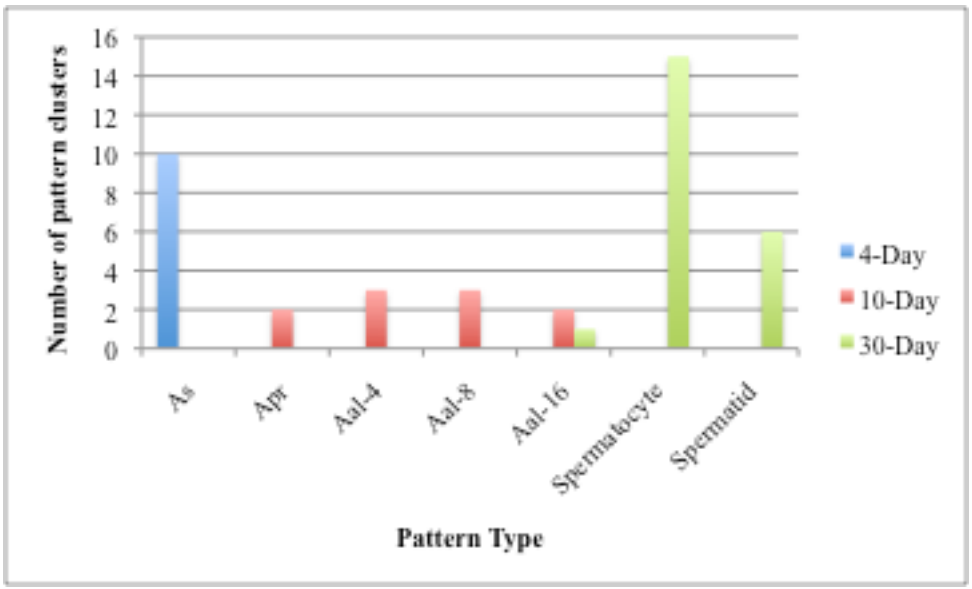


Figure 18: Whole mount adult mouse testes pattern analysis (100 tubules looked at for quantification of each time point).

The 4-day time point showed that on average only one in 30 cells per tubule were positive for GFP (Fig.19). At the 10-day time point GFP positive cells label about 2 cells per tubule on average (Fig.19). The 30-day time point labeled 17.07 cells positive for GFP per tubule on average.

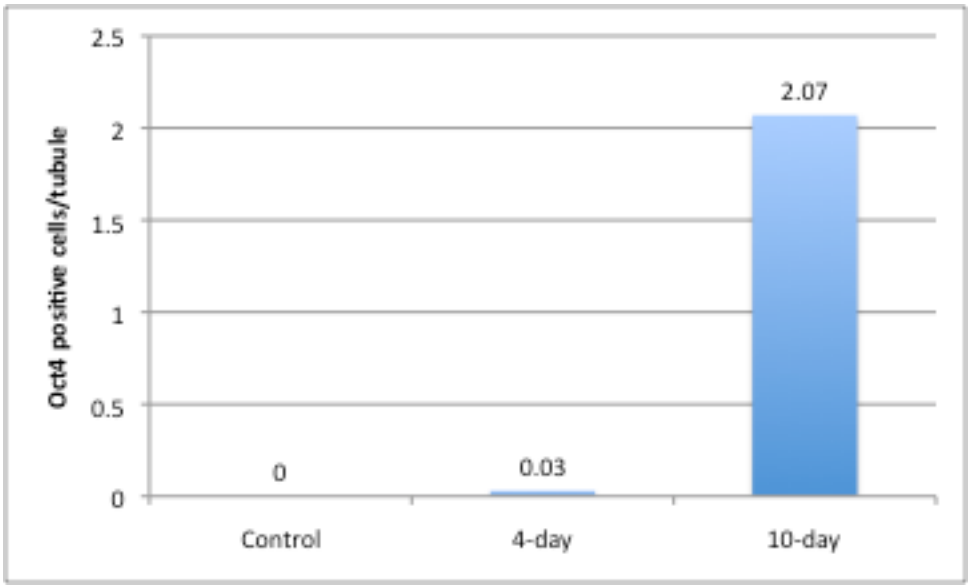


Figure 19: Quantitative comparison of Oct4 positive cells per tubule between control, 4-day time point, and 10-day time point mice at 2 months of age.

Control Testes

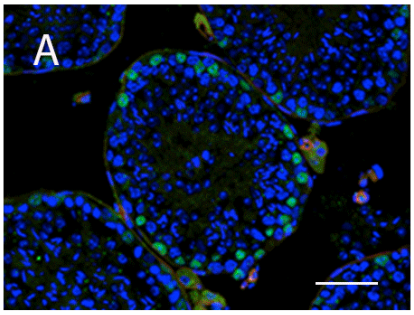
Three testicles from non-transgenic littermates from the Oct4CreER were harvested as controls. No GFP expression was seen in any of the cross section or whole mount testes observed.

Staining of SSC niche and progeny

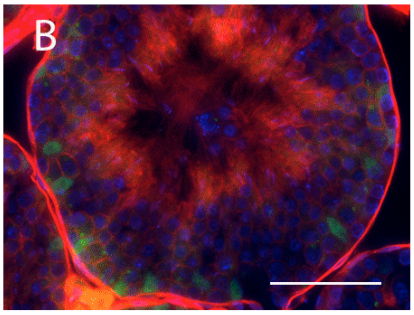
Immunohistochemistry for a proposed SSC niche marker, WT1, was conducted on non transgenic mTmG reporter mice. WT1 is expressed in Sertoli cells, an important cell type in the SSC niche closely associated with the SSC as a support cell. In figure20a and 20b WT1 positive Sertoli cells are seen around the perimeter of the tubule

membrane. Spermatogonial markers for mitotic and meiotic spermatogonia were also investigated. DMRT1 labels cells in the mitotic stage of spermatogonial development. The position of the DMRT1 expressing cells is around the tubule membrane, indicating that the A_{al} cells may be in the mitotic stage of development (Fig.20c and d). SYCP3 is a marker used to identify cells in the meiotic stage of development, when cells begin to differentiate and chromatin is tightly compacted. In figure 20e and 20f cells in the meiotic stage of development are seen. Tightly compacted chromatin can be seen indicating that these cells are beginning to differentiate (Fig.20f). The spermatocyte-like and spermatid-like spermatogonia express SYCP3 because they are dividing and migrating towards the center of the tubule. ID4 staining was attempted using the same primary antibody used by Oatley *et al* in 2011. However, no staining was observed under florescent microscopy.

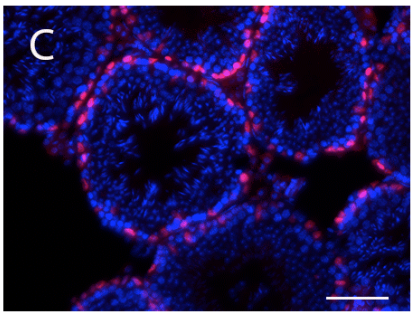
WT1 TdTomato DAPI



WT1 TdTomato DAPI



DMRT1 DAPI



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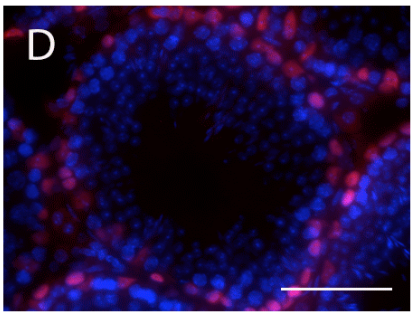
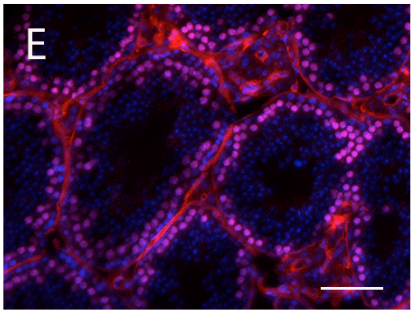


SYCP3 DAPI



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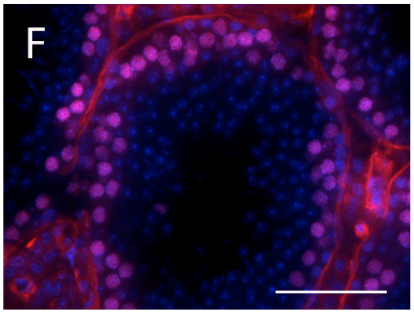

Figure 20: Control staining of niche and spermatogonial markers in the TdTomato reporter mouse A) WT1 immunohistochemistry 20X magnification, B)WT1 immunohistochemistry 40X magnification, C)DMRT1 immunohistochemistry 20X magnification, D) DMRT1 immunohistochemistry 40X magnification, E) SYCP3 immunohistochemistry 20X magnification, F) SYCP3 immunohistochemistry 40X magnification. Scale bars 50microns.

Summary

- GFP expressing cells are seen in the testes following tamoxifen administration in the Oct4:Cre^{ER} mTmG transgenic mice.
- 1 day after the tamoxifen administration only rare single cells in the basal lamina position of the tubule express GFP.
- 7 days after the last tamoxifen administration GFP expression is seen in the progeny of the Oct4 expressing cells in early mitotic cell division patterns.
- 27-days after the last tamoxifen administration A_{al}, spermatocyte-like clones, spermatid-like clones, and mature sperm were seen to express GFP.
- Because GFP is only seen in rare single cells in the 4-day time point the cells seen to express GFP in the longer chase experiments are likely the progeny of these single cells.

Discussion:

The spermatogonial stem cell population in adult mouse testes has been difficult to identify unambiguously. Here the Oct4:Cre^{ER} mTmG mouse was used to analyze the expression of Oct4 in the adult mouse testes. The 4-day time point showed only single cells flattened along the basal lamina of the tubule expressing GFP. This cell position and morphology is characteristic of the proposed spermatogonial stem cell population. The labeling of single spermatogonia in this position after the one day chase indicates that the Oct4:Cre^{ER} is not expressed in any other cell type in the adult mouse testes. This is in contrast to the both the previous Ngn3 transgenic mouse model (Yoshida et al; 2004) and the CDH1/E-cadherin labeling (Tolkunova *et al,* 2009 and Tokuda *et al,* 2007). The basal lamina position of the GFP expressing cells in the 4-day time point is also the position of Oct4 expressing cells in the testes shown by Pesce *et al* in 1998. These results are consistent with the identity of the GFP expressing cells in the 4-day time point being the Oct4 expressing SSCs in the adult testes.

The 4-day time point testes showed a very small proportion of cells that were labeled with GFP, about 1 cell in every 30 tubules, which would be 75,000 cells in 2.5meters of tubule. This rare Oct4 expression is also consistent with the hypothesized low number of cells in the SSC population. The SSC population is postulated to be a rare population of cells ranging from 100 to over 1 million SSCs per testes (Wang *et al,* 2013). Our number of Oct4 GFP expressing cells is much lower than the number of cells expressing ID4 seen by Ferguson et al in 2013 but, may be more in line with the number of ID4 expressing cells reported by Oatley et al 2011. It will be interesting to determine the number of ID4 positive cells that co-express Oct4 GFP at the 4-day time point in the Oct4:Cre^{ER} mTmG mice. To date we have not been able to co label ID4 and GFP in 4 day time point testes.

In the 10-day time point testes of the Oct4CreER adult mice A_{pr} and A_{al} cells expressing GFP were seen. The GFP positive cells in these testes had between 7 and 10 days to divide following tamoxifen administration. During the 10-day time point GFP expressing cells presumably arise as progeny of the single cells seen to express GFP at the 4-day time point. Paired cells, such as seen in figure12, could be the result of the

single cell dividing to give rise to either two new SSC single cells or one SSC and one progenitor. Further experiments will be conducted to determine if asymmetric or symmetric division has occurred. Where chains of GFP labeled undifferentiated aligned spermatogonia are seen, the single cells labeled one day post tamoxifen administration must have continued to divide to give rise to chains of 4, 8 and 16 cells. No chains of cells were seen expressing GFP at the 4-day time point, so chains of cells seen later must be the progeny of the single GFP expressing cells.

In the 30-day time point adult testes GFP labeling is seen in proliferating A_{al} patterns, spermatocytes, spermatids, and mature sperm. The GFP expressing A_{al} cells are seen next to the tubule membrane, which suggests that a single cell expressing GFP was activated and created two progenitor cells that then each divided three more times to give rise to a 16 cell linear clone. It is possible that the A_{al} cells that were derived from a single cell expressing Oct4 may break up, divide and move toward the middle of the tubule to become spermatocytes. The spermatocytes differentiate further into small round spermatids during meiosis. In cross section analysis of the 30-day time point mature sperm were seen as elongated GFP expressing cells that have completed the differentiation pathway from SSC to mature sperm in 27-30 days. The 30-day time point of Oct4:Cre^{ER} mTmG mice indicated that the single cells originally expressing GFP on day1-3 of tamoxifen injection have differentiated to give rise to all stages of spermatogenesis, including mature sperm. Throughout the 30-day time point tubules GFP expressing cells are observed at many different stages of spermatogenesis, from A_{al} to mature sperm. This indicates that the progeny of cells labeled at the 4-day time point have progressed through spermatogenesis at different rates. 27 days after the last tamoxifen administration some of the progeny of cells labeled on day 3 are still mitotic undifferentiated spermatogonia, whilst other progeny have reached the elongated mature sperm stage. These results are consistent with the Oct4 expressing cells labeled during the 4-day time point being spermatogonial stem cells.

If Oct4 expressing cells after 1-3 days of Tamoxifen administration are truly labeling just the SSC population then the Oct4CreER mouse system will be a useful tool in further investigations into how the SSC divides. To understand if the GFP expressing

cells in the 4-day time point are true stem cells further characterization will be done. For proof of principle that the 4-day time point Oct4:Cre^{ER} GFP labeled cells are the SSC they will be isolated and used for transplantation into aspermatogenic mouse testes for repopulation. The SSC population should regenerate spermatogenesis when transplanted into aspermatogenic testes.

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