University of Nevada, Reno

Gata4 is required for Testicular Development

A thesis submitted in partial fulfillment of the

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by

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Abstract

Sex differentiation occurs at embryonic day 10.5 (E10.5) in the mouse. In the XY mouse embryos, appearance of testis cords at E11.5 marks the onset of testis formation. The primary testis cords subsequently undergo further development characterized by rapid and extensive branching, elongation and coiling, resulting in hundreds of thousands of secondary or tertiary testis cords, which will develop into future seminiferous tubules during postnatal testicular development. Here we report that inactivation of *Gata4* at E12.5 in Sertoli cells does not affect testis cord formation, but blocks its further development leading to testicular dysgenesis and male infertility in adult mice. Our data demonstrate that *Gata4* is required for normal testis cord branching, elongation during embryonic development, and untimely the normal number of seminiferous tubule precursors available for the postnatal testicular development. Disruption of *Gata4* or its target genes involved in testis cord development will significantly affect the number of future seminiferous tubules, the size of the testis and fertility.

Keywords: testis cord, Sertoli cells, transcription factor, testis formation, fertility, seminiferous tubule, embryonic development, sex determination

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Chapter 1

Introduction

1. 1 GATA transcription factor family

The GATA-binding proteins are a family of zinc finger transcription factors that regulate gene expression, differentiation, and cell proliferation by binding to the consensus DNA sequence (A/T) GATA (A/G). In mammals, six GATA factors (GATA1 to GATA6) have been identified so far. All these GATA factors have two highly conserved zinc finger domains, C-X2-C-X17-C-X2-C, which are designated as N-terminal and C-terminal fingers, with different sequence outside these domains (Lowry et al., 2000; Molkentin et al., 2000). Four factors, GATA-1 (Yomogida K et al., 1994), GATA-2 (Siggers et al., 2002), GATA-4 (Arceci et al., 1993; Heikinheimo et al., 1997), and GATA-6 (Narita et al., 1996; Morrisey et al., 1996), have been shown to be expressed in the murine gonad and are thus of interest as potential regulators of testicular development and function.

1. 2 *Gata4* expression in developing and adult mice reproductive system

Gata4 is one of the six GATA transcription factors, which have been shown to be widely expressed in multiple organs and play important roles in the regulation and cell lineage-specific gene expression during mammalian development. At E11.5, *Gata4* is expressed in somatic cells of bilateral genital ridges (Viger et al., 1998). After E13.5, *Gata4* expression becomes sexually dimorphic. In XY gonads, *Gata4* expression is predominantly in somatic cells within the testis (Sertoli cells, Leydig cells and other

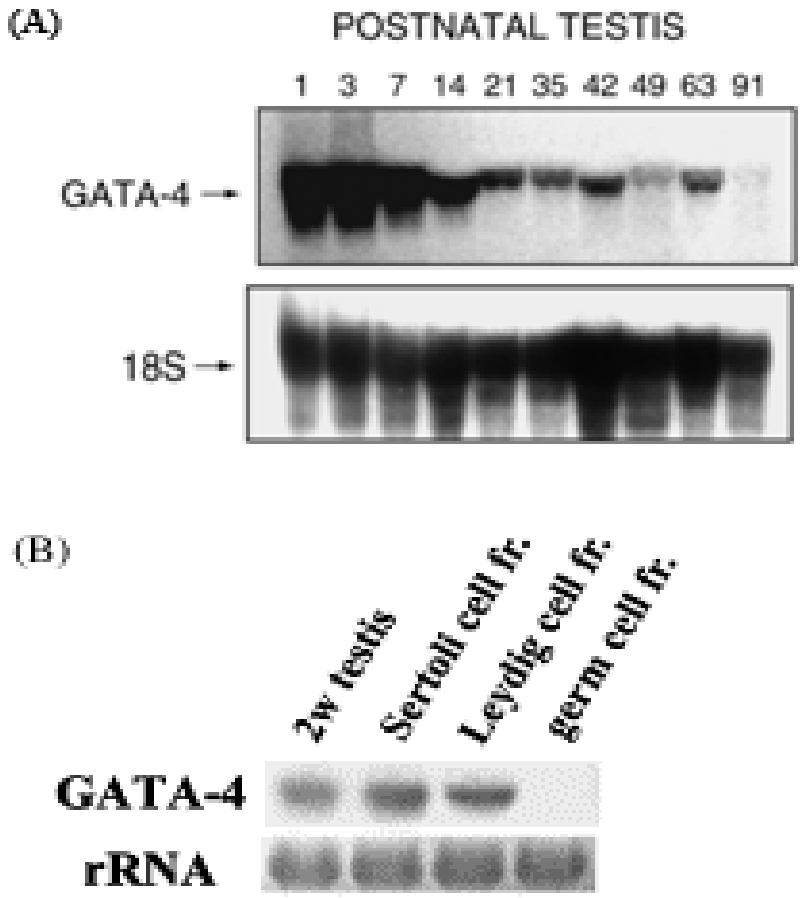

Figure. 1. *Gata4* expression in the testis. (A) *Gata4* gene expression during postnatal testicular development as determined by northern blot hybridization. The blots were rehybridized with an oligonucleotide probe specific for the 18S ribosomal RNA in order to verify the quantity and integrity of the RNA used (Robert et al., 1998). (B) Expression of mRNA in Sertoli, Leydig and germ cell fractions from the testes of 2-week-old wild mice. rRNA was working as the control (Imai et al., 2004).

interstitial cells) (Fig. 1 B), and its expression remains constantly abundant in both the primordial gonad (E11.5) and the adult testis (Fig. 1 A) (Imai et al., 2004; Viger et al., 1998). In both fetal and adult female mice, *Gata4* is expressed in granulosa cells and also be a key transcriptional regulator of the ovarian somatic cell function (Kyrönlahti et al.,

2011). The expression of *Gata4* transcripts has been shown to decrease in Figure 1A during normal postnatal development, which is probably due to the increasing proportion of germ cell during postnatal testicular development.

1. 3 *Gata4* function in the testicular development based upon *Gata4* knock-out and knock-in studies

Neither the global knockout nor the knock-in lines can be used to comprehensively analyze the role of *Gata4* in gonadal development due to embryonic lethality. A global homozygous deficiency of *Gata4* leads to $Gata4^{-/-}$ embryonic death between 7.5 and 9.5 dpc because of a defect in cardiac morphogenesis, which precludes the analyses of the *Gata4* function during gonadal development and spermatogenesis (Kuo et al., 1997; Molkentin et al., 1997). Another knock-in mouse line ($Gata4^{ki/ki}$) expresses a mutant form of *Gata4* that cannot bind *Fog2*. Its homozygous embryos cannot survive beyond E13.5 (Crispino et al., 2001). However, analyses of $Gata4^{ki/ki}$ primordial gonads revealed a lack of testis cord formation, suggesting that interactions between *Gata4* and *Fog2* are essential for the formation of the testis cord.

1. 4 Origin of the testis

In mice, gonadal development starts at embryonic days 9.5-10.5 (E9.5-E10.5) with the thickening of surface epithelium and underlying mesenchyme of the mesonephros followed by formation of the primordial gonad indistinguishable between the male and

the female. The formation of primordial gonad coincides with the arrival of extra-embryonically derived primordial germ cells (PGCs). Activation of the *Sry* gene induces the program leading to the formation of the testis (Gubbay et al., 1990; Sinclair et al., 1990). Otherwise, the gonad will develop into the ovary (Koopman et al., 1991).

The hallmark of testis formation is the differentiation of surface epithelial cell-derived supporting cells into Sertoli cells, which form the testis cord by enclosing the primordial germ cells and excluding other somatic cells types (e.g. mesenchymal cells-derived endothelial and interstitial cells). *Sry* gene expression is believed to be the trigger of this differentiation (Capel review, 1998). *Sry* is expressed between E10.5 and E12.5 in supporting epithelial cells and later in Sertoli cells (Koopman et al., 1990). *Sox9* (Sekido et al., 2004) and *Gata4* (Viger et al., 1998) have been identified as the downstream genes of *Sry* expression. As a result of the activation of the male pathway of gonadal development, Sertoli cell polarity and the Sertoli-Sertoli cell interactions occurred (Kanai et al., 1991), the testis cord is formed at E11.5 followed by rapid and extensive branching, elongation, and coiling between E12.5 and birth, during which ~10-12 primary testis cords are transformed into hundreds and thousands of secondary or tertiary testis cords, representing precursors for the seminiferous tubules in the postnatal testes (Nel-Themaat et al., 2009). Soon after the testis cord formation (E11.5), some Sertoli cells migrate to the periphery of the cords and become polarized such that their nuclei are adjacent to the basal membrane and their cytoplasm extending to the interior of the cord and enveloping germ cells (Gassei et al., 2008). This specific cellular arrangement between Sertoli cells and germ cells is established at ~E13.5 and is maintained as such in the postnatal testis. Despite that many Sertoli cells become basally

located in the testis cords starting at E13.5, there are still numerous Sertoli cells remaining in the center of the cords. It is likely that these Sertoli cells are subsequently become polarized and basally localized as well once they are partitioned into newly formed testis cords through active branching. In the newborn mouse testis, hundreds and thousands of testis cords with both Sertoli cells and PGCs located on the basal membrane represent the precursors of the future seminiferous tubules in the adult testis. It appears that the final number of seminiferous tubule precursors is established during embryonic testis development and the postnatal development represents mainly the further growth of the already established testis cords/seminiferous tubules during embryonic development. Therefore, early embryonic development determines the number of final seminiferous tubules and thus the size of the adult testis.

1. 5 The Cre-loxP system in the study of gene function in a cell-specific manner

Gene-targeted mice, derived from embryonic stem cells, are useful tools to study gene function during development. However, if the inactivation of the target gene results in embryonic lethality, the post-developmental function of the gene cannot be further studied.

Lox P (locus of X-over P1) sequence consists of 34bp with an asymmetric 8 bp sequence in between with two sets of 13 bp sequences flanking it.

13bp 8bp 13bp

ATAACTTCGTATA - GCATACAT -TATACGAAGTTAT

Cre is a 38 kDa recombinase protein, which cause the site-specific recombination

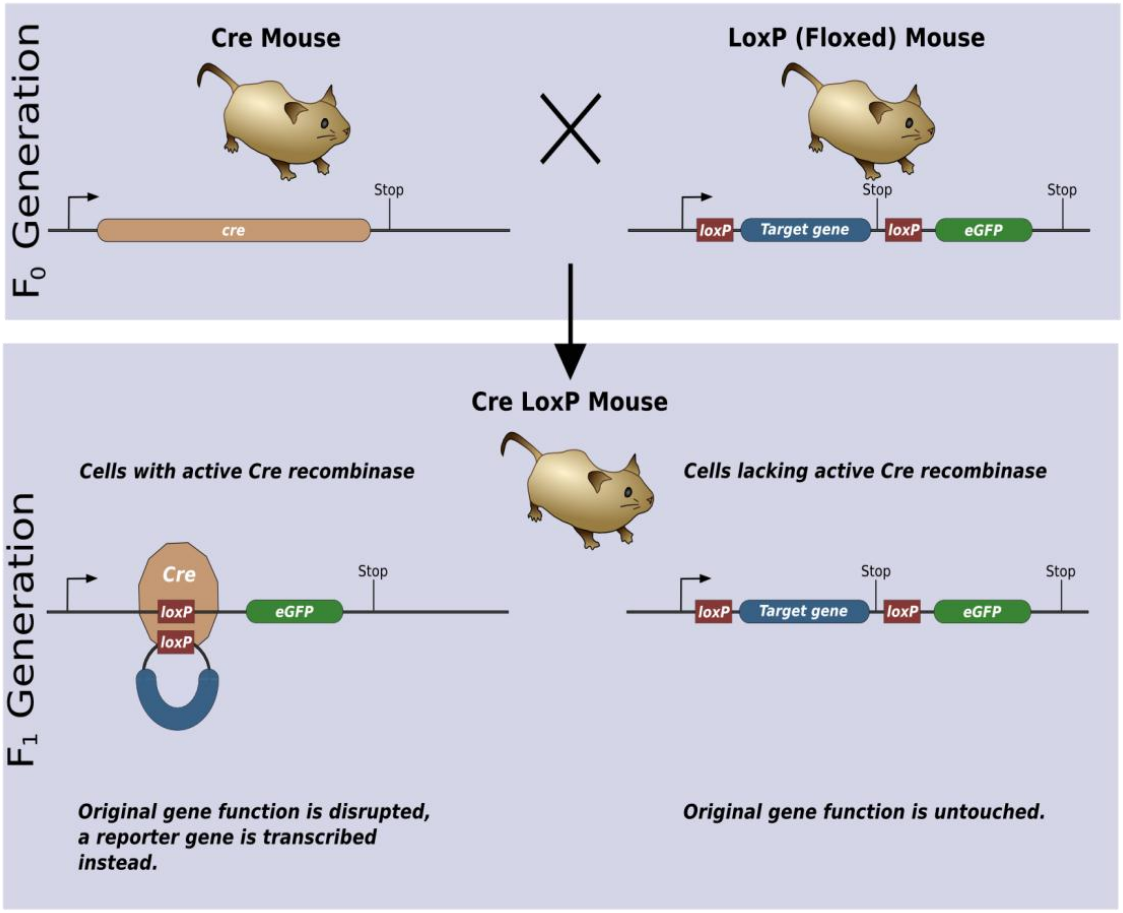

Figure. 2. **Cre-loxP system.** Cre recombinase-mediated recombination between two 34 bp loxP sites and then excise the target gene.

From: http://upload.wikimedia.org/wikipedia/commons/5/58/CreLoxP_experiment.png

between loxP sites. The Cre recombinase loxP (Cre-loxP) (Sternberg et al., 1981; Sauer et al., 1988) system was a site-specific recombination, which was developed to overcome the limitation of embryonic lethality as well as to confine the inactivation of any gene to a specific tissue or cell type at any stage of development in vivo, thereby allowing the analysis of physiological and pathophysiological consequences of the genetic alteration in mature animals.

1. 6 *mT/mG* double fluorescent Cre recombinase reporter mouse

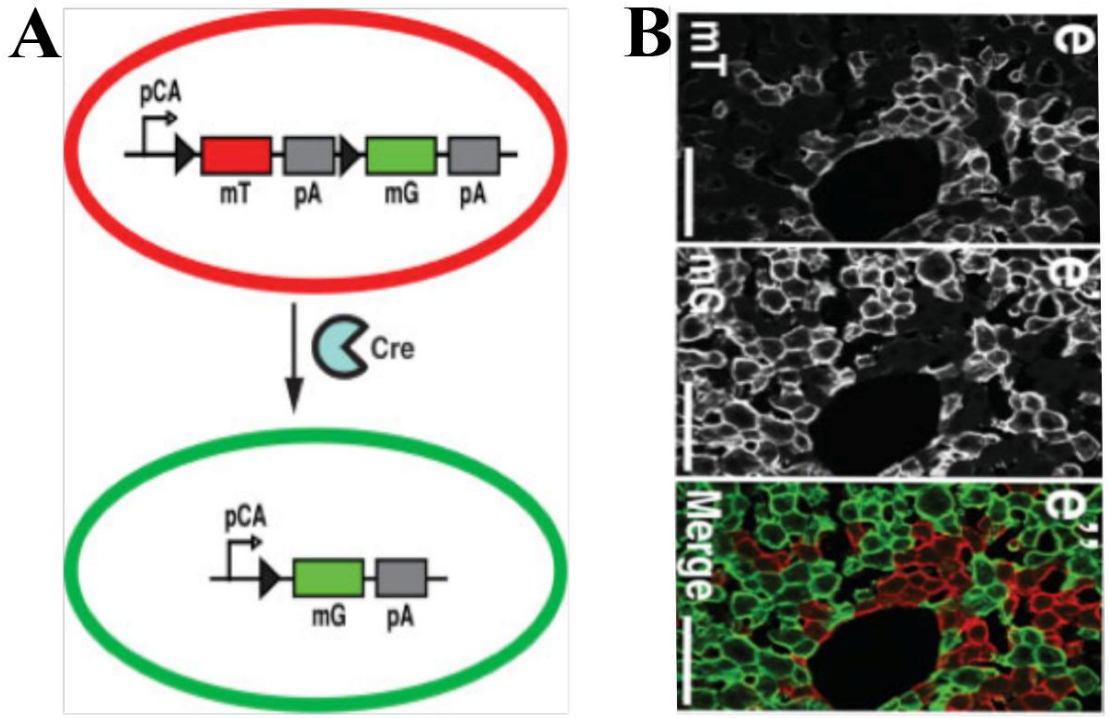

Figure. 3. *mT/mG* double fluorescent Cre recombinase repoter mouse. (A) Schematic diagram of the *mT/mG* construct before and after Cre-mediated recombination. (B) Complementary fluorescent membrane labeling at single cell resolution in the liver (Muzumdar et al., 2007)

In the Cre recombinase-loxP system, the activity of Cre plays an important role. To assess the range of tissues in which the Cre is active, many Cre reporter transgenic lines has been designed with a marker gene (e.g. *LacZ, GFP, CFP, or YFP*), which will be active following Cre-mediated recombination (Mao et al., 2001; Soriano et al., 1999; Srinivas et al., 2001). Later, several double fluorescent reporter transgenic lines that express an extra marker in non-recombined cells were generated (Novak et al., 2000; Baird et al., 2000). However, there were several problems for these double reporter lines. Its double reporters have been difficult to obtain together or the homozygous mice have

not been generated successfully because of the toxicity (Baird et al., 2000). In the present study, we use a global double fluorescent Cre recombinase reporter mouse line, *mT/mG*, which utilizes a strong, ubiquitous promoter (pCA) to drive mT/mG coupled with knock-in at the well-characterized ROSA26 locus with high levels of expression in all cells(Fig. 3 A) (Niwa et al., 1991). *mT/mG* expresses membrane-targeted tdTomato ('mT') prior to Cre excision and membrane-targeted EGFP ('mG') following Cre excision (Muzumdar et al., 2007). Therefore, the mG labeling is Cre-dependent and complementary to mT (Fig. 3 B). Also, heterozygous and homozygous *mT/mG* mice are fully viable and fertile.

Chapter 2

Generation of Sertoli cell-specific *Gata4* knockout mice with reporter capability

2. 1 Design of *Gata4*-Loxp and *Amh-Cre* mice

A *Gata4*-loxP conditional allele has been generated (Watt et al., 2004) and the loxP sites flank exons 3- 5 of *Gata4*, which encode both zinc finger DNA-binding domains and the nuclear localization signal essential for *Gata4* function (Fig. 4 A). Several cell-specific *Gata4* knockout lines using this *Gata4*-loxP allele have proven that after Cre-mediated recombination, *Gata4*-loxP allele is converted into a null allele, leading inactivation of *Gata4* gene (Watt et al., 2004, Kyrönlahti et al., 2011, Battle et al., 2008).

The Müllerian inhibitor currently known as Müllerian-inhibiting substance (MIS) or anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) family of growth and differentiation factors. AMH is a dimeric glycoprotein that is expressed specifically in the Sertoli cells of fetal and adult testis and granulose cells of the ovary after birth (Behringer review, 1995). The AMH-Cre transgenic mice express the Cre recombinase under the control of the anti-Müllerian hormone (AMH) gene promoter (Fig. 4 B). In the male, the Cre-mediated recombination only occurs in Sertoli cells in the testis (Lécureuil et al., 2002).

2. 2 Generation of Sertoli cell-specific *Gata4* knockout mouse line

To overcome the embryo lethality, we generated a Sertoli cell-specific *Gata4* knockout mouse line (*Amh-Cre; Gata4*$^{lox/lox}$, called cKO herein) by crossing a Sertoli

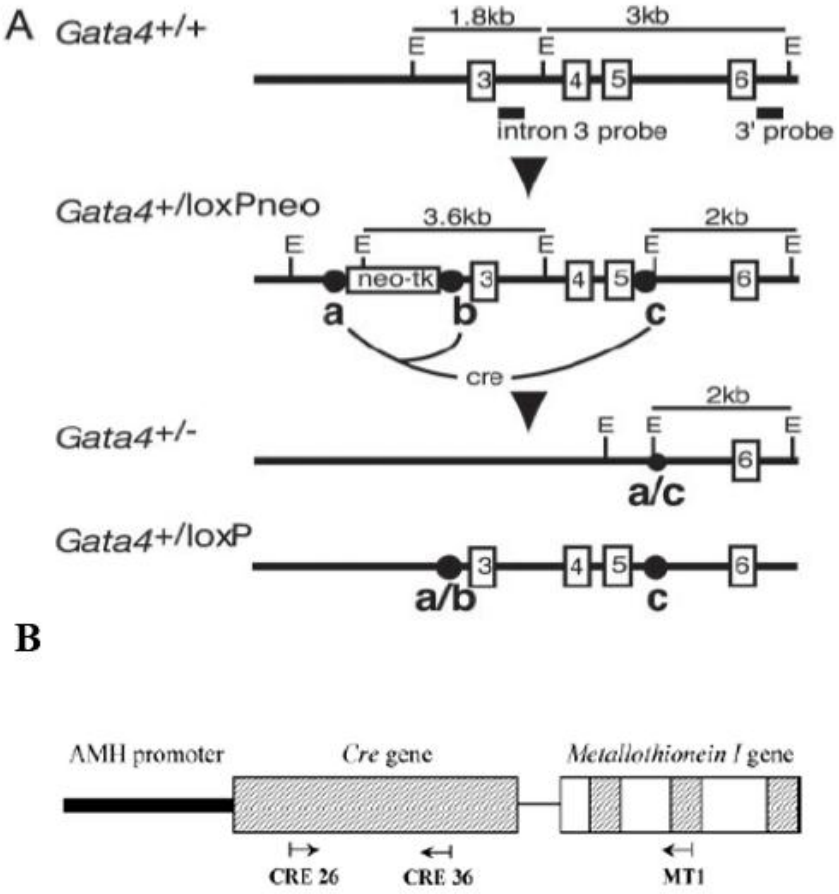

Figure. 4. Design of *Gata4*-Loxp and *Amh-Cre* mice. (A) Schematic of a two-step Cre-loxP strategy to delete exons 3–5 of the Gata4 gene in ES cells. The targeting vector was designed to place a loxP-flanked thymidine kinase/neomycin phosphotransferase expression (tk/neo) cassette upstream of exon 3, and a third loxP site just downstream of exon 5 of the targeted gene (Watt et al., 2004). (B) Gonad-specific expression of Cre recombinase mRNA in AMH-*Cre* transgenic mice. The black bar figures 3.6 kbp of the human AMH promoter. The *Cre* and *Metallothionein I* gene, derived from pBS185 plasmid (thin line), are denoted by dashed (exons) or by white (introns) boxes (Lécureuil et al., 2002).

cell-specific Cre line (AMH-Cre) (Lécureuil et al., 2002) with *Gata4*-loxP mouse line (Watt et al., 2004). The *Gata4*-loxP and AMH-Cre mice we used were both from the Jackson Laboratory (*Gata4*$^{tm1.1Sad/J}$), and the breeding scheme used is shown in Figure 5.

Mice homozygous for this *Gata4*-loxP (*Gata4*$^{lox/lox}$) conditional allele, both male and female, were viable and fertile. We mated transgenic AMH-Cre female mice with male mice carrying two loxp *Gata4* alleles in order to generate 50% *Gata4*$^{+/lox}$; *Amh-Cre* and 50% *Gata4* $^{+/lox}$ mice. These animals were then intercrossed to produce *Amh-Cre*; *Gata4* $^{lox/lox}$ as well as control littermates, namely *Gata4* $^{lox/lox}$ and *Amh-Cre; Gata4* $^{+/lox}$ mice.

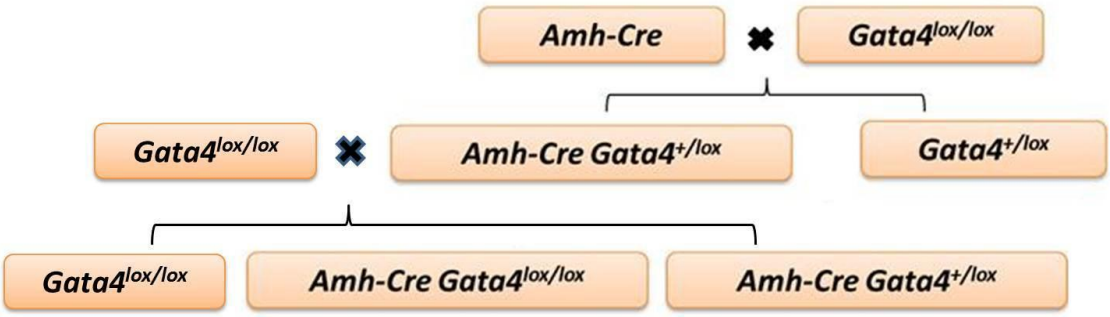

Figure. 5. Generation of *Amh-Cre*; *Gata4* $^{lox/lox}$ mice.

2. 3 Generation of a reporter line with all Sertoli cells deficient of *Gata4* expressing EGFP

We introduced a ROSA26mTmG allele into the cKO background so that those Cre-expressing cells can be monitored based upon the membrane-targeted EGFP. The *mT/mG* homozygous mouse line used was purchased from the Jackson Laboratory, and both male and female were viable and fertile. All tissues and cell types expressed strong red fluorescence prior to Cre-mediated excision and the mG expression was Cre-dependent and complementary to mT. To achieve the selective mG expression together with the inactivation of *Gata4* in Sertoli cells, we mated mice carrying two loxp *Gata4* alleles

with *mT/mG* homozygous mice to generate *Gata4* $^{lox/lox}$; *mT/mG* $^{tg/tg}$ mice. We then mated male *Gata4* $^{lox/lox}$; *mT/mG* $^{tg/tg}$ mice with female *Amh-Cre; Gata4* $^{lox/lox}$ mice to get the *Amh-Cre; Gata4* $^{lox/lox}$*; mT/mG* $^{+/tg}$ mice (Fig. 6).

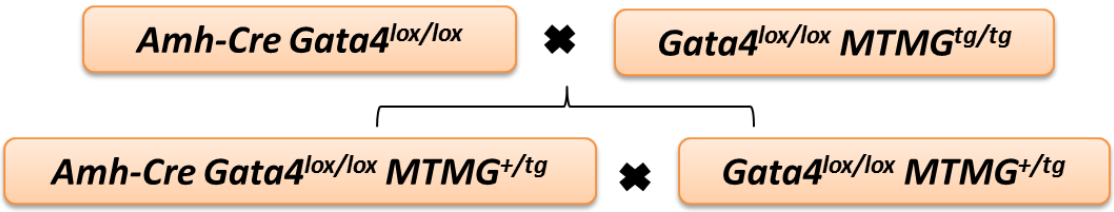

Figure. 6. Generation of *Amh-Cre; Gata4* $^{loxP/loxP}$*; mT/mG* $^{+/tg}$ mice.

2. 4 The control for *mT/mG Gata4* conditional knock-out mice

Because the heterozygous and homozygous *mT/mG* mice are fully viable and fertile, we mated *Amh-Cre* mice with *mT/mG* $^{tg/tg}$ mice in order to generate the *Amh-Cre; mT/mG* $^{+/tg}$ mice as the control, which marked the normal Sertoli cells green. We detected these *mG* fluorescently labeled Sertoli cells by fluorescence-activated cell sorting (FACS) as the control of cKO, to analyze the molecular changes in purified Sertoli cells.

Chapter 3

Materials and Methods

3. 1 Fertility tests and sperm analysis

Amh-Cre; Gata4 $^{lox/lox}$ males (n=5) or control littermates (*Amh-Cre; Gata4* $^{+/lox}$, n=2 and *Gata4* $^{lox/lox}$, n=3) were each bred with two 6-week-old wild type C57BL/6J female mice for 6 months. The number of litters and pups/litter were recorded. Epididymal sperm count was performed with sperm extracted from the caudal epididymis and ductus deferens of adult male mice.

3. 2 Histology

Testes and epididymides of adult mice were dissected, fixed overnight in Bouin's fixative and embedded in paraffin for hematoxylin and eosin (H&E) and PAS staining. Five-µm sections were used. After deparaffinization and hydration, hemotoxalin (HHS32, Sigma) stained 6 minutes and eosin (314-630, Fisher) stained 1 minute. For PAS staining, the sections were immersed in 0.5% periodic acid solution (P-5463, Sigma) for 10 minutes and were subsequently treated in Schiff's reagent (3952016, Sigma) for 15–30 minutes. All procedures were performed at room temperature, and stained sections were observed under the Zeiss Axioscop 2 microscope and images were aquired using a digital camera and the Capture SE software (PixelLink).

3. 3 Apoptosis assays

Testes of adult mice were dissected, fixed overnight in Bouin's fixative and embedded into paraffin. Five-μm sections were used for the detection of apoptotic cells using the Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (S7101, Millipore). After deparaffinization and hydration, the sections were pretreated with 20 μg/mL of proteinase K for 15 min, and then treated with 3% hydrogen peroxide in PBS for 5 min followed by rinsing twice with PBS. Equilibration buffer was then applied on the sections for 10 min followed by incubation with working strength TdT enzyme at 37 °C for 1 h in a humidified chamber. After three times of washing with 1×PBS, room temperature anti-digoxygenin peroxidase conjugate was applied on the sections and the sections were then incubated in a humidified chamber for 30 minutes at room temperature. Wash the sections in 4 changes of PBS and then apply peroxidase substrate to develop color. Counterstaining was carried out with Mayer's hematoxylin (MHS32, Sigma). All the stained sections were observed under the Zeiss Axioscop 2 microscope and images were aquired using a digital camera and the Capture SE software (PixelLink).

3. 4 Immunofluorescent staining and proliferation assays

Testes for immunofluorescent staining were fixed 2-4 hours in 4% paraforlmaldehyde (PFA) at room temperature. The fixed testes were cryoprotected in serial sucrose solutions with increasing concentrations by mixing 5% sucrose in 1XPBS and 20% sucrose in 1XPBS in ratios of 2:1, 1:1 and 1:2, respectively. Then, the testes

were immersed in 20% sucrose in 1XPBS at 4◦C overnight followed by embedding into the mixture of Aqua-Mount (13800, Thermo Scientific) and 20% sucrose in 1XPBS (1:1). Ten-μm sections were used for fluorescent staining. The slides were blocked in buffer X (1% BSA in 1X PBS) with normal goat and fetal bovine sera at room temperature for 1 h in a humidity box. Two antibodies, anti-Wnt 1 (sc-192, Santa Cruz Biotechnology, 1:500) and anti-GCNA1 (1:20), a monoclonal IgM antibody against the mouse germ cell nuclear antigen kindly provided by G. Enders (The University of Kansas medical Center), were added onto the sections together and sections were incubated overnight at 4 ˚C. The slides were washed three times with 1×PBS and then incubated with AlexaFLuo488-conjugated goat-anti-rabbit secondary antibody (Molecular Probes, 1:1000) and AlexaFLuo594-conjugated goat-anti-mouse secondary antibody (Molecular Probes, 1:1000) for 1h. After three times of washing with 1×PBS, cover slips were applied using aqueous mounting medium containing DAPI. All images were obtained with a Zeiss Axioscop 2 microscope and processed using our in-house imaging software (VolumetryG7mv) (Bao et al., 2010).

3. 5 RNA extraction and cDNA synthesis

Total RNA was isolated from control and *Gata4* knockout E13, E17, Newborn, P8 and adult mice testes, using TRIZOL (15596-018, Invitrogen). RNA quality and concentration were determined using a NanoDrop ND-2000 Spectrophotometer (Thermo Scientific). Samples with A260: A280 values greater than 1.8 were accepted for subsequent use. To avoid DNA contaminations, DNA free kit (AM1906, Invitrogen) was used for removal of contaminating DNA from total RNA samples. SuperScript III

Reverse Transcriptase (18080-044, Invitrogen) was used for cDNA synthesis. Reverse transcription was performed using 1.5μg DNA free total RNA, 3μg random primers (48190-011, Invitrogen) and 2μl 10 mM dNTP. After heating the mixture to 65°C for 5 minutes and incubating on ice for 2 minutes, add 2μl 0.1M DTT, 80 units RNaseOUT Recombinant Ribonuclease Inhibitor (10777-019, Invitrogen), SuperScript III Reverse Transcriptase (18080-044, Invitrogen) and 5X First-Strand Buffer. The mixture was incubated at 50°C for 1 h, and then the reaction was inactivated by heating at 70°C for 15 minutes. Using a NanoDrop ND-2000 Spectrophotometer (Thermo Scientific) to measure the concentration of each sample and diluted to 25 ng/μl for qRCR.

3. 6 Quantitative Reverse Transcription Polymerase Chain Reaction

qPCR was carried out on the 7900 Fast Real-Time PCR System (Life Technologies) using SYBR Green PCR Master mix (Life Technologies) according to the manufacturer's instructions. The primer pairs are listed in Table 2. *GAPDH* was used as a control.

3. 7 Microarray Analysis

Total RNA was purified from 2 groups of control and knockout E13 mice testes (14 mice in each group) separately, using *mir*Vana^{TM} miRNA Isolation Kit (AM1561,

ambion). 0.125 μg of total RNA from each sample was send to the Nevada Genomics Center (NGC) for the microarray analysis.

Chapter 4

Results and Conclusion

4. 1 Generation of Sertoli cell-specific *Gata4* knockout mice

We cross the *Gata4*$^{lox/lox}$ line with the AMH-Cre line (Fig. 7A) and the resultant *Amh-Cre; Gata4*$^{lox/lox}$ (cKO mice) and control (*Amh-Cre; Gata4*$^{+/lox}$ or *Gata4*$^{lox/lox}$) male mice were used for the entire study. PCR genotyping (Fig. 7 B, Table 1) analyses showed the genotype results of WT, *Gata4*$^{lox/lox}$, *Amhcre*, *Amhcre Gata4*$^{+/lox}$ and *Amhcre Gata4*$^{lox/lox}$ using the DNA collected from adult mouse-tail. Using the P8 *Amhcre; Gata4*$^{lox/lox}$ testis cDNA, primers E2 and E6 showed the recombination between two loxP sites and primers E3 and E4 showed the 3,4,5 exons had been deleted only in Sertilo cells (Fig. 7 C, Table 1). The P8 testis cDNA of *Gata4*$^{lox/lox}$ was using as the control. The genotyping results confirmed that after Cre-mediated recombination exons 3-5 were indeed deleted in the cKO testes.

Taking advantage of a dual fluorescence reporter mouse line (ROSA26mTmG line from JAX), we also generated a compound conditional *Gata4* cKO line by crossing *Amh Cre; Gata4*$^{+/lox}$ males with *Gata4*$^{lox/lox}$*; mT/mG*$^{tg/tg}$ females. The resultant *Amh Cre; Gata4*$^{lox/lox}$*-mT/mG*$^{+/tg}$ (cKO) mice displayed membrane-bound EGFP (mG) in all Cre-expressing Sertoli cells, whereas non-Cre-expressing cells expressed membrane-targeted tomato Red (mT). This compound cKO reporter line allowed us to 1) monitor the onset of Cre expression, 2) to visualize and track the behavior of *Gata4*-deficient Sertoli cells during testicular development and 3) to isolate true *Gata4*-null Sertoli cells for molecular analyses.

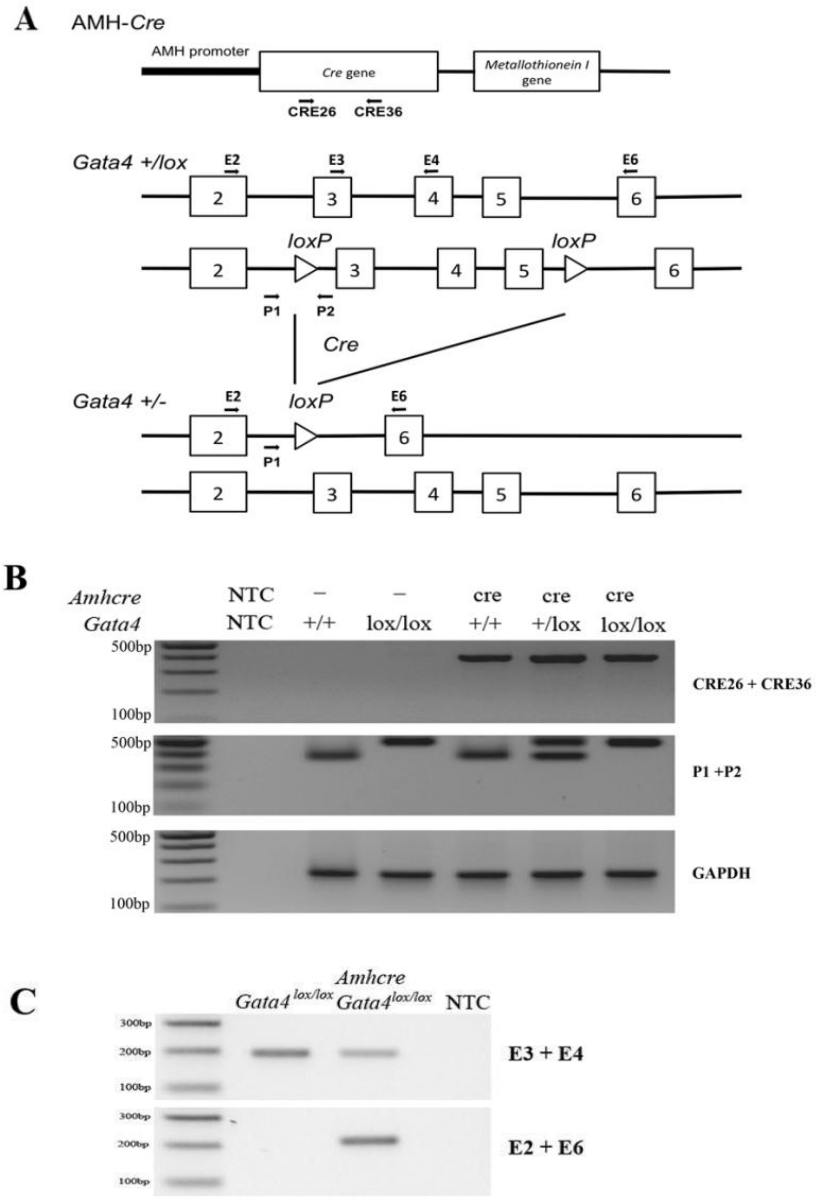

Figure. 7. Generation of Sertoli cell-specific *Gata4* knockout mice. (A) Design of *Gata4* loxp conditional KO mice. (B) PCR genotyping analysed of WT, *Gata4*$^{lox/lox}$, *Amhcre*, *Amhcre Gata4*$^{+/lox}$ and *Amhcre Gata4*$^{lox/lox}$. All DNA were collected from adult mouse-tail. (C) Recombination and deletion tested using the *Amhcre Gata4*$^{lox/lox}$ P8 testis cDNA and *Gata4*$^{lox/lox}$ as the control. E3+E4 showed the 3,4,5 exons had been deleted only in Sertilo cells. E2+E6 showed the recombination between two loxP sites.

4. 2 *Amh-Cre; Gata4$^{lox/lox}$* males are infertile due to severe testicular dysgenesis

Fertility tests by breeding the cKO males with adult WT females resulted in no litter born over a period of six months (Table 2). The adult cKO testes were ~1/20 of the

Table. 1. Primers for Genotype

Primer	Sequence	Size
Cre26	5'-CCT GGA AAA TGC TTC TGT CCG-3'	400 bp
Cre 36	5'-CAG GGT GTT ATA AGC AAT CCC-3'	
P1	5'-CCC AGT AAA GAA GTC AGC ACA AGG AAA C-3'	WT: 355bp
P2	5'-AGA CTA TTG ATC CCG GAG TGA ACA TT-3'	Mutant: 455bp
E3	5'-CTG TGG CCT CTA TCA CAA GAT G-3'	189bp
E4	5'- CCA TGG AGC TTC ATG TAG AG -3'	
E2	5'-TTC GAC AGC CCA GTC CTG CA-3'	173bp
E6	5'-CTG TGC CCA TAG TGA GAT GAC AG-3'	
GAPDH F	5'-TCC ATG ACA ACT TTG GCA TTG-3'	206bp
GAPDH R	5'-CAG TCT TCT GGG TGG CAG TGA-3'	

Table. 2. Results of testis weight, sperm count and litter size analysis

(n=4-9 animals per genotype).

	Gata4$^{lox/lox}$	Amh-Cre-Gata4$^{lox/lox}$
Testis weight (mg) at P5	5.0±0.8 (n=6)	1.4±0.3 (n=6)
Testis weight (mg) Adult	115.8±11.7 (n=6)	6.8±1.1 (n=6)
Litter size (pups/litter)	7.6±2.0 (n=20)	0
Sperm count (sperm/ml)	20X10^6 (n=10)	0

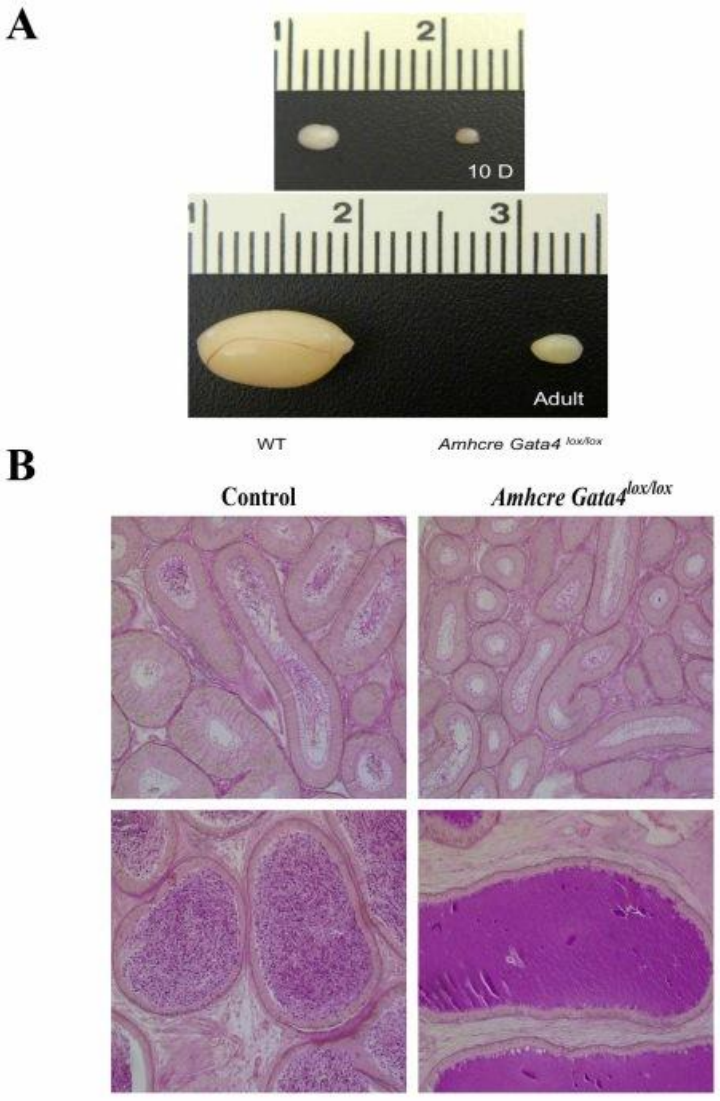

Figure. 8. Dramatic size reduction and complete absence of sperm in *Amh-Cre; Gata4*$^{lox/lox}$ testes. (A) Testis from *Amh-Cre; Gata4*$^{lox/lox}$ mice showed a drastic reduction at P10 and in the adult showed 90% reduction in size compared to control (n=8-14 animals per genotype). (B) PAS staining of adult epidydimides revealed complete absence of sperm.

control (*Amh-Cre; Gata4*$^{+/lox}$ or *Gata4*$^{lox/lox}$) in wet weight (Fig. 8 A and Table 2). In the epididymis of the cKO males, no sperm were present, whereas the control epididymis is filled with sperm (Fig. 8 B). The testis of the cKO males weighted ~1/4 of the controls at postnatal day 5 (P5) and the difference in testis weight increased thereafter (to 1/20 of the controls in adults). It is clear that the cKO males are completely infertile due to

azoospermia. Among all *Amh-Cre; Gata4*$^{lox/lox}$ mice, the ratio of male to females was 1:1 (n=20), suggesting that there was no male-to female sex reversal.

Histological analyses revealed that the size of the cKO testes was similar to the controls at birth, but there appeared to be many more seminiferous tubules in the control testis than in the cKO testis (Fig. 9, upper 1st panel). Given the total volume of the control and the cKO testes was roughly the same, the diameter of the WT seminiferous tubules was much smaller than that of the cKO seminiferous tubules/cords (double-headed arrows in inserts of Fig. 9). In the newborn testes of control mice, Sertoli cells had migrated to the basal membrane of the seminiferous tubules and were in close contact with primordial germ cells (PGCs), which were also seen on the basal membrane. Because of a lack of cells in the center of the testis cords, the lumen of the future seminiferous tubules was discernable. In contrast, the cKO testis contained a few much larger testis/seminiferous cords, in which numerous cells were evenly distributed across the cords although cells resembling Sertoli cells and PGCs appeared to be concentrated along the basal membrane (Fig. 9, upper 1st panel to the right). At P4, PGCs resumed proliferation and differentiated into spermatogonia. Increasing number of proliferating spermatogonia took the positions in the basal or adluminal compartments (Fig. 9, arrows in upper 2nd panel) to give the looking of stratified seminiferous epithelium. The cKO testis, however, remained to be cord-like with numerous cells scattered across the cord without any sign of regulated arrangement, as seen in the WT testis at P4. Similarly, the testis cords diameter in *Gata4* cKO was much greater than that of the seminiferous tubules in the control testis. While the testes in the controls undergo rapid postnatal growth, the cKO testis increased in size at a much lower rate. At p7, type B

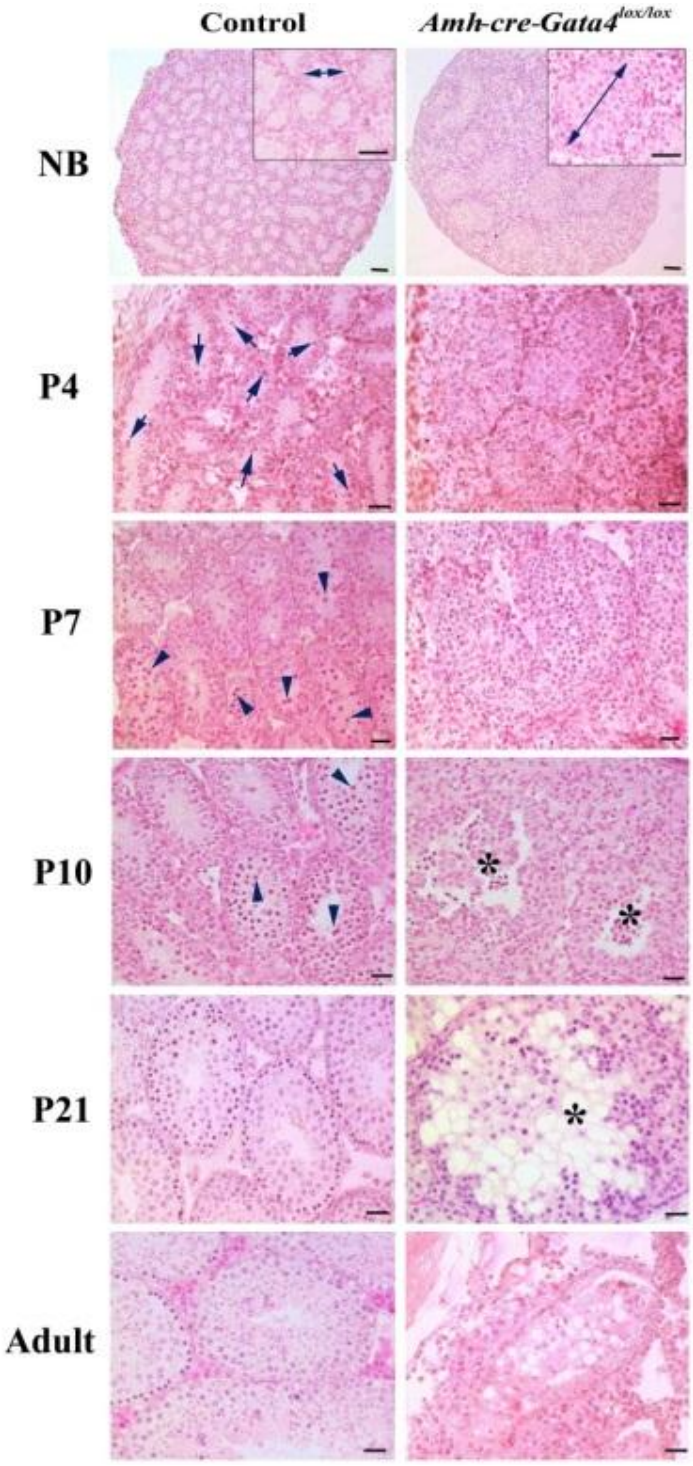

Figure. 9. Histological analyses of development testes. Control (left column) and cKO (right column) were stained with H&E. In the newborn testes, the diameter (double-headed arrows) of the WT seminiferous tubules was much smaller than that of the cKO seminiferous tubules/cords. At P7, type B spermatogonia start to enter meiosis and become preleptotene spermatocytes (arrow heads) in the control testis. In the cKO testis, there is no lumen formation and the cells started depletion leaving numerous vacuoles within the cord structures after P10. (See *). Scale bar: 50μm.

spermatogonia start to enter meiosis and become preleptotene spermatocytes (Fig. 9, arrow heads in upper 3rd panels) in the control testis, whereas in the cKO testis there appeared to be no sign of lumen formation and the testis cords remained with numerous

cells evenly distributed across the cord. At P10, early pachytene spermatocytes were seen on the seminiferous tubules of the control mice. In contrast, the cKO testis contained only testis cords with much larger diameter comparing to the controls. Cell clamps and clusters started to be seen, which may well be the early sign of cell depletion. Indeed, severe depletion of those centrally located cell populations can be detected at P21, when the first wave of spermatogenesis just finished the first meiosis and haploid male germ cells called round spermatids started to appear in the seminiferous tubule epithelium. The adult control testis weights 20 times more than that of the cKO males (Table 2) and constant depletion of cells within the testis cord was ongoing, leaving numerous vacuoles within the cord structures, which represent a hallmark of active germ cell depletion (see * in Fig. 9, 2nd panels from the bottom). These histological analyses suggest that the cKO testes failed to develop normally during the postnatal testicular development. In the cKO testes, the seminiferous tubules were never formed and testis cords persisted until P10, when the cells within the cords started to be depleted. Spermatogenesis never occurred in the cKO testes and the development of the testis cords into seminiferous tubules and further development of spermatogenic cells appeared to be severely slowed down or halted in these cKO testes.

To further reveal the cell identity, we performed immunofluorescent staining for a Sertoli cell marker WNT1 and a germ cell maker GCNA (Fig. 10). WNT1 and GCNA antibodies stained the nuclei of Sertoli cells and germ cells, respectively. AT P8, both Sertoli cells and developing spermatogonia (up to type B spermatogonia) were located in the basal compartments and few or no cells were found in the center of the seminiferous tubules and the lumen was thus obvious and distinct. In the cKO testis at P8, the testis

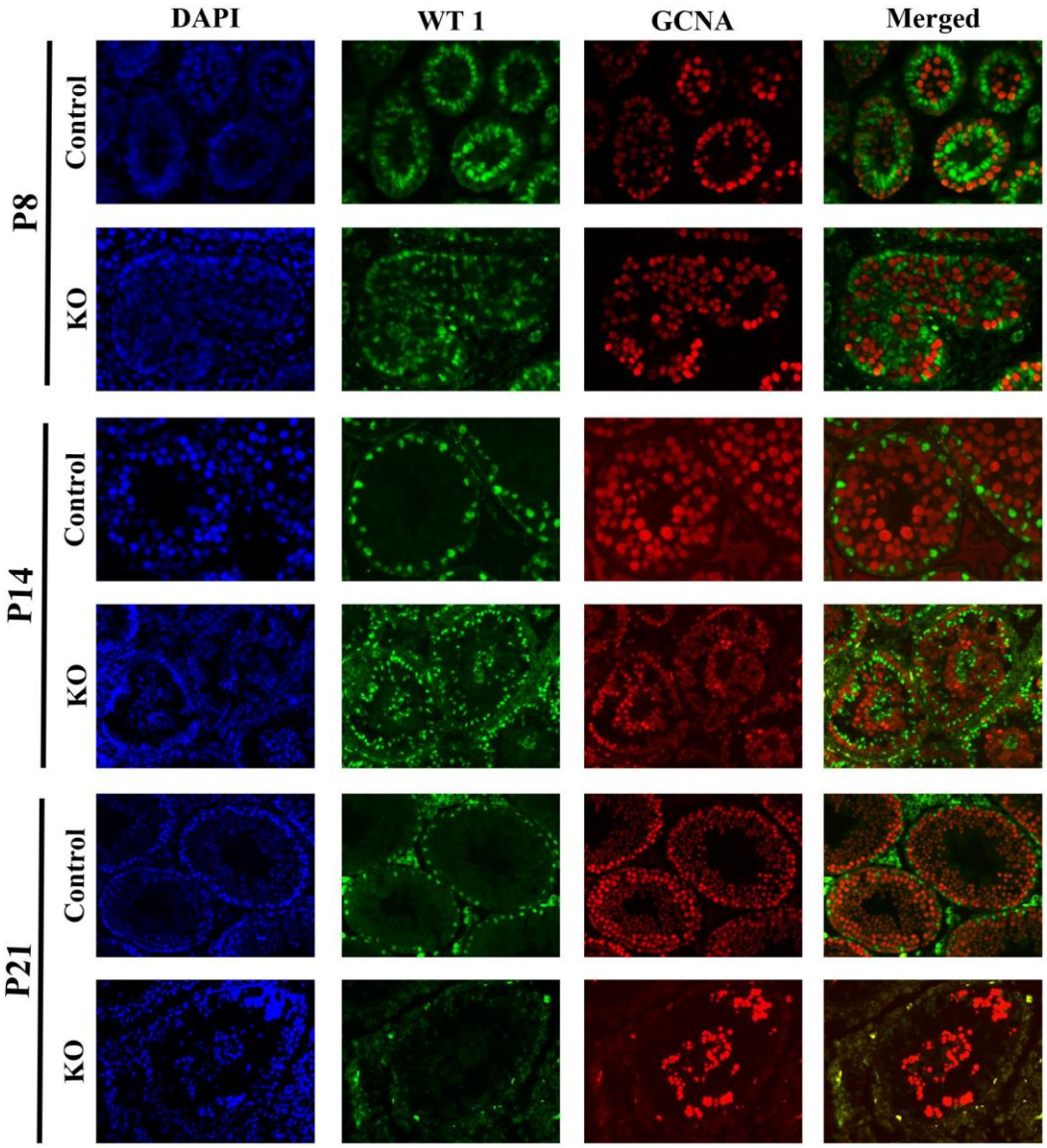

Figure. 10. Immunofluorescent staining of development mouse testis. DAPI (blue) was used for nuclear staining. WNT1 (WT1, green) and GCNA (red) antibodies stained the nuclei of Sertoli cells and germ cells, respectively. At P8, both Sertoli cells and germ cells seemed proliferation, and evenly distributed across the cord although a layer of Sertoli cells appeared to have assumed their normal position along the basal membrane as the control. The Sertoli cells appeared dramatically depleted at P14 in *Amh-Cre; Gata4*$^{lox/lox}$ testis and were almost completely depleted at P21, leaving some germ cells in the center of the testis-cord. The germ cell depletion seemed as an effect secondary to Sertoli cell depletion.

cords persisted and displayed much greater diameters. Both Sertoli cells and germ cells were evenly distributed across the cord although a layer of Sertoli cells appeared to have assumed their normal position along the basal membrane. Moreover, the size of the nuclei of both Sertoli cells and germ cells in the cKO testis appeared to be smaller and the intensity of the WNT1 immunoreactivity appeared to be weaker comparing to that in the control testis. At P14, the number of Sertoli cells per tubule decrease dramatically and the position of Sertoli cell nuclei appeared to shift towards the basal membrane. Further development of germ cells had generated multiple layers of spermatogenic cells up to mid-pachytene spermatocyte stage and the meiotic germ cells (preleptotene, leptotene, zygotene, pachytene spermatocytes) were located in the adluminal compartment and the lumen was clearly visible and distinguishable in the control testis at P14. In contrast, the cKO testis contained testis cord-like structures filled with both Sertoli cells and germ cells and no lumen was visible and Sertoli cells appeared to be depleted, leaving seemingly more germ cells than Sertoli cells inside the cords. When the first meiotic cell division was accomplished at P21, germ cells became dominant and Sertoli cell nuclei were only detected along the basal membrane. In the cKO testis Sertoli cells were almost completely depleted leaving some germ cells in the center of the testis-cord. These data further confirm the histological analyses, suggesting that the precursor of the seminiferous tubules failed to form appropriately in the cKO testes. Pre-Sertoli cells and PGCs appeared to be confined to a limited number of testis cords that failed to differentiate into precursor seminiferous tubules. These cells are most likely pre-Sertoli cells and PGCs And they continue to proliferate in postnatal testis development until ~P10, when Sertoli cells that failed to migrate and differentiate get depleted followed by

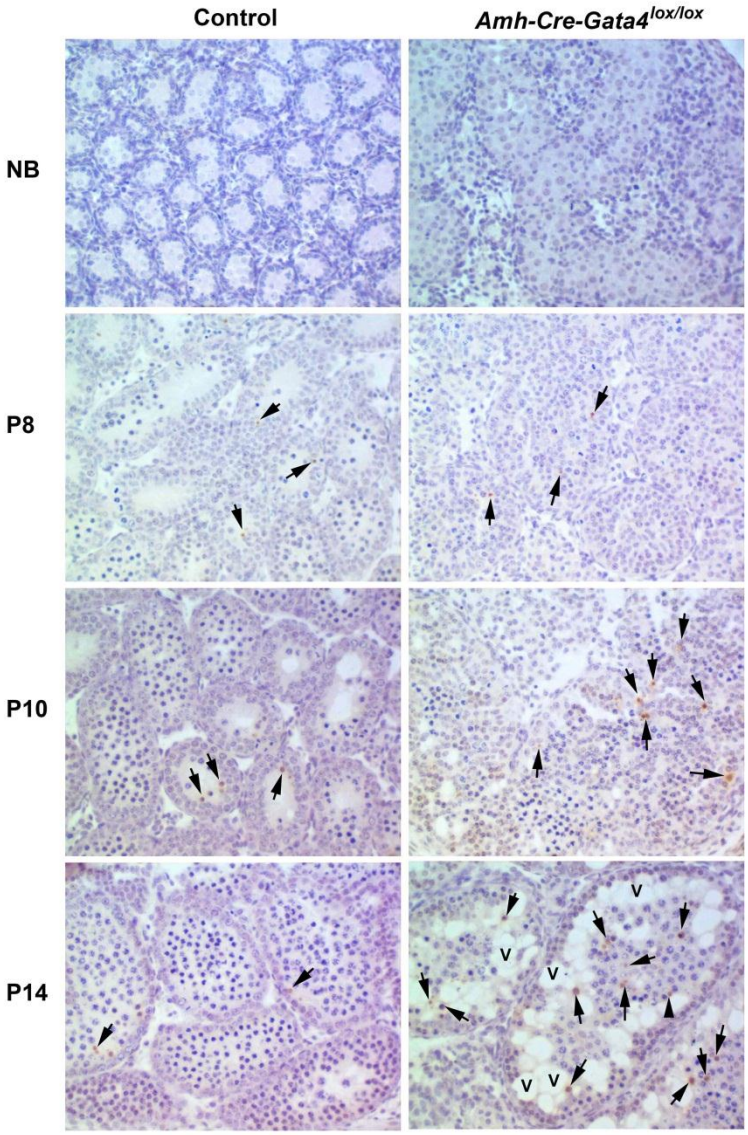

Figure. 11. TUNEL staining showed the depletion of cells in mouse testicular. Massive cell apoptosis in prepubertal *Amh-Cre Gata4*$^{lox/lox}$ testis. TUNEL labeled cells (arrow head) revealed a dramatic increase in apoptosis in P7 and P14 mutant testis.

germ cell depletion as an effect secondary to Sertoli cell depletion. TUNEL staining showed that the depletion of Sertoli cells with an onset at ~P10 was at least partially mediated through apoptosis (Fig. 11). Given that many germ cells persisted in the absence of Sertoli cell in the cKO adult testis, the Sertoli cell depletion appeared to be the primary cause for the germ cell depletion.

4. 3 Postnatal testis dysgenesis results from aberrant testis cord formation during embryonic testicular development

The analyses above pointed to a fact that the defects in the cKO testes are derived from abnormal development of the testis cord during embryonic testicular development

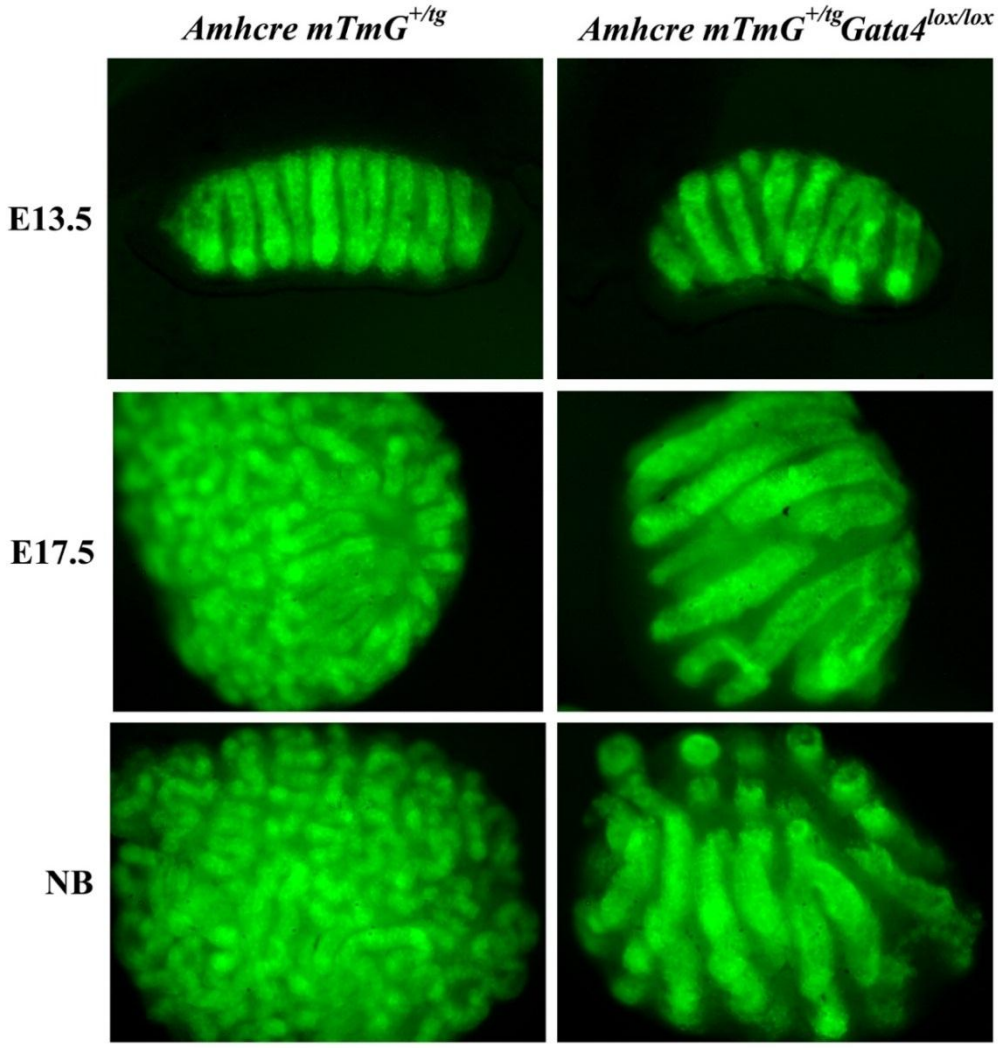

Figure. 12. Testis cord expressing EGFP (*mT*) in normal and cKO Sertoli cells at different stages of development under the control of *Amh-Cre* experssion. At E13.5, the total number of testis cords was similar between control and the cKO. Between E17.5 and newborn, the cKO testis cords showed aberrant testis cord formation and display enlarged testis cord diameters, whereas the control undergo elongation, coiling, branching processes with smaller diameters.

because in the cKO mice, the testis cords persist postnatally and fail to develop into seminiferous tubules. Given that the earliest Cre expression was detected at E12.5, the total ablation of GATA4 protein would be ~E13.5 considering the preexisting protein may last for a period of time. We dissected both control (*Amh-Cre; mTmG*$^{+/tg}$) and cKO reporter mouse embryonic testes and observed the testis cord morphology under a fluorescent dissection scope (Fig. 12). At E13.5, the total number of testis cords was similar between control and the cKO testes. But the morphology of the testis cord in cKO mice appeared to be heterogeneous comparing to the of the control testes at E13.5, which display well placed smooth-looking and homogeneous groups of testis cords. At E17.5, the control testis cords had undergone elongation, coiling, branching and formed numerous coiled individual testis cords with smaller diameters. In contrast the cKO testis cords failed to undergo these elongation, coiling, branching processes and display and display enlarged testis cords at a similar number to that in E13.5. At birth, the control testis contained countless number of smaller testis cords that were highly coiled. At a similar size, the cKO testis contained slightly elongated, a limited number of large testis cords with much greater diameters comparing to those of the testis cord of the controls (Fig. 12). These results revealed that the primary defects in the cKO testis lies in failure of testis cords to elongate and branch to form highly coiled testis cords during embryonic testis cord development. All postnatal defects represent the consequential development of the primary defects. These defects occur as soon as *Gata4* is inactivated at E12.5, suggesting that *Gata4* is absolutely required for the testis cord development during the embryonic testicular development.

4. 4 Genes affected by the absence of *Gata4*

Although the detailed pathway for male sex dedifferentiation during embryonic development remains elusive, several genes have been identified to be required or involved in male sex differentiation. These genes include *Sox8* (Schepers et al. 2003), *Sox9* (Sekido et al., 2004), *Amh* (Viger et al., 1998; Arango et al., 1999), *Dhh* (Clark et al., 2000; Yao et al., 2002), *Fog2* (Svensson et al., 1999), *Sf1* (Arango et al., 1999; Ikeda et al., 1994), *Wt1* (Miyamoto et al., 2008; Gao et al. 2006) and *Gata6* (Ketola et al., 1999). The cellular composition in postnatal cKO and control testes differs significantly. Direct comparison of levels of these genes using the postnatal testes is therefore meaningless. However, the size and total number of Sertoli cells and Germ cells are comparable between the cKO and control testes during embryonic development. We therefore chose to analyze levels of these genes known to be involved in early testis formation and testis cord development. Because of the very limited materials available from embryonic testes, we did not sort the green Sertoli cells, but used total testes to examine the levels of these genes. Among these genes, *Sox8*, *Sox9* and *Amh* are expressed in Sertoli cells. The other genes are not Sertoli cell specific, but also expressed in other cell types within the testis, including Leydig cells, Sertoli cells peritubular myoid cells and endothelial cells. The semi-qPCR analyses revealed that *Sox9, Wt1* and *Sf1* were significantly down-regulated in the cKO testes compared with control testes at E13 (Fig. 13). *Sox8* seemed to be affected later than the others. *Sox9* and *Wt1* showed a little increase in the cKO testis between E17 and NB maybe due to the abnormal Sertoli cell proliferation. In contrast, *Fog2* showed significantly increase in the cKO testes from E17.

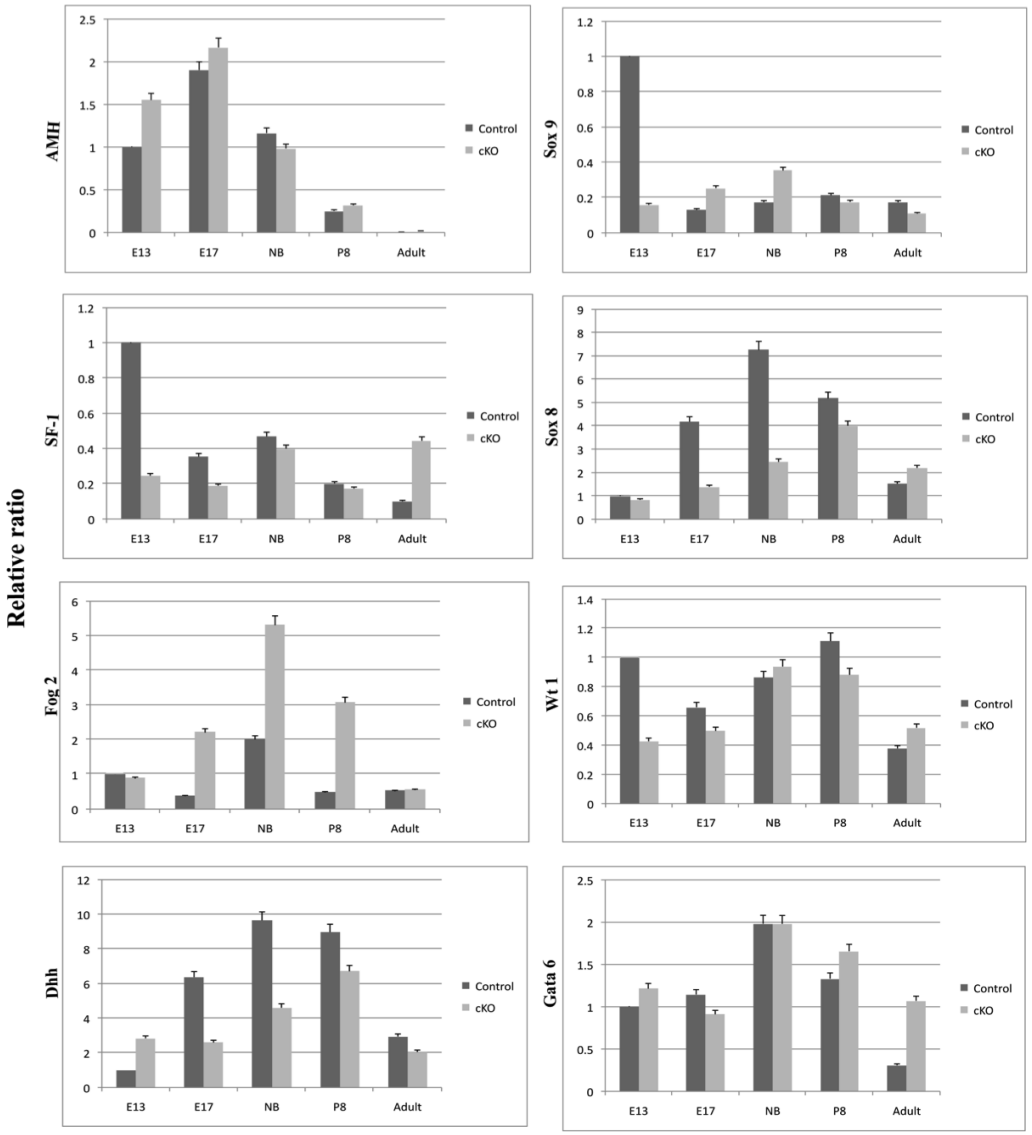

Figure. 13. Several genes are affected by the absence of Gata4. semi-qPCR was used to determine mRNA levels in E13, E17, Newborn (NB), 8 day (P8) and adult testis. The data were represented as the ratio to the expression level of control E13 testis. The results revealed that the expression *Sox9, SF-1, Sox8, Fog2, Wt1* and *Dhh* were significantly different from the control. For each sample, semi-qPCR was done three times. Error bars represent the standard errors of the means.

Dhh were upregulated in the cKO testis at E13, but down-regulated from E17, which is

probably due to the increasing proportion of other cell types during testis development. *Amh* and *Gata6* showed little difference between the cKO and control. Therefore, *Gata4* seems both the suppressor and activator to different genes. From our results, the lack of *Gata4* expression in Sertoli cells may affect the genes which were essential for the male sex differentiation, Sertoli cell differentiation or testis cord formation and development. *Gata4* is important for the mouse testicular development and testis formation.

Table 3: Primers for Semi-PCR

Primer	Sequence	Reference
Amh F	5'- GCA GTT GCT AGT CCT ACA TC -3'	Salmon et al., 2005
Amh R	5'- TCA TCC GCG TGA AAC AGC G -3'	
Sox9 F	5'- CCT ACA CTG GCA GTT ACG G -3'	Salmon et al., 2005
Sox9 R	5'- CGT CTT GAT GTG CGT TCG C -3'	
SF1 F	5'- ACA AGC ATT ACA CGT GCA CC -3'	Babu et al., 2002
SF1 R	5'- GCT GGC ATA GGG CTC TGG ATA C -3'	
Sox8 F	5'- TCC TAC TCG CAC TCC GGG -3'	Salmon et al., 2005
Sox8 R	5'- GCC GAC GGG ATG AAT GGA -3'	
Fog2 F	5'- CAG GGA ACC GTA GCG GGA -3'	Salmon et al., 2005
Fog2 R	5'- CTC CAG TGG AAA GTC GCC -3'	

Wnt1 F	5’- CAG AGA GCA AGG CAC CAG -3’	Salmon et al., 2005
Wt1 R	5’- TAA GAG CCC AGT GCT AGT G -3’	
Dhh F	5’- CCA TCG CGG TGA TGA ACA TG -3’	Takabatake et al., 1997
Dhh R	5’- TTA TCA GCT TTG ACC GAT AC -3’	
Gata6 F	5’- CAC CGG TCA TTA CCT GTG CAA TG -3’	
Gata6 R	5’- GAG GTG GTC GCT TGT GTA GAA G -3’	
GAPDH F	5’- AAC TTT GGC ATT GTG GAA GG -3’	Song et al. 2011
GAPDH R	5’- CAC ATT GGG GGT AGG AAC AC -3’	

Chapter 5

Discussion and Future direction

Those constitutive KO mice lack *Fog2* or *Fog2* binding to *Gata4* from E10.5, the normal onset of their expression (Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999), whereas in the present study *Gata4* was inactivated at ~E12.5. It has been well documented that it is between E10.5 and E12.5 that Sertoli cells are formed followed by the formation of testis cords. This time line is consistent with our observation in this study showing that in cKO males, Sertoli cells were fully formed (based upon the morphology and marker gene expression including *Wnt1*, *Sox9, Gata6, Amh* and *Fog2*) and the testis cord was also fully formed when *Gata4* is inactivated at ~E12.5 (Miyamoto et al., 2008; Gao et al. 2006; Sekido et al., 2004; Ketola et al., 1999; Svensson et al., 1999). Although this study cannot suggest an essential role of *Gata4* in the formation the testis cord, our data indeed demonstrate that *Gata4* is required for the further development of the testis cord after E12.5.

Despite intensive studies on the genetic control of sex differentiation during gonadal development, the exact pathway that leads to the male differentiation program remains unclear. *Sry* appears to be the master switch in initiating the male program (Capel review, 1998), but its expression time window seems to be overlapping with many of the transcription factors expressed around E10.5, e.g., *Gata4*, *Sox9, Amh*, *SF1*, *Wt1*, *Fog1* and *Fog2* (Koopman et al., 1990). The specific sequence of events that are mediated by these genes during sex differentiation remains controversial largely due to the conflicting expression data on the exact timing of their expression and a lack of

animal models that can reveal the interactions among these transcriptional regulators and their targets. Nevertheless, each of these genes had been individually shown to be involved in the sex differentiation during gonadal development. In our cKO testes between E13.5 and birth, levels of these genes appear to be downregulated in the absence of *Gata4 i*n Sertoli cells, with the most significant decrease in levels of *Sox9*. This finding is consistent with numerous previous studies, which have demonstrated that *Gata4* can affect expression of *Sry*, *Amh* and *Sox9* (Tevosian et al. 2002; Viger et al., 1998; Manuylov et al., 2007). A thorough transcriptome and proteomic analyses using purified Sertoli cells collected and pooled from E12.5 - E13.5 cKO and WT testes may shed light on the target genes of *Gata4*, both direct targets (containing *Gata4* binding motif) or indirect ones (without *Gata4* binding motif).

The primary defects in our cKO males point to one thing that has not been described previously, that is, the total number of testis cord developed in the embryonic testes determines the final number of seminiferous tubules in the adult testes and consequently the size of the adult testes. Using the Sox9-EGFP mouse line, the other lab observed that the testis cord is formed soon after the surface epithelial cells differentiate into Sertoli cells (Nel-Themaat at al., 2009). At E12.5 the testis cords start taking into shape and the entire testis contains ~8-12 well organized, smooth-looking testis cords. Thereafter testis cords start to elongate and meanwhile also undergo branching, resulting in a much larger number of individual cords. When elongation reaches certain levels, those testis cords are coiling to maximize the net volume and surface area within the testis. At birth the testis contains hundreds of thousands of highly coiled cords with much smaller diameters comparing to those at E12.5. During postnatal testicular development

these cords undergo canalization and the appearance of lumen marks the transition from testis cords to seminiferous tubules. Our data revealed that both the cKO and the control testes display similar number of testis cords and cell morphology at E12.5. The fact that the testis volume did not differ between cKO and control mice from E13.5 up to birth suggests that the total cell number remain the same as well. Although both cKO and control Sertoli cells were proliferating, the control cords can elongate, branch and form a large number of cords with smaller diameters and thus dilute the rapidly increased number of Sertoli cells and PGCs during the cord development. In contrast, in the cKO testis, Sertoli cells and PGCs kept proliferating but were all confined to a limited number of large cords (increased from 8-10 to ~15-20 in total number) because these cord did not undergo rapid elongation and branching and thus no coiling was observed. Consequently, a large number of Sertoli cells and PGCs are trapped within those "big and fat" testis cords instead of numerous thinner and longer cords as seen in the controls. Interestingly, although the size differences at birth between cKO and control testes are not significant, the rapid elongation of the existing fully developed cords resembling precursor seminiferous tubules in the control testes soon set them apart from those in cKO testes because those large and undifferentiated cords do not undergo further elongation. Meanwhile germ cell development was arrested and severe apoptosis depletes the undifferentiated Sertoli cells. As a result, the cKO testes were 1/5 of the controls at P10, and by P60 the cKO testes weighted only 1/20 of the controls. Our data suggest that the testis cord development after the cord formation determines the final number of seminiferous tubules available for the postnatal development. The more fully differentiated testis cords available upon birth, the more seminiferous tubules will be

produced during the postnatal development and the bigger volume the testis will have. In this sense, the testis size is largely determined during the embryonic development depending upon the number of fully differentiated testis cords formed between E12.5 and birth. After birth no further branching occurs and thus the total number of seminiferous tubules will not increase. The postnatal development of the seminiferous tubules is thus a process of further elongation of the pre-existing tubules that are meanwhile being loaded with developing germ cells. *Gata4* apparently regulates the multiplication of testis cords through branching, elongation and coiling. Without *Gata4*, these events will not occur and the testis will be destined to be tiny due to limited number of testis cords/seminiferous tubule precursors. Overall, it is apparent that branching of the testis cords between E12.5 and birth plays an essential role in increasing the total number of individual cords, which serve as the precursor seminiferous tubules during postnatal development. Genes that regulate the branching events need to be identified and these are clearly under the control of *Gata4*.

Based upon a recent report (Nel-Themaat et al. 2011), as early as E13.5, Sertoli cells start to migrate outwards the testis cord and form the basal layer of the cord although numerous Sertoli cells and PGCs remain in the center of the cords. Those Sertoli cells and PGCs are further allocated to newly form individual cords resulting from branching and become basally located. At E17.5 almost all Sertoli cells have already assumed the basal location and PGCs gradually migrate to the basal layer as well. At birth the cords already appear to have lumen since no cells are located in the center and thus these cords form precursor seminiferous tubule at birth. However, the fate of those Sertoli cells that fail to migrate and assume basal localization (herein called

undifferentiated Sertoli cells) has not been described. Our cKO mice also gave us an opportunity to track down the fate of those undifferentiated Sertoli cells and PGCs, which are present in large quantity in our cKO testes after birth. Our data demonstrate that those undifferentiated Sertoli cells actually can continuously proliferate between E13.5 and birth and after birth up to postnatal day 10. No enhanced apoptosis was observed until P10, suggesting Sertoli cells do not normally undergo apoptosis during embryonic development and early postnatal development. Sertoli cells cease proliferation and start terminal differentiation ~P10. At this point the total number of Sertoli cells theoretically is fixed and therefore the total number of germ cell that they can support is also determined as a result. The depletion of those centrally localized, probably undifferentiated Sertoli cells at P10 suggest that the signals that support proliferation and survival of developing Sertoli cells are no longer available at this time point and those that have not differentiated into mature Sertoli cells are thus depleted. It would be interesting to analyze the differences between Sertoli cells that are localized to the basal membrane and those that are centrally localized. It has been well documented that Sertoli cells mediate the clearance of apoptotic germ cells through phagocytosis (Russell and Clermont, 1977; Chemes, 1986; Pineau et al., 1997). When massive germ cell apoptosis occurs, Sertoli cells display numerous vacuoles within their cytoplasm. Those Sertoli cell vacuoles have thus been regarded as a hallmark of massive depletion of germ cells within the seminiferous epithelium. Given that numerous vacuoles exist when massive depletion of those undifferentiated Sertoli cells occurs after P10, the differentiated Sertoli cells localized to the basal membrane appear to be clearing those apoptotic undifferentiated Sertoli cells. It is not surprising that germ cells that fail to migrate to the basal membrane

and form multiple layers along the seminiferous epithelium, but instead are localized to the center of the cords can also initiate development, as evidence that many spermatocytes are present in P14 and P21 cKO testes. Given the precise coordination between the development of Sertoli cells and germ cells during the first wave of spermatogenesis in WT testes, the signals for germ cell development from mitotic to meiotic and then to haploid phases at least partially come from Sertoli cells. It would be interesting to see the proportions of mitotic germ cells versus meiotic germ cells in cKO testes when normal Sertoli cell signaling is at the minimum.

Our cKO mice do not display male to female sex reversal largely due to the fact that Cre is expressed at E12.5, when Sertoli cell and the testis cord formations have completed. Also, only Sertoli cell *Gata4* is inactivated and the interstitial Leydig cells remain untouched and thus should work normally and produce testosterone, which is known to be required for proper formation of both the internal and external genitalia. Normal sex ratio and normal male accessory organs further confirm that *Gata4* inactivation is confined to Sertoli cells.

A recent report using a mouse line with dual fluorescent reporters demonstrate that Sertoli cells undergo polarization, migration and become localized to the basal layer of the testis cord at E12.5 and this localization remains throughout the postnatal life (Nel-Themaat et al. 2011). The fact that many Sertoli cells manage to migrate and localize to the basal layer of the undifferentiated testis cords in both embryonic and postnatal testes in the cKO mice suggest that *Gata4* is not required for the Sertoli cell polarization, migration and final localization onto the basal membrane during embryonic development.

In contrast, the absence of *Gata4* results in failure in the development of testis cord into elongated and coiled precursor seminiferous tubules through branching.

Chapter 6

Conclusion

In summary, inactivation of *Gata4* at E12.5 does not affect Sertoli cell formation and formation of testis cords, but severely hinders further development of the testis cords characterized by failure in multiplication of the testis cords through branching, elongation followed by coiling. Consequently, the testis with *Gata4* deficiency in Sertoli cells at and after E12.5 contains only a few undifferentiated testis cords with large diameters, which undergo limited elongation postnatally, followed by severe depletion of both the undifferentiated Sertoli cells and germ cells. These males display miniature testes and no sperm production and thus complete infertility. *Gata4* appears to be a master gene upstream of an important pathway that controls the testis cord development and thereby the number of precursor seminiferous tubules during embryonic testicular development.

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