Reciprocal regulation between Nanos2 and Sox2 for sexual differentiation of male germ cells in mice

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Contents

1. Abstract

Germ cells, which are male gamete or spermatozoa and the female gamete or oocytes, are specialized cells to transmit genetic information to the next generation. In mice, spermatozoa and oocytes are differentiated from primordial germ cells (PGCs) through the process called sexual differentiation that takes place in the embryonic gonads. The germ cell sex is determined by signaling cues from the surrounding somatic cells. In female gonads, PGCs enter meiosis and proceed to meiotic prophase I, whereas PGCs in male gonads enter mitotic arrest at G_0/G_1 stage and maintain the quiescence until spermatogensis begins after birth.

One of the key factors required for promoting male pathway in germ cells is Nanos2, an evolutionally conserved RNA-binding protein. Nanos2 is a male-specific factor essential for the fertility of male mice. Nanos2 plays a crucial role in sexual differentiation of male germ cells because meiosis is abnormally induced as similar to female germ cells and male-type genes are down-regulated in the *Nanos2-deficient* male germ cells. In addition, the expression of pluripotency genes (*Oct3/4, Nanog* and *Sox2*), which is a hallmark of undifferentiated PGCs, is maintained in *Nanos2-null* male germ cells. Nanos2 is involved in RNA degradation, therefore target mRNAs are up-regulated in the absence of Nanos2, which may provide molecular base of Nanos2 deficiency including abnormal cell cycle regulation. However, it is unclear how Nanos2 promotes the male differentiation pathway. Our laboratory has been addressing this question based on a hypothesis that Nanos2 might promote male genetic program by repressing the events abnormally induced in the *Nanos2-null* germ cells, such as mitosis, meiosis and maintaining PGCs properties. The effect of abnormal meiotic initiation on the Nanos2 deficiency had been tested by analyzing *Nanos2/Stra8* double knockout (dKO) male germ cells. However, the male differentiation pathway was not rescued in the dKO male germ cells, even though meiosis was prevented. These results indicate that Nanos2 is required for promoting male differentiation pathway other than suppressing meiosis.

In my thesis research, I focused on other two events compromised in *Nanos2-null* germ cells, mitotic resumption and up-regulation of pluripotency genes to understand the mechanism by which male genetic program is promoted in mouse germ cells. My thesis is composed of two parts. I describe analyses of downstream events of Nanos2 in the Chapter I, and I extended my research to the upstream event of Nanos2 in the Chapter II.

In the Chapter I, I re-examined *Nanos2-null* phenotype and found that mitotic resumption rather than meiotic entry was frequently occurred in *Nanos2-null* male germ cells. I expected that male pathway would be recovered if I could inhibit the abnormal mitotic resumption of *Nanos2-null* germ cells. Since retinoic acid (RA) is implicated in the mitotic regulation in addition to meiotic initiation, I investigated the involvement of RA signaling in the defect in *Nanos2-null* germ cells. Interestingly, cell cycle abnormalities, mitotic resumption and meiotic entry, were completely prevented by treating *Nanos2-null* male gonad with RA receptor antagonist AGN193109, suggesting a role for Nanos2 in the suppression of RA signaling. Next, I examined expression of Dnmt3L, a representative downstream factor of Nanos2 required for male specific genomic imprinting. However, I could not observe any recovery of the Dnmt3L expressions. These results indicate that Nanos2 is required for the expression of Dnmt3L independently of maintaining mitotic quiescence.

Next I asked whether the prolonged expression of the pluripotency genes in *Nanos2-null* male germ cells influence the male-type gene expression. To address this question, I employed conditional *Sox2* knocked-out mice ($Sox2^{\Delta/\Delta}$). Because Sox2 is a component of the core transcriptional network in embryonic stem cells (ESCs), I expected that the deletion of *Sox2* in male germ cells would affect the expression of other core transcription factor, *Oct3/4* and *Nanog.* As expected, the additional deletion of *Sox2* in the *Nanos2*-knockout background decreased *Oct3/4* and *Nanog*. I found that the mitotic quiescence was retained in the *Sox2/Nanos2* double mutant male germ cells, indicating that Nanos2-mediated repression of pluripotency genes is required for the maintenance of mitotic quiescence in male germ cells. Furthermore, these gene expressions were repressed in the AGN193109 treated *Nanos2-null* male gonads. These data suggest that Nanos2 maintains the mitotic quiescence through preventing RA signaling which otherwise promotes pluripotency gene expression in male germ cells. However, Dnmt3L expression was not recovered regardless of knocking out of *Sox2* either with or without AGN193109. This result indicates that Nanos2 is required for Dnmt3L expression in addition to repressing RA signaling. Surprisingly, I found that male type-genes including *Nanos2* and *Dnmt3L* were highly expressed in $Sox2^{\Delta/\Delta}$ male germ cells. This observation led me hypothesize that Sox2 is involved in the suppression of the *Nanos2* expression in PGCs.

Based on my hypothesis, in the Chapter II, I conditionally knocked-out the

Sox2 gene from PGCs before *Nanos2* expression begins by injecting tamoxifen at E10.5, which is 2 days earlier than conducted in Chapter I. As expected from the result shown in Chapter I, I observed the Nanos2 up-regulation in the *Sox2-null* germ cells at E12.5 compared with that in the control embryos. The elevated Nanos2 expression was not due to the increased expression of nodal signaling pathway, which is known to induce *Nanos2*. However, the precocious Nanos2 up-regulation had a marginal effect on the expression of male-type genes including Dnmt3L. Next I focused on the cell cycle state, since mitotic arrest is another sign of the male differentiation pathway. In normal male gonads, most of PGCs (70%) were still proliferative at E12.5. I found that more than 90% of *Sox2-null* PGCs entered mitotic arrest at E12.5, indicating that Sox2 is involved in the entry of mitotic quiescence. Since *Nanos2* is precociously expressed in the *Sox2-null* PGCs, it is possible to speculate that the Nanos2 might have induced the mitotic arrest. However, the ectopic Nanos2 expression had moderate effect on the induction of mitotic arrest. To further explore the reason why mitotic arrest is prematurely induced in the absence of Sox2, I focused on a cell cycle regulator, $p15^{INK4b}$ since $p15^{INK4b}$ is strongly up-regulated when PGCs enter mitotic arrest via Larp7-Brd4-mediated pathway. I found that $p15^{INK4b}$ and its upstream activator, *Brd4*, were up-regulated in *Sox2-null* germ cells, indicating that Sox2 regulates $p15^{INK4b}$ via suppressing Brd4.

Based on these results, I finally tested the possibility that male pathway is prematurely promoted if higher dose of Nanos2 is expressed in the absence of Sox2. To this end, I induced exogenous Nanos2 together with knocking out *Sox2* in male germ cells (Tg-Nanos2/$Sox2^{\Delta/\Delta}$). As expected, I found Dnmt3L expression in Tg-Nanos2/$Sox2^{\Delta/\Delta}$ male germ cells earlier than the wild-type, which supported my hypothesis.

Taken together, my study proposed that reciprocal regulation between Nanos2 and Sox2 plays a key role in the male germ cell differentiation pathway. I elucidated that the down-regulation of Sox2 triggers initiation of mitotic arrest and then *Nanos2* is highly expressed in the mitotic quiescent cells. Nanos2 regulates both maintenance of mitotic arrest and down-regulation of Sox2 expression via repressing retinoic acid signaling. These results provide important information to understand the gene regulatory network for male sexual differentiation in mouse germ cells.

2. Gene Symbols

Blimp1: B-lympocyte-induced maturation protein 1

Brd4: Bromodomain containing 4

CAG: A cimbination of the cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter

CAT: Chloramphenicol Acetyltransferase

CDK2: Cyclin-dependent kinase2

Cyp26b1: Cytochrome P450, family 26, subfamily B, polypeptide 1

Dazl: Deleted in AZoospermia

Dmrt1: Doublesex and mab-3 related transcription factor 1

Dnd1: Dead end homolog 1

Dnmt3L: DNA Methyltransferase 3-like

Elk1: ELK1, member of ETS oncogene family

Gapdh: Glyceraldehyde-3-phosphate dehydrogenase

GCNF: Germ cell nuclear factor,Nr6a1

GFP: Green fluorescence protein

Larp7: La ribonucleoprotein domain family, member 7

Miwi2 / Piwi4: Piwi-like RNA-mediated gene silencing 4

Oct3/4: POU domain, class 5, transcription factor 1

pH3 : Phosphorylation of histone H3 (Ser10)

Plzf: Promyelocytic leukaemia zinc finger

Prdm14: PR domain-containing protein 14

PTEN: Phosphatase and Tensin Homolog Deleted from Chromosome 10

Sycp3: Synaptonemal complex protein 3

Sox2: Sex determining region Y box 2

SP1: Trans-acting transcription factor 1

SSEA-1: Stage-specific embryonic antigen-1

Stra8: Stimulated by retinoic acid gene 8

Tdrd1: Tudor domain containing 1

Vasa/Mvh: Mouse vasa homolog

Chapter I

3. Introduction

Germ cells have an important biological role to transmit genomic information from one generation to the next. In the mouse, primordial germ cells (PGCs), the precursor of spermatozoa and oocytes, are identified as a cluster of cells at the base of allantois at around embryonic day (E) 7.25 (Fig 1). Subsequently, PGCs migrate through the gut wall and reach to the genital ridges at E10.5. PGCs have abilities to become either sperm or oocyte until E11.5 (McLaren, 1984). Once entering embryonic gonads, PGCs initiate sexual differentiation toward spermatogenesis or oogenesis around E12.5 depending on the gonadal sexes (Kafri et al., 1992); sexual differentiation of PGCs is instructed by extrinsic cues from the surrounding somatic cells (Colvin et al., 2001; Koopman et al., 1991; Ottolenghi et al., 2007; Uhlenhaut et al., 2009). However the mechanism leading to germ cell sex differentiation is not uncovered yet.

Sexual differentiation of PGCs is associated with distinct cell cycle regulations. In female gonads, retinoic acid (RA) secreted from mesonephros, induces Stra8 expression in PGCs, a key factor required for the induction of premeiotic DNA replication (Lin et al., 2008). Therefore, the female germ cells enter meiosis from E13.5, proceed to meiotic prophase I (leptotene, zygotene and pachytene stages), and subsequently arrest their meiotic cell cycle in the diplotene stage by E17.5 (Borum, 1967). On the other hand, in male gonads, Cyp26b1 that is involved in the inactivation of RA is expressed in somatic cells via Fgf9/Sox9 feed-forward loop from E10.5 (Kashimada et al., 2011). In the *Cyp26b1-deficient* mice, male germ cells abnormally initiate meiosis by the exposure to increased RA (MacLean et al., 2007). Therefore, male germ cells are normally protected from RA. Instead, they enter mitotic arrest after E13.5, and most of male germ cells are arrested in the G_1/G_0 stage at E15.5. The arrested cell cycle status is maintained until birth (Parker and Sheth, 2007).

The factor(s) that induces the mitotic arrest in male germ cells has not been identified. However, two recent studies reported cell cycle regulators involved in the mitotic arrest in male germ cells. Rb1 is a major cell cycle regulator involved in cell proliferation, apoptosis, and cell differentiation, and *Rb1-defecient* male germ cells do not enter mitotic arrest at E14.5 (Spiller et al., 2010). Inhibitors of Cyclin-CDK complex, $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ are up-regulated in the arrested male germ cells at E13.5. Once the CyclinE expression is down-regulated, it remains in

a low level from E14.5 to E15.5 (Western et al., 2008). In particular, the expression of $p15^{INK4b}$ is activated specifically in PGCs from E13.5 and then PGCs undergo mitotic arrest (Okamura et al., 2012).

In addition to cell cycle regulators, a critical factor involved in the sexual differentiation is an RNA-binding protein, Nanos2. The *Nanos* gene is evolutionally conserved and plays important roles in germ cell development (Koprunner et al., 2001; Kurokawa et al., 2006; Mochizuki et al., 2000; Mosquera et al., 1993; Pilon and Weisblat, 1997; Subramaniam and Seydoux, 1999; Tsuda et al., 2003). In the mouse, three Nanos homologs (*Nanos1-3*) are identified (Tsuda et al., 2003). Although *Nanos1* is expressed in the neural system (Haraguchi et al., 2003), *Nanos2* and *Nanos3* are expressed in fetal and postnatal germ cells (Tsuda et al., 2003). While *Nanos3* is expressed in both female and male germ cells, *Nanos2* is expressed only in male germ cells from E12.5 to E15.5 and spermatogonial stem cells (Sada et al., 2009; Tsuda et al., 2003). Nanos2 is required for the survival and differentiation of male germ cells (Suzuki and Saga, 2008). Nanos2 interacts with CCR4-NOT complex (Bartlam and Yamamoto, 2010), and this Nanos2-CCR4-NOT complex localizes to P-bodies, which are known as a site for RNA degradation (Parker and Sheth, 2007; Suzuki et al., 2010; Suzuki et al., 2012), indicating that Nanos2 is involved in the RNA degradation in male germ cells. *Nanos2-null* male germ cells fail to proceed normal male differentiation pathway and lose male specific gene expression including Dnmt3L, which is required for methylation of imprinted genes in germ cells (Suzuki and Saga, 2008). Many genes involved in cell cycle regulation, female germ cell differentiation and maintenance of PGC properties are up-regulated, whereas male-specific genes are down-regulated in *Nanos2-null* germ cells. In addition, it is also shown that Nanos2 has an instructive function to convert germ cell sex; forced expression of Nanos2 in female germ cells results not only in the suppression of female pathway but also in the induction of male-specific genes such as Dnmt3L. Therefore Nanos2 is a necessary and sufficient factor to promote male sexual differentiation. However, the mechanism how Nanos2 promotes male pathway is unknown.

I aimed to uncover the mechanism leading to the male differentiation pathway via understanding events downstream of Nanos2. If the major function of Nanos2 is to repress events observed in *Nanos2-null* germ cells via RNA degradation, we might be able to rescue the *Nanos2-null* phenotype by repressing such events induced by the lack

of Nanos2. Our laboratory has already started this strategy. To prevent abnormal meiosis in *Nanos2-null* male germ cells, *Nanos2/Stra8* double knock out (dKO) mouse was generated, since the prominent phenotype observed in *Nanos2-null* germ cells are reported to enter meiosis (Suzuki and Saga, 2008). This strategy worked well to suppress meiosis abnormally induced in *Nanos2-null*. However, the expression of male-type genes including Dnmt3L was not recovered in the *Nanos2/Stra8 double-null* germ cells (Saba et al., 2013). In addition, the dKO male germ cells resumed mitotic cell cycles even though meiotic entry was suppressed. These data suggest that Nanos2 may promote male differentiation pathway by preventing mitotic activity and/or pluripotency gene expression.

Therefore, I decided to examine the phenotype of *Nanos2-null* male germ cells in more detail, especially by focusing on the cell cycle regulation. Subsequently, I tried to repress mitosis, meiosis and up-regulation of pluripotency genes to ask whether the suppression of these events is sufficient to promote male pathway in the absence of Nanos2.

4. Results

4-1. Most of *Nanos2-null* male germ cells failed to undergo meiotic progression.

It is reported that *Nanos2-null* male germ cells enter meiosis by expressing meiosis-specific proteins, Stra8 and Sycp3 (Suzuki and Saga, 2008). However, I found that the chromosomal localization of Sycp3 observed in *Nanos2-null* male germ cells was different from those in female germ cells (Fig I-2E,H, Suzuki and Saga, 2008). Thus I decided to examine the progression of meiosis in *Nanos2-null* male germ cells in more detail by the means of chromosome spreading methods. Meiotic prophase I is composed of leptotene, zygotene, pachytene, and diplotene stages, and those can be identified by their characteristic chromosomal morphologies (Fig I-2A-D). In addition to Sycp3, I used an antibody to detect phosphorylated histone H2AX (γH2AX) as a marker of DNA double-strand breaks (DSBs) that is a hallmark of leptotene and zygotene stages (Kuo and Yang, 2008). The results of immunocytochmical analyses showed that, while γH2AX signals were observed in female germ cells, the γH2AX signal was never observed in *Nanos2-null* male germ cells at E15.5 (Fig I-2E-J). Furthermore, while female germ cells proceed to the pachytene stage, over 90% of *Nanos2-null* male germ cells exhibited abnormal localization of Sycp3 at E16.5, although I could detect very few numbers of cells showing zygotene stage (Fig I-2K). These data suggest that most of *Nanos2-null* male germ cells do not undergo meiosis.

I did not observe the meiotic progression in *Nanos2-null* male germ cells. However, *Nanos2-null* male germ cells die by apoptosis from E15.5 (Suzuki and Saga, 2008). This raised the possibility that *Nanos2-null* male germ cells died after the initiation of meiosis or during meiotic progression. To prevent apoptosis, I crossed *Nanos2*$^{+/-}$ mouse with *Bax*$^{+/-}$ mouse. Bax is known as a pro-apoptotic gene, and apoptosis can be repressed in the *Bax-null* genetic background (Knudson et al., 1995). I observed that, whereas female germ cells proceeded to the diplotene stage by E17.5, over 90% of *Nanos2/Bax* dKO male germ cells did not show normal meiotic progression and still exhibited abnormal staining pattern of Sycp3 as observed in *Nanos2-null* male germ cells (Fig I-2L). These data suggest that apoptosis is not a cause of the failure of meiotic progression in *Nanos2-null* male germ cells.

4-2. Nanos2 is required for maintaining quiescent state of male germ cells

Because I found that most of *Nanos2-null* male germ cells failed to undergo meiosis, I

suspected that they failed to maintain mitotic arrest and resumed mitosis without entering meiosis. It is previously reported that signal of pH3, an M phase marker in the mitotic cells (Van Hooser et al., 1998), is observed in *Nanos2-null* male germ cells, indicating that *Nanos2-null* male germ cells resumed mitosis (Suzuki and Saga, 2008). However, detailed quantitative analyses to distinguish mitosis and meiosis were not conducted yet.

To address the issue, I employed two markers: Rec8, an early marker of meiosis and pH3, a marker of mitosis. Rec8 is known as meiosis-specific kleisin and is required for proper chromosome disjunction in meiosis (Watanabe, 2004). First I examined staining patterns of Rec8 and pH3 in wild-type female and male germ cells at E15.5. All female germ cells were positive for Rec8 but negative for pH3, indicating that these cells entered meiosis (Fig I-3A-C’’’). In male germ cells, I observed two staining patterns: Rec8/pH3 double negative and pH3 single positive. I considered the Rec8/pH3 double negative cells to be quiescent cells (Fig I-3D-D’’’) and the pH3 single positive cells as proliferating cells (Fig I-3E-E’’’). Then I analyzed *Nanos2-null* male germ cells and found four staining patterns (Fig I-3F-I’’’). In addition to the quiescent cells (Fig I-3F-F’’’) and proliferating cells (Fig I-3G-G’’’), I also observed Rec8 single positive (Fig I-3H-H’’’) and Rec8/pH3-double positive cells (Fig I-3I-I’’’). I considered the former as cells that had entered meiosis as expected from previous results. However, I never observed REC8/pH3 double positive cells in neither control male nor female germ cells. Next, I counted the number of each type of cells at E14.5 and E15.5 for both wild-type and *Nanos2-null* male germ cells. I found that more than 80 % of cells were Rec8/pH3 double negative in either genotype at E14.5. However, while the higher ratio of quiescence (Rec8/pH3 double negative) was kept in control male germ cells (Fig I-3J), the ratio was decreased, and instead the pH3-single positive (proliferating) population was elevated at E15.5 in *Nanos2-null* male germ cells (Fig I-3K). Meiotic cells were observed in the *Nanos2-null* at E14.5 but the ratio was not increased at E15.5 and thereafter (Fig. I-3J, K). These data indicate that *Nanos2-null* male germ cells entered mitotic arrest by E14.5 but they resumed mitosis from E15.5, suggesting that the lack of Nanos2 induces mitosis rather than meiosis.

4-3. Nanos2 indirectly controls mitotic arrest through transcriptional regulation of cell cycle inhibitors.

To understand the mechanism that triggers mitotic resumption in *Nanos2-null* male germ cells, I examined expression levels of CyclinE, Cip/Kip and INK4 family genes in germ cells, because these regulators were implicated in the mitotic arrest in male germ cells (Trautmann et al., 2008; Western et al., 2008) (Fig I-4A). For this purpose, I introduced Oct4-dPE-GFP reporter transgene in the *Nanos2*-hetero and -homo genetic background via crossing with Oct4-dPE-GFP transgenic mouse as a germ cell marker (Yoshimizu et al., 1999). GFP-positive cells were isolated by fluorescence activated cell sorter from control (wild-type or *Nanos2*-hetero) and *Nanos2-null* male gonads, and the efficiency of the cell sorting estimated was 95-98% according to the GFP signals. I also confirmed that the expression of a germ cell-specific marker, Vasa, was detected only from RNAs prepared from GFP-positive cells by quantitative real time polymerase chain reaction (qRT-PCR) (Fig I-4B). I then examined expression levels of the cell cycle regulators by qRT-PCR. The expression level of *CyclinE1* did not change between control and *Nanos2-null* male germ cells from E13.5 to E15.5 (Fig I-4C). However, *CyclinE2* was significantly increased in *Nanos2-null* male germ cells at E14.5 (Fig I-4D). Furthermore, $p15^{INK4b}$ and $p27^{Kip1}$, negative regulators of CyclinE-CDK2 complex activation, were significantly down-regulated in *Nanos2-null* male germ cells compared with control male germ cells (Fig I-4E-I).

Considering that Nanos2 is involved in the RNA degradation, target RNAs should be up-regulated in the *Nanos2-null* germ cells. To investigate the possibility that cell cycle regulator(s) is directly regulated by Nanos2, I searched the list of Nanos2 interacting RNAs from RNA-sequence data analyzed in our laboratory. Although I could not find out CyclinE family in the list, I found three genes (*Sp1*, *Max*, and *Elk1*) involved in cell cycle regulation. Sp1 promotes the transition from G_1/G_0 to S phase by releasing pRB from E2F family through the directly interaction with E2F1, E2F2 and E2F3 (Karlseder et al., 1996). Max interacts with Myc, and the hetero dimmer regulates cell cycles and apoptosis (Amati et al., 1993). Elk-1 directly activates the $p21^{Cip1}$ and is involved in p53-independent Bax gene expression (Shin et al., 2011). I tested whether these RNAs were up-regulated by a post-transcriptional regulation using specific primer sets to distinguish the unspliced and spliced RNAs, because Nanos2 binds to target RNA(s) for RNA degradation in the cytoplasm. However, I did not observe up-regulation of those mRNA for either spliced or unspliced products (Fig I-4J-O). Therefore, I could not find possible candidates for Nanos2 target in cell cycle regulators,

suggesting that Nanos2 indirectly regulates cell cycle regulator.

4-4. Retinoic acid signaling is responsible for the resumption of mitosis in male germ cells.

Although I could not find direct involvement of Nanos2 in the cell cycle regulation, a major and primary function of Nanos2 could be the maintenance of cell cycle arrest. Therefore, I expected that if I could suppress cell cycle progression of *Nanos2-null* germ cells, these cells would recover the ability to differentiate to prospermatogonia. Since previous reports revealed that RA prevented mitotic arrest and induced meiotic entry in male germ cells *in vitro* and *in vivo* (Koshimizu et al., 1995; Trautmann et al., 2008), I hypothesized that I might be able to rescue *Nanos2-null* phenotype by repressing RA signaling.

To test this possibility, I prepared control and *Nanos2-null* male gonads from embryos at E12.5 and cultured them for 3 days in the presence and absence of RA receptor antagonist, AGN193190 (Carmell et al.; Trautmann et al., 2008). I analyzed cell cycle status by using anti-KI67 antibody that detects all active phases of the cell cycle except for G_0 phase (Endl and Gerdes, 2000). Immunofluorescence analysis revealed that control male germ cells were negative for KI67, indicating the arrested cell cycles regardless of the presence or absence of AGN193190 (Fig I-5A-F). As expected, *Nanos2-null* male germ cells resumed mitosis when they were cultured without AGN193190 (Fig 5G-I). Interestingly, I found that *Nanos2-null* male germ cells maintained mitotic arrest as similar to control male germ cells in the presence of AGN193190 (Fig I-5J-M). These data indicate that RA induces the resumption of mitosis in *Nanos2-null* male germ cells.

4-5. Male-type gene expression was not rescued by the repression of RA signaling.

I successfully rescued mitotic arrest in *Nanos2-null* male germ cells by culturing *Nanos2-null* male gonads with AGN193190 (Fig I-5). This raises the possibility that male differentiation pathway might also be rescued in *Nanos2-null* male germ cells in this culture condition.

I then examined Dnmt3L expression by immunofluorescence and qRT-PCR analyses. Dnmt3L was expressed in control male germ cells cultured with or without AGN193190 (Fig I-6A-F). As predicted, Dnmt3L was not expressed in *Nanos2-null*

male germ cells cultured without AGN193190 (Fig I-6G-I). However, the Dnmt3L signal was not observed even when *Nanos2-null* male germ cells were cultured with AGN193190 (Fig I-6J-L). qRT-PCR analysis also showed that *Dnmt3L* expression was not rescued in *Nanos2-null* male germ cells cultured with AGN193190 (Fig I-6M). These data showed that suppression of both mitosis and meiosis was not sufficient to promote male-type gene expression in the absence of Nanos2.

4-6. Sox2 negatively regulates mitotic cell cycles in male germ cells.

Previous studies indicated that the expression of pluripotency genes, *Oct3/4, Nanog*, and *Sox2*, were maintained in *Nanos2-null* male germ cells compared with those in control (Saba et al., 2013) (Fig I-7A-C). Therefore, I assumed that this prolonged pluripotency gene expression might have prevented male type-gene expression including Dnmt3L in *Nanos2-null* male germ cells. To test this possibility, I analyzed *Sox2/Nanos2* dKO male germ cells by using Sox2 conditional knockout mice with *Rosa26-CreER*^{T2} (Cre recombinase can be induced ubiquitously) (*Sox2*$^{\Delta/\Delta}$). I injected tamoxifen by intraperitoneal administration to pregnant female mice at E12.5, collected male gonads at E13.5, and then cultured them for 48 hours (Fig I-8A), because Sox2 knockout mice are embryonic lethal (Campolo et al., 2013). I checked mRNA expression levels of *Oct3/4* and *Nanog* because these pluripotency genes reciprocally regulate each other in ESCs (Boyer et al., 2005; Loh et al., 2006). Indeed, *Oct3/4* and *Nanog* were down-regulated in *Sox2*$^{\Delta/\Delta}$ male germ cells, although the expression of these genes was not so altered at this cultured condition in *Nanos2-null* gonads (Fig I-8C,D). Therefore, I successfully suppressed all pluripotency genes examined by knocking out the *Sox2* gene.

I then examined whether cell cycle state was influenced by the down-regulation of pluripotency genes. I found that control and *Sox2*$^{\Delta/\Delta}$ male germ cells had entered mitotic arrest as similar to those observed *in vivo* at E15.5 by using KI67 marker (Fig I-8E-H), whereas *Nanos2-null* germ cells were proliferative as shown before (Fig I-5G-L). Interestingly, the mitotic resumption of *Nanos2-null* male germ cells was suppressed by the additional removal of Sox2 (Fig. I-8H), indicating that up-regulation of Sox2 could be responsible for the mitotic resumption observed in the *Nanos2-null* germ cells. I have already showed that RA receptor antagonist, AGN193190, also prevented mitotic resumption of *Nanos2-null* germ cells (Fig. I-5L),

indicating that RA might be responsible for the up-regulation of Sox2 in *Nanos2-null* germ cells.

Since I succeeded to repress mitotic resumption and up-regulation of pluripotency genes by knocking out Sox2, I hypothesized that *Nanos2-null* germ cells might have entered male differentiation pathway. However, Dnmt3L expression was not recovered at all in *Nanos2/Sox2* dKO (Fig. I-8O), indicating that simultaneous suppression of mitosis and up-regulation of pluripotency genes are not sufficient to rescue the *Nanos2* deficiency. I next added AGN193190 to the culture medium to ask whether any additive effect of the suppression of RA signaling in addition to the lack of Sox2 is observed. In control male germ cells, Dnmt3L was expressed even if gonads were cultured with or without AGN193190 (Fig. I-8I,J). However, I could not observe Dnmt3L expression in *Sox2/Nanos2* dKO male germ cells cultured with AGN193190 (Fig I-8P). I also used qRT-PCR to examine other male-type gene expressions such as *Tdrd1* and *Miwi2*. Both Tdrd1 and Miwi2 are required for repression of transposable elements and also implicated in Piwi-interacting RNA metabolism during meiosis in spermatogenesis (Bao et al., 2014; Carmell et al., 2007; Chuma et al., 2006; Wang et al., 2009). But no clear rescue was observed in the absence of Nanos2 (Fig. I-8S,T). Interestingly, however, I noticed that *Nanos2, Dnmt3L* and *Tdrd1* gene expressions were up-regulated in the $Sox2^{\Delta/\Delta}$ male germ cells (Fig I-8Q-S), suggesting that Sox2 negatively regulates male-type gene expression. I also found that similar up-regulation of *Nanos2* and *Tdrd1* was observed in the control gonad cultured with AGN193190 (Fig. I-8Q,S). These data suggest that RA signaling might influence germ cell differentiation through controlling *Sox2* gene expression.

5. **Discussion**

The aim of this Chapter was to understand mechanisms leading to male germ cell differentiation pathway by adopting a strategy to rescue the phenotypes of *Nanos2-null* germ cells. In the absence of Nanos2, mitosis is resumed, meiosis is initiated, and pluripetency genes are up-regulated. I succeeded to suppress all these abnormalities in *Nanos2-null* germ cells. However, I could not observe the promotion of male-type gene expression in these conditions. Therefore I concluded that Nanos2 is an indispensable factor for germ cells to become prospermatogonia. Nevertheless, this analysis uncovered important issues regarding how Nanos2 works. I found that Nanos2 maintains male germ cell quiescence via suppressing RA signaling. In addition, I obtained evidence indicating that RA signaling regulates Sox2 expression, and the down-regulation of Sox2 is required to maintain the quiescent state of male germ cells.

Nanos2 maintains mitotic arrest by suppressing retinoic acid signaling

Previous report suggested that Nanos2 is required for promoting male differentiation pathway through maintenance of mitotic arrest other than suppressing meiosis (Saba et al., 2013). In my thesis study, I found that Nanos2 regulates mitotic arrest via suppressing RA signaling in male germ cells.

It has been unknown how Nanos2 regulates the mitotic arrest. I hypothesized that Nanos2 suppresses some activator(s) of mitosis. However, I could not find possible cell cycle regulators among mRNAs associated with Nanos2. Therefore it is likely that Nanos2 represses some factors acting upstream of such cell cycle regulators. It is reported that normal mitotic arrest is disrupted in male germ cells if those are cultured with RA at E11.5, E12.5 or E13.5 (Trautmann et al., 2008). In addition to the *in vitro* (culture) assay, mitotic arrest was also prevented when RA was administrated at E13.5 *in vivo* (Trautmann et al., 2008). In male gonads, Cyp26b1 is expressed in somatic cells from E10.5 to E12.5 and degrades RA (MacLean et al., 2007). *Cyp26b1-deficient* male germ cells show premature expression of meiotic markers instead of male-type gene expression due to the exposure to increased RA (MacLean et al., 2007). These experiments had confirmed that RA disrupted mitotic arrest of male germ cell and also induced meiotic initiation, indicating that RA has negative effect on male differentiation pathway. I also confirmed that RA signaling is involved in the mitotic resumption in *Nanos2-null* germ cells. How Nanos2 represses RA signaling is an open question.

Previous report elucidated that RA induces PI3K activity in F9 cells (Bastien et al., 2006) . In addition, PTEN, a lipid phosphatase antagonist of PI3K function, appears to be required for mitotic arrest and undergo mature characters of PGCs, because *PTEN-null* PGCs failed to mitotic arrest and then die from apoptosis after E14.5 (Kimura et al., 2003). One of the Nanos2 targets might be involved in the PI3K signaling pathway.

Nanos2 may suppress Sox2 via repressing RA signaling

One of important findings in this chapter is that mitotic resumption in *Nanos2-null* germ was also repressed by knocking out of Sox2 even without RA antagonist. This suggests that increased expression of *Sox2* is a cause of the mitotic resumption in *Nanos2-null* germ cells. Interestingly, the *Sox2* expression was repressed in the male gonads treated with RA receptors antagonist, suggesting that RA signaling and Sox2 are closely linked to resume cell cycles. Before discussing this possibility, I would like to discuss about relationship between Nanos2 and Sox2.

Sox2 is one of pluripotency-conferring genes and is expressed in PGCs from early state of embryos. The functional importance of Sox2 in PGC maintenance is reported previously. In the absence of Sox2, PGC number is dramatically reduced and PGCs die by apoptosis, indicating that Sox2 is involved in the proliferation of PGCs (Campolo et al., 2013). However, the function of Sox2 in the germ cells after sexual differentiation has not been addressed. Previous studies conducted in my laboratory demonstrated that Nanos2 works in the RNA degradation pathway and major Nanos2 targets must be up-regulated in the absence of Nanos2. Based on the data, Sox2 expression in *Nanos2-null* germ cells is maintained higher than that of wild-types. In addition, Nanos2-immunoprecipitation study indicates that Sox2 RNA has affinity to Nanos2-RNA complex (data not shown). However, I could not test whether Sox2 RNA is regulated by a post-transcriptional mechanism since Sox2 is encoded by a single exon, which makes difficult for me to distinguish primary transcript from the mature one. Nevertheless, I favor to consider that Nanos2 indirectly regulates *Sox2* gene expression via repressing RA signaling pathway, because pluripotency gene expression was down-regulated in male germ cells cultured with RA receptor antagonist (Fig I-8B-D), in which mitotic activity was also suppressed. In addition, at least *Sox2* and *Nanog* expression is up regulated in the *Cyp26b1-null* gonads (Saba et al., 2014). I speculate

that male germ cells entered quiescent state in the absence of RA by the function of Cyp26b1. Once Nanos2 is induced, Nanos2 functions to protect male germ cells from RA signaling, thereby the expression of pluripotency gene is suppressed and it contributes to the maintenance of quiescent state.

Nanos2 is essential for promoting male differentiation pathway

The aim of this study was to determine the conditions for promoting male differentiation pathway in mouse germ cells. To test male pathway promotion, I examined Dnmt3L as a male representative gene. Nanos2 can promote *Dnmt3L* expression independently of the somatic environment, because Dnmt3L is induced in Nanos2 over-expressing female germ cells (Suzuki and Saga, 2008). One of possible explanation is that Nanos2 regulates negative regulator(s) of Dnmt3L expression. The regulatory mechanism of Dnmt3L in male germ cells is not uncovered and no transcriptional regulator is identified. Recent study indicates that Dnmt3L transcription is tightly regulated by multiple layers of repressive epigenetic modifications (Zamudio et al., 2008). I suspect such an epigenetic factor may be included in the Nanos2 targets. Identification of such target RNAs and further functional analyses of genes downstream of Nanos2 are required to fully understand how Nanos2 promotes Dnmt3L expression.

Although I could not observe promotion of male-type gene expression in *Nanos2/Sox2* double null germ cells, I found that *Nanos2* is highly expressed in $Sox2^{\Delta/\Delta}$ male germ cells, when I deleted Sox2 expression from E12.5 using conditional-KO strategy. In addition, I found that Sox2 deficiency induced cell cycle arrest in *Nanos2-null* germ cells, which inspire me to consider an important possibility; there is some condition favorable for *Nanos2* induction and Sox2 might be a factor regulating such a condition and it may also contribute to promoting male pathway. In the Chapter-II, I will deal with this issue to clarify the function of Sox2 as one of upstream factor of Nanos2.

Chapter II

6. Introduction

While Oct3/4, Sox2, and Nanog are transcriptional factors that play crucial roles in the maintenance of pluripotency in ESCs through governing a transcriptional network (Avilion et al., 2003; Takahashi and Yamanaka, 2006; Zuccotti et al., 2008), these factors are also involved in various developmental processes. Oct3/4 is required for mouse tooth development and spermatogonial stem cell self-renewal (Dann et al., 2008; Nakagawa et al., 2012). Sox2 is essential for maintaining self-renewal of trophoblast stem cells, cancer stem cells and neural stem cells and is also required for forming eye lens development together with transcription-factor Pax6 (Adachi et al., 2013; Kamachi et al., 2001; Leis et al., 2012; Pevny and Nicolis, 2010). Nanog is required for primitive endoderm formation and the gateway to the pluripotent ground state (Dann et al., 2008; Messerschmidt and Kemler, 2010).

These transcription factors also play indispensable roles in the development of PGCs. Oct3/4 is expressed in the embryo proper until E7.5, thereafter, is specifically expressed in germ cells (Kehler et al., 2004). Oct3/4 is required for PGCs survival because conditional knock out of Oct3/4 in migrating PGCs result in the death of PGCs by apoptosis (Okamura et al., 2008). Nanog is expressed in the epiblast, primitive streak and PGCs (Chambers et al., 2007; Yamaguchi et al., 2009). Nanog plays a key role in the proliferation and survival of migrating PGCs (Chambers et al., 2007). The expression of Sox2 in PGCs begins from E6.75 in PGC precursors (Avilion et al., 2003; Masui et al., 2007; Western et al., 2010), and Sox2 is required for the survival of PGCs (Campolo et al., 2013). After PGCs reach the gonads, these factors show distinct expression profiles depending on the sex of PGCs. In female gonads, these gene expressions quickly decline concomitant with the initiation of meiosis. On the other hand, in male gonads, the expression of *Oct3/4* continues throughout embryonic stages, and its expression is maintained in spermatogonial stem cells in the postnatal testis. *Sox2* and *Nanog* retain higher expression until E13.5 but its expression gradually decrease thereafter (Western et al., 2010). Whereas the male-specific extended expression of the genes implies the involvement of the genes in male germ cell development, its significance remains unknown.

In the Chapter I, I demonstrated that the male-type gene expression was not recovered even though the abnormal mitotic resumption was prevented in *Nanos2* and

Sox2 dKO male germ gonads. Surprisingly, however, I found increased expression of male-type genes in $Sox2^{\Delta/\Delta}$ male gonads than control male gonads. These results motivated me to test a possibility that pluripotency genes are involved in the negative regulation of the male-type gene expression to control the timing of the initiation of male germ cell development.

To address this issue, I conditionally knocked-out Sox2 in male gonads in the earlier stage and examined the influences of precocious deletion of Sox2 on the timing of male-type gene expression and the entry into the mitotic quiescence. I show that Sox2 is involved in the negative regulation of *Nanos2* and of the entry into the mitotic quiescence.

7. Results

7-1. Sox2 represses the *Nanos2* expression independently of Nodal/Activin signaling pathway.

To examine whether Sox2 represses male-type gene expression *in vivo*, I generated $Sox2^{\Delta/\Delta}$ embryos by crossing $Sox2^{flox/flox}$ mice with $Sox2^{flox/flox}$;$Rosa26\text{-}CreER^{T2}$ mice. Tamoxifen was administrated into the pregnant females at E10.5, and male gonads were harvested at E12.5 and E13.5. The results of qRT-PCR showed that mRNA expression of *Sox2* was successfully down-regulated at E12.5 and the low level of *Sox2* expression was retained until E13.5 in $Sox2^{\Delta/\Delta}$ male gonads (Fig II-1A). I found that mRNA expression of *Nanos2* was increased more than eight-fold in $Sox2^{\Delta/\Delta}$ male gonads than control male gonads at E12.5, and the elevated expression was retained at E13.5 (Fig II-1B). Immunofluorescence analysis showed that Nanos2 protein was weakly detected in male germ cells from E12.5 only in $Sox2^{\Delta/\Delta}$ male germ cells (Fig II-1C, D), whereas it became detectable in control male germ cells from E13.5 (Fig II-1E, F). These results suggest that Sox2 is involved in the repression of Nanos2 in male germ cells.

Our laboratory previously showed that nodal signaling is involved in the induction of *Nanos2* expression (Wu et al., 2013). To ask whether Sox2 represses *Nanos2* through affecting nodal signaling, I examined the expression of *Nodal* and the target genes *Lefty1/2* in $Sox2^{\Delta/\Delta}$ male germ gonads. However, both *Nodal* and *Lefty1/2* were not significantly changed in $Sox2^{\Delta/\Delta}$ male germ gonads (Fig II-2A-C). These results suggest that Sox2 represses Nanos2 independently of Nodal/Activin signaling.

7-2. Deletion of Sox2 gene marginally up-regulates male-type genes.

To examine whether the precocious Nanos2 expression in $Sox2^{\Delta/\Delta}$ male gonads promotes male-type differentiation earlier than normal male gonads, I analyzed the expression of male-type genes in $Sox2^{\Delta/\Delta}$ male gonads. The results of qRT-PCR showed that *Dnmt3L, Tdrd1* and *Miwi2* were increased in $Sox2^{\Delta/\Delta}$ male gonads at E12.5, although the levels were lesser than that of *Nanos2*. At E13.5, *Dnmt3L* and *Tdrd1* were similarly expressed between control and $Sox2^{\Delta/\Delta}$ male gonads, whereas the mRNA level of *Miwi2* was slightly up-regulated in the mutant (Fig II-3A-C). As expected from qRT-PCR data, Dnmt3L and Tdrd1 protein were not observed until E13.5 in $Sox2^{\Delta/\Delta}$ male gonads, even though its mRNA was precociously induced in the mutant (Fig II-3D-M). Because $Sox2^{\Delta/\Delta}$ mice die by E14.5 probably due to the somatic defects

induced by the deletion in embryo, I could not examine their expression at this stage. These results indicate that loss of Sox2 has marginal effect on the male-type gene expression despite its deletion significantly induces the *Nanos2* expression.

7-3. Deletion of Sox2 may promote *Nanos2* expression via inducing precocious mitotic entry.

Next I examined whether $Sox2^{\Delta/\Delta}$ male germ cells precociously enter the mitotic arrest or not. Immunofluorescence analysis using anti-KI67 antibody (Fig II-4A,C) revealed that the proportion of mitotic cells was dramatically decreased to 8.9% in $Sox2^{\Delta/\Delta}$ male germ cells, whereas 69.0% of germ cells were KI67-positive in control male germ cells (Fig II-4E). These results indicate that down-regulation of Sox2 is involved in the entry of the mitotic quiescence. The precocious entry to mitotic arrest in $Sox2^{\Delta/\Delta}$ male germ cells may be due to the stronger expression of *Nanos2*. To test this possibility, I conducted in situ hybridization to detect *Nanos2* mRNA together with anti-KI67 staining (Fig II-4F-I). I found that while *Nanos2* was expressed in 12.5% of mitotic cells, it was highly expressed in 27.6 % of quiescent cells in the control (Fig II-4J). Intriguingly, 88.8% of the quiescent cells expressed *Nanos2* in $Sox2^{\Delta/\Delta}$ male germ cells (Fig II-4J). These results indicate that cell cycle state is correlated with *Nanos2* transcription initiation.

Since I observed the *Nanos2* expression even in the KI67-positive mitotic cells in the control gonads, I suspected that *Nanos2* might be induced independently of mitotic state, but Nanos2 may function to repress mitosis once it is induced. To examine this possibility, I conducted a forced expression experiment of Nanos2 using *CAG-floxed-CAT-floxed-3xFlagNanos2* transgenic mouse line (*Tg-Nanos2)* crossed with $Rosa26\text{-}CreER^{T2}$ (Suzuki et al., 2007). I injected tamoxifen at E10.5 and confirmed *Nanos2* expression regardless of KI67 signals (Fig II-4G,J). I observed that 42.9% of germ cells did not enter to mitotic arrest even though *Nanos2* expression was observed in 80.4% of KI67-positive germ cell (Fig II-4G,J), indicating that Nanos2 has only minor effect for the induction of mitotic entry. The ratio of KI67-positive cells was reduced up to 3.6% of germ cells in $Sox2^{\Delta/\Delta}$*/Tg-Nanos2* embryos (Fig II-4I,J). In any genetic backgrounds, more than 93.6% of KI67-negative cells exhibited Nanos2 expression（Fig II-4I,J）. These data indicate that deletion of Sox2 rather than the expression of Nanos2 triggers initiation of mitotic arrest in male germ cells. These data

also suggest that Sox2 deficiency promotes premature mitotic arrest, and this quiescence state may promote *Nanos2* expression.

7-4. Sox2 negatively regulates mitosis by suppressing p15^{INK4b} pathway.

To investigate the cause of precocious mitotic quiescence in *Sox2*$^{\Delta/\Delta}$ male germ cells, I focused on a cell cycle regulation mechanism involved in the mitotic arrest in male sexual differentiation. I examined the *p15*^{INK4b} expression because it is reported that p15^{INK4b} is activated specifically in PGCs from E13.5 resulting in the entry into the mitotic arrest (Okamura et al., 2012). The results of qRT-PCR showed that mRNA expression of *p15*^{INK4b} was significantly increased in *Sox2*$^{\Delta/\Delta}$ male gonads at E12.5 (Fig II-5A). Furthermore, immunofluorescence analysis revealed that P15^{INK4b} was intensively detected in the *Sox2*$^{\Delta/\Delta}$ male germ cells, whereas P15^{INK4b} was hardly detectable in control male germ cells (Fig II-5B,C). These data indicate that Sox2 negatively regulates *p15*^{INK4b} expression.

On the other hand, I had observed that *p15*^{INK4b} was suppressed in the *Nanos2-null* germ cells (Fig I-4H). To examine a possibility that the increased expression of *p15*^{INK4b} in the *Sox2*$^{\Delta/\Delta}$ germ cells is mediated by premature induction of Nanos2, I analyzed the *p15*^{INK4b} expression in male gonads, in which Nanos2 was induced using *Tg-Nanos2* mice crossed with *Rosa26-CreER*^{T2}. I injected tamoxifen at E10.5, collected male gonad from E12.5 to E14.5. I confirmed higher *Nanos2* expression in comparison with control from E12.5 male germ cells (Fig II-5D). However, the *p15*^{INK4b} expression was not up-regulated in the *Tg-Nanos2* (Fig II-5E). Consistent with this, *Sox2* was not repressed in the *Tg-Nanos2*, whereas other two pluripotency genes were decreased (Fig. II-5G,H). These data indicate that Nanos2 is not involved in the regulation of p15^{INK4b}. Since the Sox2 expression is up-regulated in the *Nanos2-null* germ cells, I also checked the possibility that Nanos2 has any direct effect on the expression of prulipotency genes by conducting a forced expression experiment of Nanos2. The results showed that the *Tg-Nanos2* did not affect expression levels of *Sox2*, suggesting that *Sox2* up-regulation observed in the *Nanos2-null* germ cells was not direct effect of Nanos2 which is consistent with idea that Sox2 acts upstream of Nanos2 (Fig II-5F). However, the other pluripotency genes including *Oct3/4* and *Nanog* were down-regulated in the *Tg-Nanos2* gonads (Fig II-5G,H), indicating that *Oct3/4* and *Nanog* might be suppressed by Nanos2. These data suggest

that up-regulation of *p15*^{INK4b} is induced by the lack of Sox2 that is the cause of precocious entry to mitotic arrest in male germ cells.

7-5. Sox2 regulates p15^{INK4b} expression via control of Brd4 expression.

To understand the mechanism leading to the up-regulation of p15^{INK4b} in *Sox2-null* germ cells, I examined expression of Brd4, an activator of *p15*^{INK4b} through direct binding to the transcription start site (Okamura et al., 2012). If Sox2 regulates p15^{INK4b} expression via Brd4, *Brd4* expression is expected to be up-regulated in *Sox2*$^{\Delta/\Delta}$ male germ cells. To address this question, I performed qRT-PCR in *Sox2*$^{\Delta/\Delta}$ male germ cells. The result of qRT-PCR showed that the amount of *Brd4* mRNA was significantly increased at E12.5 (Fig II-6A). Again I confirmed that the *Tg-Nanos2* had no effect on the *Brd4* expression (Fig II-6B). These data suggest that Sox2 negatively regulates *p15*^{INK4b} expression via suppressing *Brd4* expression independently of Nanos2.

7-6. Both mitotic arrest and Nanos2-dependent pathway are required for promoting male-type gene expression.

The above observations including the results of the Chapter-I strongly suggest that sexual differentiation of male germ cells are promoted by, at least, two distinct molecular cascades; the entry into the mitotic quiescence induced by the down-regulation of Sox2 and following male-type gene expression induced by the up-regulation of Nanos2. To test this hypothesis, I generated a condition for male germ cells, in which Sox2 is deleted after E10.5 to induce precocious entry to mitotic arrest and ectopic Nanos2 induction. For this purpose, I crossed *Sox2*$^{flox/flox}$; *CAG-floxed-CAT-floxed-3xFlagNanos2* female with *Sox2*$^{flox/flox}$*; Rosa26-CreER*^{T2} male (Fig II-7A). I injected tamoxifen to pregnant female mice at E10.5, collected gonad at E13.5. The results of qRT-PCR analyses showed that expressions of *Nanos2, Tdrd1* and *Miwi2* were significantly increased in *Tg-Nanos2* compared with control (Fig II-7B,D). However, the expression levels of *Miwi2* and *Tdrd1* were not altered between *Tg-Nanos2* and *Sox2*$^{\Delta/\Delta}$*/Tg-Nanos2* (Fg II-7D, E). Although qRT-PCR analysis of *Dnmt3L* showed no significant difference between control and *Tg-Nanos2*, the significant difference was observed between *Tg-Nanos2* and *Sox2*$^{\Delta/\Delta}$*/Tg-Nanos*2 (Fig II-7C). Furthermore, immunofluorescence analysis revealed that Dnmt3L signal was detected in *Sox2*$^{\Delta/\Delta}$*/Tg-Nanos2*, whereas the protein was not observed in control,

Tg-Nanos2, and *Sox2*$^{\Delta/\Delta}$ male germ cells (Fig II-7F-M). These data suggest that the increased Nanos2 expression in the absence of Sox2 further promotes male differentiation process associated with premature Dnmt3L expression.

8. Discussion

Sox2 and Nanog play an essential role in the maintenance of PGCs before sexual differentiation takes place (Avilion et al., 2003; Chambers et al., 2007; Kehler et al., 2004; Masui et al., 2007; Western et al., 2010; Yamaguchi et al., 2009). Concomitant with the onset of sexual differentiation of male germ cells, these gene expressions are gradually down-regulated after E13.5 and reaches to a low level at E15.5. Therefore, I suspected that down-regulation of the pluripotent genes correlates with the commitment to male-type differentiation of PGCs. In this Chapter, I aimed to clarify the role of the pluripotency genes in sexual differentiation of male germ cells by analyzing the conditional $Sox2^{\Delta/\Delta}$ male germ cells. I found that the deletion of *Sox2* precociously induced the *Nanos2* expression. Moreover, $Sox2^{\Delta/\Delta}$ male germ cells enter the mitotic arrest earlier than normal male germ cells. The precocious mitotic arrest is likely achieved by the elevated expression of $p15^{INK4b}$ and *Brd4*. Hence the controlled down-regulation of Sox2 determines the timing of the entry leading to the mitotic quiescence in male germ cells, which in turn may affect the male reproductivity because decreasing numbers of embryonic male germ cells will result in the decreased spermatogonial stem cell reserve in postnatal testis.

How does Sox2 regulate cell cycle in male germ cells?

The most striking discovery in my thesis is that *Sox2-defecinet* male germ cells prematurely initiated mitotic arrest, one of the first events in the sexual differentiation of male germ cells. As I described in the Chapter-I, PGC proliferation is positively regulated by RA signaling and the decreased RA signaling by the function of Cyp26b1 in the male gonad is responsible for germ cells to enter mitotic arrest. The question is how RA signaling promotes PGC proliferation and how Sox2 is involved in this process. It is known that Kit (a tyrosine-protein kinase receptor) plays important roles in the cell cycle regulation via activating PI3K/AKT signaling pathway during spermatogenesis. Kit is also expressed in PGCs (Whyley, 1997). Interestingly, it was shown that the transcription of *Kit* is regulated by Sox2 through the direct binding to *Kit* enhancer at E8.0 (Rossi, 2013). I thus speculate that Sox2 promotes PGC proliferation by activating *Kit* transcription under the control of RA signaling. It is already known that Kit expression is positively correlated with KI67 signals in male germ cells (Saba et al. 2014). Very recently, it is reported that a transcription factor, DMRT1 directly inhibits

Sox2 expression in the male germline stem cells (Krentz et al., 2009) (Krentz et al., 2009). DMRT1 is also expressed in the embryonic germ cells but the lack of this gene in PGCs results in different phenotype depending on the genetic background. It induced teratoma formation in 129 mice but it also induced germ cell-loss phenotypes in C57BL/6J mice (Krentz et al. 2009). Further analyses using different genetic background might be required to clarify the relationship between DNMT1 and Sox2 in the mitotic arrest.

Mitotic arrest may trigger activation of *Nanos2*.

I found that mitotic arrest prematurely occurred in $Sox2^{\Delta/\Delta}$ male germ cells (Fig II-5). In addition, *Nanos2* transcripts were detected in the most of KI67-negative quiescent cells in $Sox2^{\Delta/\Delta}$ male germ cells (Fig II-4J), indicating that the *Nanos2* transcription is induced as a consequence of mitotic arrest and the quiescent state might be required for *Nanos2* induction. However, though I observed *Nanos2* transcripts in most of KI67-negative cells in $Sox2^{\Delta/\Delta}$ male germ cells, the transcription was also observed in some KI67-positive cells in the wild-type germ cells. This observation indicates that the quiescent state is not the required condition for the induction of *Nanos2* but some cell cycle state may be involved in the transcriptional activation. Previous report indicates that fluorescence intensity of KI67 signal was classified into low and high groups depending on the cell cycle state (Scalzo et al., 1998). Indeed, I also noticed that KI67-high or KI67-low population in the control gonads and I found the tendency of higher *Nanos2* mRNA in the KI67-low cells but I did not distinguish the difference in my current quantitative results. I need to use other markers to examine whether any relationship exists between cell cycle state and transcriptional initiation of *Nanos2*. I also tested a possibility that Sox2 might suppress *Nanos2* transcription via direct binding. However, I could not find Sox2 binding consensus sites in the promoter and enhancer region of *Nanos2*. I need Sox2 over-expression studies to further examine this possibility.

What is required to promote male germ cell differentiation?

In the $Sox2^{\Delta/\Delta}$ male germ cells, I observed precocious cell cycle arrest and *Nanos2* up-regulation, but I did not observe up-regulation of other male-type genes that were generally induced under the control of Nanos2. The reason can be ascribed to the

insufficient amount of Nanos2 protein induced in *Sox2*$^{\Delta/\Delta}$ male germ cells at E12.5, although the precocious Nanos2 protein was observed only in *Sox2*$^{\Delta/\Delta}$ male germ cells at E12.5 (Fig II-1C,D). This idea was supported by my experiment with Nanos2 over-expression. I succeeded to induce Dnmt3L protein expression only when Nanos2 is induced excess amount in the absence of Sox2 (Fig II7-F-M). I speculate that some threshold level of Nanos2 protein is required to make a condition for inducing other male-type gene expression in mouse germ cells, which is achieved after E14.5 in the wild-type condition.

The function of Sox2 in male sexual differentiation in mouse germ cell: combined discussion for Chapter I and II.

The aim of my study is to elucidate the regulatory mechanism of male sexual differentiation in mouse germ cells. I focused on Nanos2 because it was an essential gene for promoting male differentiation pathway. In Chapter I, I tried to find the key factor that is promoted in the downstream of Nanos2. Whereas I successfully prevented abnormal mitotic resumption, meiosis, and pluripotency gene expression, I could not rescue the male-type gene expression in *Nanos2-null* male germ cells. Therefore I failed to find any downstream factor of Nanos2 involved in the promotion of male differentiation pathway. However, I found that Nanos2 suppresses RA signaling, by which Sox2 expression was suppressed and the quiescent state of germ cells was maintained. In addition, I discovered that Sox2 is an important factor, acting upstream of Nanos2. In Chapter II, I investigate how Sox2 works in the male differentiation pathway and I found that Sox2 is involved in the mitotic arrest, the first step of male differentiation pathway. Finally, I would like to discuss my idea why the reciprocal relationship between Nanos2 and Sox2 is important in male germ cell differentiation pathway

Germ cells differentiation is a complicated developmental process (Fig. II-8). In the early stage of embryogenesis (E10.5), RA synthesized and secreted from mesonephros activates Sox2 and Kit pathway that supports PGC proliferation. In the male gonad, Cyp26b1 is up-regulated after E12.5 in somatic cells and degrades RA, therefore, activity of Sox2-Kit pathway is down-regulated. As a result, germ cells enter mitotic arrest and *Nanos2* is induced through nodal signaling in male germ cells from E12.5. Once Nanos2 is expressed, Nanos2 maintains the mitotic arrest by suppressing

RA signaling which results in the down-regulation of pluripotency gene expression such as Sox2. This condition is essential to promote male germ cell differentiation leading to up-regulation of other male-type genes including Dnmt3L.

9. Conclusion

Understanding the regulatory mechanisms for male differentiation pathway is an important matter in spermatogenesis. In Chapter I of my thesis, I revealed that Nanos2 is essential for promoting male differentiation pathway through maintenance of mitotic arrest rather than initiation of meiosis. Furthermore, retinoic acid promotes *Sox2* expression that is negatively regulated by Nanos2 via suppression of retinoic acid signaling. In Chapter II, I showed that Sox2 prevents mitotic arrest via regulating cell cycle regulator. I found that Nanos2 and Sox2 reciprocally regulate the male-type gene expression during male differentiation pathway. My study may provide a new insight to further understanding of the regulatory mechanism that governs sexual differentiation of male germ cells.

10. Materials and Methods

Mice

The *Nanos2* knockout, and CAG-CAT-3xFlag-*Nanos2* mouse lines were established in our laboratory (Suzuki and Saga, 2008; Tsuda et al., 2003). *Oct4*-dPE-GFP mouse line was provided from Yasuhisa Matsui (University of Tohoku, Japan) (Yoshimizu et al., 1999). The *Bax*^{tm1sjk} mutant mouse was purchased from the Jackson Laboratory (Knudson et al., 1995). The *Sox2* conditional knock out mouse was kindly provided by Hisato Kondoh (University of Osaka, Japan).

Genotyping

Genomic DNA was isolated from tails (2μg ProK./50μl TE, 55^{o}C 30min, 95^{o}C 10min) and mouse genotypes were determined by PCR. The primers used were as follows:
3'lacZ-1 (5'-GGAGCCCGTCAGTATCGGCGGAATT-3')
N2-LA-R1 (5'-TCCCAGTCAGACGACTTG-3')
for the *Nanos2* LacZ-knock-in allele;

N2-F (suzu) (5'-GGCAGAGAAGAATGCCAGTT-3')
N2-R (suzu) (5'-TGTTCCCAGTCAGACGACTT-3')
for the Nanos2 wild-type allele;

3FLAG-F1 (5'-CTACAAAGACCATGACGGTG-3')
N2-3'U-R2 (5'-CCGAGAAGTCATCACCAG-3')
for the Flag-tagged Nanos2 transgene;

Rosa26-CreERT2 F (5'-CTGGGAGTTCTCTGCTGCCTCCT-3')
Rosa26-CReERT2 R (5'-CGCATAACCAGTGAAACAGCATTGC-3')
for the Rosa26 CreERT2 mice,

GFP R1 (5'-TCACGAACTCCAGCAGGACCATG-3')
GFP L1 (5'-CCTGGTCGAGCTGGACGGCGAC-3')
for the Oct4-dPE-GFP transgenic mice

Sox2-2 (5'-GCATTGTGCGCTCTCATCACATTTGGAC-3')

Sox2-3 (5'-CCTTGTGGTCAGTGTTCATCTGCTGACTG-3')
for the Sox2 floxed allele,

Sox2-2 (5'-GCATTGTGCGCTCTCATCACATTTGGAC-3')
Sox2-4 (5'-TGGATAGAAGATGACCGAAACCAGGAGC-3')
for the Sox2 wild-type allele.

Preparation of meiotic germ cell spread

Oocyte-spreadings were prepared from embryonic day 13.5 to 17.5. I collected female gonads in 1.5ml tube containing 0.7ml of M2 medium. Ovary was passed through 18G needle under a 1ml syringe and spin down for 3min at 400g. After discarding the supernatant, 1ml of PBS(-) was added. After spin down for 3min at 400g, PBS(-) was discarded. 1mg/ml collagenase Type I (invitrogen) was added to cells and the suspension was incubated at 37°C for 15-30min. After spin down for 3min at 400g, the supernatant was discarded. 500µl of 0.05% Trypsin-EDTA was added and the lysate was pipetted gently for 1min and incubated for 1min at 37 ^{o}C. 500µl of DMEM containing 10% (v/v) FCS was added. After spin down for 3min at 400g, suspension was discarded. Cells were resuspended in 1ml of PBS(-) by pipetting and centrifuged for 3 min at 400g. Hypotonic buffer (30mM Tris-HCl (pH 7.5), 50mM sucrose, 17mM trisodium crirate dehydrate, 5mM EDTA, 0.5mM DTT, 0.5mM PMSF) was added and incubated for 30 min at room temperature. After spin for 3 min at 400g, the supernatant was discarded. Cells were resuspended in 100mM sucrose containing 1mM sodium hydroxide. Cell suspension was spread on slide grass with 1% PFA containing 0.5% Triton X-100. Slide grasses were placed in a humidity slide chamber and incubated in 37^{o}C incubator for overnight.

Immunostaining

The mouse embryos and gonads were fixed in 4% PFA for 30 minute at 4^{o}C and washed three times for 5 min each with in PBS containing 0.1% TritonX-100 (PBS-T). After blocking with PBS-T containing 3% skim milk for 1 hour at RT, samples were rinsed and incubated overnight with primary antibodies in PBST at 4^{o}C. The following day, samples were washed 6 times for 15 min each in PBS-T and were incubated for 2 hours at RT with secondary antibodies in PBS-T. After the samples had been washed 3

times for 5 min each with PBS-T, they were mounted on MAS-coated slide glasses or a glass-bottom dish (Matsunami) and enclosed with PBS by manicure. The samples were then analyzed by confocal laser microscopy (FV12000, OLYMPUS). Primary antibodies were used at the following dilutions: 1:2000 for mouse anti-SYCP3 (Abcam), 1:2000 for rabbit anti-γ.H2AX (Abcam), 1:100 for anti-rabbit REC8 (provide from Dr. Watanabe, Tokyo university in Japan), 1:200 for mouse anti-phospho-histone H3 (ser10) (provide from Dr. Kimura, Oosaka university in Japan), 1:10000 for mouse anti-FLAG (M2) (SIGMA), 1:1000 for anti-rabbit KI67 (SP6) (Thermo Fisher Scientific) and 1:5000 for rat TRA98 (provide from Dr. Nishimune, Osaka University in Japan), 1:200 for rabbit NANOS2, 1:500 for rabbit TRDR1 (provide from Dr. Chuma, Kyoto University in Japan), 1:400 for rabbit DNMT3L (provide from Dr. Yamanaka, Kyoto University in Japan), 1:400 for guinea pig DAZL (provide from Dr. Suzuki, Yokohama National University in Japan) and 1:200 for rabbit $p15^{INK4b}$ (Cell signaling). Secondary antibodies were all used at a 1:500 dilution; Alexa-488 conjugated donkey anti-rabbit IgG, Alexa-488 conjugated donkey anti-mouse IgG, Alexa-488 conjugated donkey anti-rat IgG, Alexa-594 conjugated donkey anti-mouse IgG and Alexa-594 conjugated donkey anti-rat IgG (Invitrogen) and Cy5 conjugated goat anti-mouse IgG (Rockland).

Germ cell sorting

For germ cell sorting, single cell suspensions were prepared from a pair of gonads by incubation with 0.15% trypsin-EDTA, 37^{o}C, 10min and filtered through a 35μm sieve (BD BioScience, San Jose, CA). All measurements and sorting were performed on a JSAN Desktop Cell sorter.

Organ culture

Medium for serum-free culture of embryonic gonad contained KnockOut Dulbecco's modified Eagle's medium (KO-DMEM, Invitrogn), 15% KO serum (Invitrogn), 500μg/ml Penicillin/Streptomycin (Invitrogn), 0.1mM MEM non-essential amino acid (Invitrogen). Additives to culture were 5mM Retinoic acid receptor antagonist (AGN 193109) (Santa Cruz). Embryonic gonads were cultured on filters (Millipore, TMTP01300, 3 μm) in 24 well dishes. All cultures were maintained at 37°C in 5% CO_2.

Quantitative RT-PCR

Total RNAs were prepared with RNeasy (Qiagen) for gonads and with TRIzol and then PureLink RNA Mini Kit (life technology Japan) for sorted germ cells, and used for reverse transcription by Super script III (Invitrogen). Samples were prepared as a pool of cDNA derived from 1～2 gonad and each sample was analyzed in triplicate. Quantitative RT-PCR was performed on the TP800 thermal cycler (TAKARA) using SYBR Green (Takara). mRNA levels were calculated with an absolute quantification method and normalized by the amount of Vasa and Gapdh for each sample. Results are represented as mean ± SD. Statistical analysis was performed using Excel (Microsoft). Pairwise comparisons were analyzed using Student's *t*-test. The primers used were as follows;

Gapdh F (5'-ACCACAGTCCATGCCATCAC-3')
Gapdh R (5'-TCCACCACCCTGTTGCTGTA-3')

Vasa F (5'-GTTGAGTATCTGGACATGATGCAC-3')
Vasa R (5'-CGAGTTGGTGCTACAATAATACACTC-3')

Nanos2 F (5'-ACAGCAGTCAGTCAGTCTC-3')
Nanos2 R (5'- CCGAGAAGTCATCACCAG -3')

Dnmt3L F (5'-CTATGCGGGTGTGGAGCAAC-3')
Dnmt3L R (5'-TCACCAGGAGGTCAACTTTCG-3')

Tdrd1 F (5'-CGTCGGAATCTGATGTGAAAGT-3')
Tdrd1 R (5'-TGGTTCAGCATGGCATTTCTCA-3')

Miwi2 F (5'-CGGGACGGTGTAGGCAATGG-3')
Miwi2 R (5'-GTCAGCTTGTGTGCATATTGGC-3')

Oct3/4 F (5'-TCACCTTGGGGTACACCCAG-3')
Oct3/4 R (5'-CAGTTCTTAAGGCTGAGCTGC-3')

Nanog F (5'-CCGCTTGCACTTCATCCTTTG-3')

Nanog R (5’-CCTCAGCCTCCAGAGATGC-3’)

Sox2 F (5’-GGCGGGTGGCTTTTGTCC-3’)
Sox2 R (5’-CGGGAAGCGTGTACTTATCCTT-3’)

Cyclin E1 (Ccne1) F (5’-TGCTAGGTGTTTTAACTATAGGGTCA-3’)
Cyclin E1 (Ccne1) R (5’-TCTGGAGCACTCAGTGGTGT-3’)

Cyclin E2 (Ccne2) F (5’-CGAGCTGTGGAGGGTCTG-3’)
Cyclin E2 (Ccne2) R (5’-AAACGGCTACTGCGTCTTGA-3’)

$p15^{INK4b}$ (Cdkn2b) F (5’-AATAACTTCCTACGCATTTTCTGC-3’)
$p15^{INK4b}$ (Cdkn2b) R (5’-CCCTTGGCTTCAAGGTGAG-3’)

$p18^{INK4c}$ (Cdkn2c) F (5’-CAGATTAACCATCCCAGTCCTT-3’)
$p18^{INK4c}$ (Cdkn2c) R (5’-CCCCTTTCCTTTGCTCCTAA-3’)

$p19^{INK4d}$ (Cdkn2d) F (5’-AATGTGACCCAAGGCCACT-3’)
$p19^{INK4d}$ (Cdkn2d) R (5’-TTTCCTCTTTTGTTGACAAGTAACC-3’)

$p21^{Cip1}$ (Cdkn1a) F (5’-CTTCTGCTGTGGGTCAGGAG-3’)
$p21^{Cip1}$ (Cdkn1a) R (5’-CACACAGAGTGAGGGCTAAGG-3’)

$p27^{Kip1}$ (Cdkn1b) F (5’-TGGAGGGCAGATACGAATG-3’)
$p27^{Kip1}$ (Cdkn1b) R (5’-CGGGGGCCTGTAGTAGAACT-3’)

$p57^{Kip2}$ (Cdkn1c) F (5’-TAGAGGCTAACGGCCAGAGA-3’)
$p57^{Kip2}$ (Cdkn1c) R (5’-CCCAGAGTTCTTCCATCGTC-3’)

Rb1 F (5’-CAGGGTCTTTTGAACTTGCAG-3’)
Rb1 R (5’-GCTGGACTAGAAATGGTGTGG-3’)

Nodal F (5’AGCCAAGAAGAGGATCTGGTATGG -3’)

Nodal R (5'-GACCTGAGAAGGAATGACGGTGAA-3')

Lefty1 F (5'-GACCTGAGAAGGAATGACGGTGAA-3')
Lefty1 R (5'-AGTCCTGGACAAGGCTGATGTG-3')

Lefty2 F (5'-CGAACACTAGCAGGTGAGTGGA-3')
Lefty2 R (5'-ATCGACTCTAGGCTCGTGTCCATC

Stra8 F (5'-CCTAAGGAAGGCAGTTTACTCCCAGTC-3')
Stra8 R (5'-GCAGGTTGAAGGATGCTTTGAGC-3')

Sycp3 F (5'-GCATTCTGGGAAATCTGGGA-3')
Sycp3 R (5'-TGGAGCCTTTTCATCAGCAA-3')

Brd4 F (5'-CCACTTCAGACTCCTTCACC-3')
Brd4 F (5'-TGGTATCTGCTTTCCTCTTCAC-3')

These primers are already described (Hayashi et al., 2012; Saba et al., 2013; Western et al., 2008; Wu et al., 2013).

In situ hybridization

Gonads isolated from E13.5 embryo were fixed in 4% paraformaldehyde/PBS for 30min at 4°C and then incubated in 20% sucrose/PBS for overnight. Fixed gonads were embedded in O.C.T compound (Sakura). The section was cut for 7µm. They were then treated for 5 min PBS and followed by tissue acetylation (1xTAE/MQ, 0.5% acetic anhydride) for 10 min. 20 ml of prehybridization buffer and hybridization buffer contain 2 mL of 50% dextran sulfate (Sigma D253), 4ml of 20xSSC, 10ml formamide, 400µl denhaedt's (Sigma D2532), 1mL salmon sperm DNA. After wash with PBS, sections were incubated in prehybridization buffer for 2h at RT followed by hybridization overnight at 68°C in the hybridization solution containing 1 mg probe/ml. After hybridization, sections were washed with 0.2xSSC for 1h at 68°C; cooled at RT; washed with 0.2xSSC for 5min; 1xPBS for 5min and incubated with blocking Buffer (Roche 11096176001) for 1h at RT. After blocking, sections were incubated in

anti-DIG/blocking buffer (1:400) for 1h at RT; mounted with the tyramid Cy3/tyramide buffer (1:100) for 10min at RT. After conjugation with tyramid, sections were processed for immunohistochemistry. The sequence information of RNA probe for Nanos2 is already described (Tsuda et al., 2003).

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12.Figures

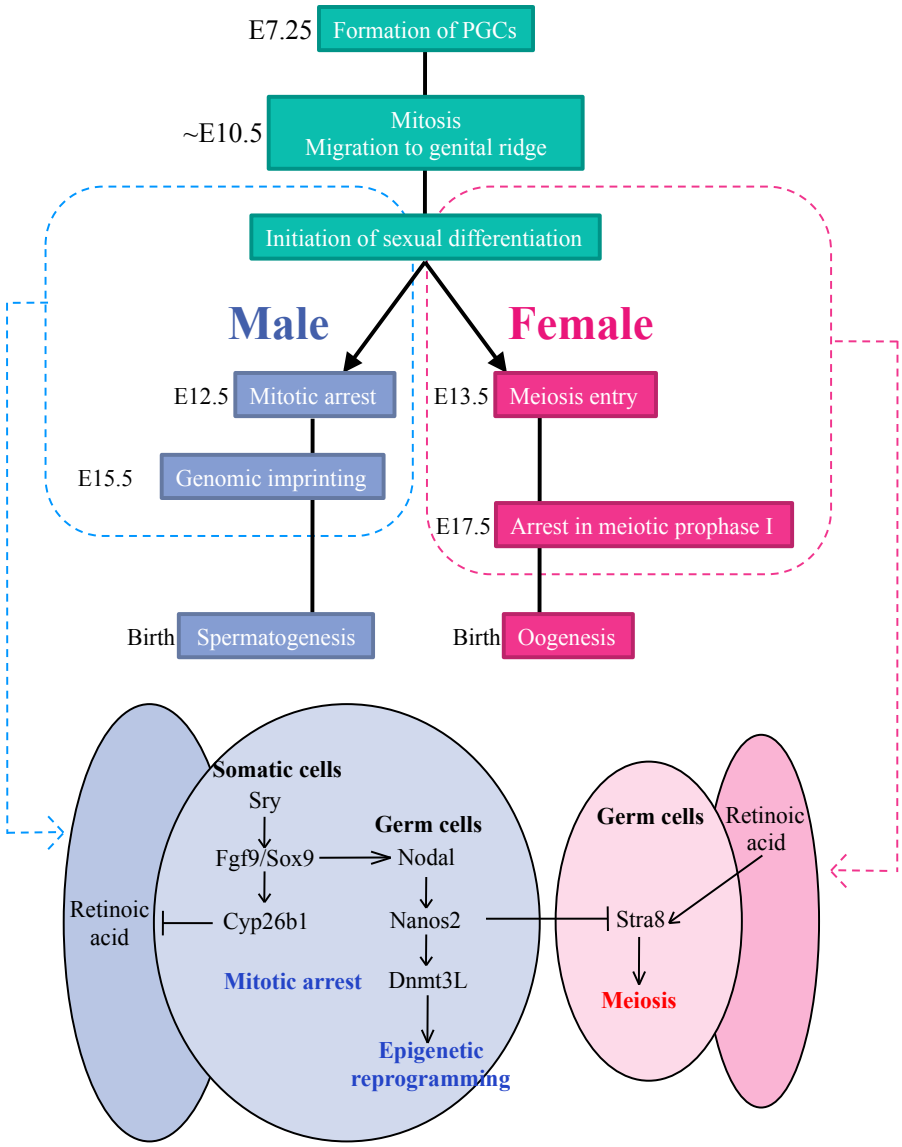


Figure I-1. Schematic representation of sexual differentiation cascades of germ cells.

PGCs entered male and female gonads take different genetic pathways to start spermatogenesis and oogenesis. In the male pathway, Sry activates Sox9 and induces primary fate determination of somatic cells at E10.5. Activation of Sox9 promotes Fgf9 expression. In the second step, activation of Sox9/Fgf9 promotes *Cyp26b1* expression. CYP26B1 is involved in the inactivation of retinoic acid in male gonads. Activation of Sox9/Fgf9 also promotes *nodal* expression in male germ cells. This nodal signaling promotes *Nanos2* expression at E13.5, allowing for induction of Dnmt3L expression. In female gonad, retinoic acid promotes *Stra8* expression, which induces meiosis at E13.5.

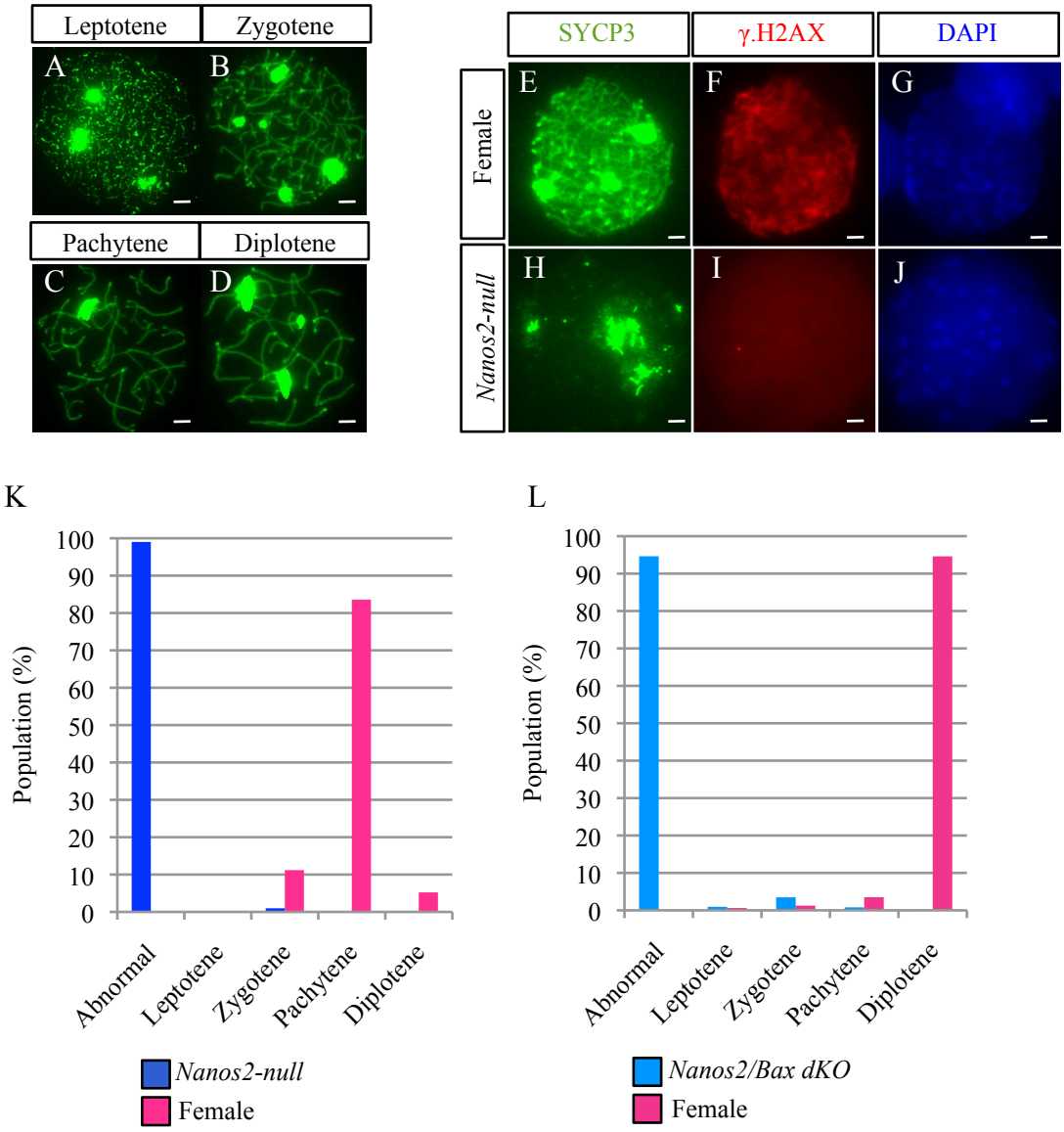


Figure I-2. Most of *Nanos2-null* male germ cells failed to undergo meiotic progression.

(A-D) Immunofluorescence staining patterns of anti-SYPC3 antibody indicating typical meiotic progression in female germ cells at E15.5.

(E-J) Immunofluorescence analysis of female germ cells (E-G) and *Nanos2-null* male germ cells (H-J) using meiotic specific markers at E15.5.

(K) Composition of meiotic cells observed in female germ cells (n=215) and *Nanos2-null* male germ cells (n=228) at E16.5.

(L) Composition of meiotic cells observed in female germ cells (n=798) and *Nanos2/Bax* dKO male germ cells (n=1380) at E17.5. Scale bar: 1μm

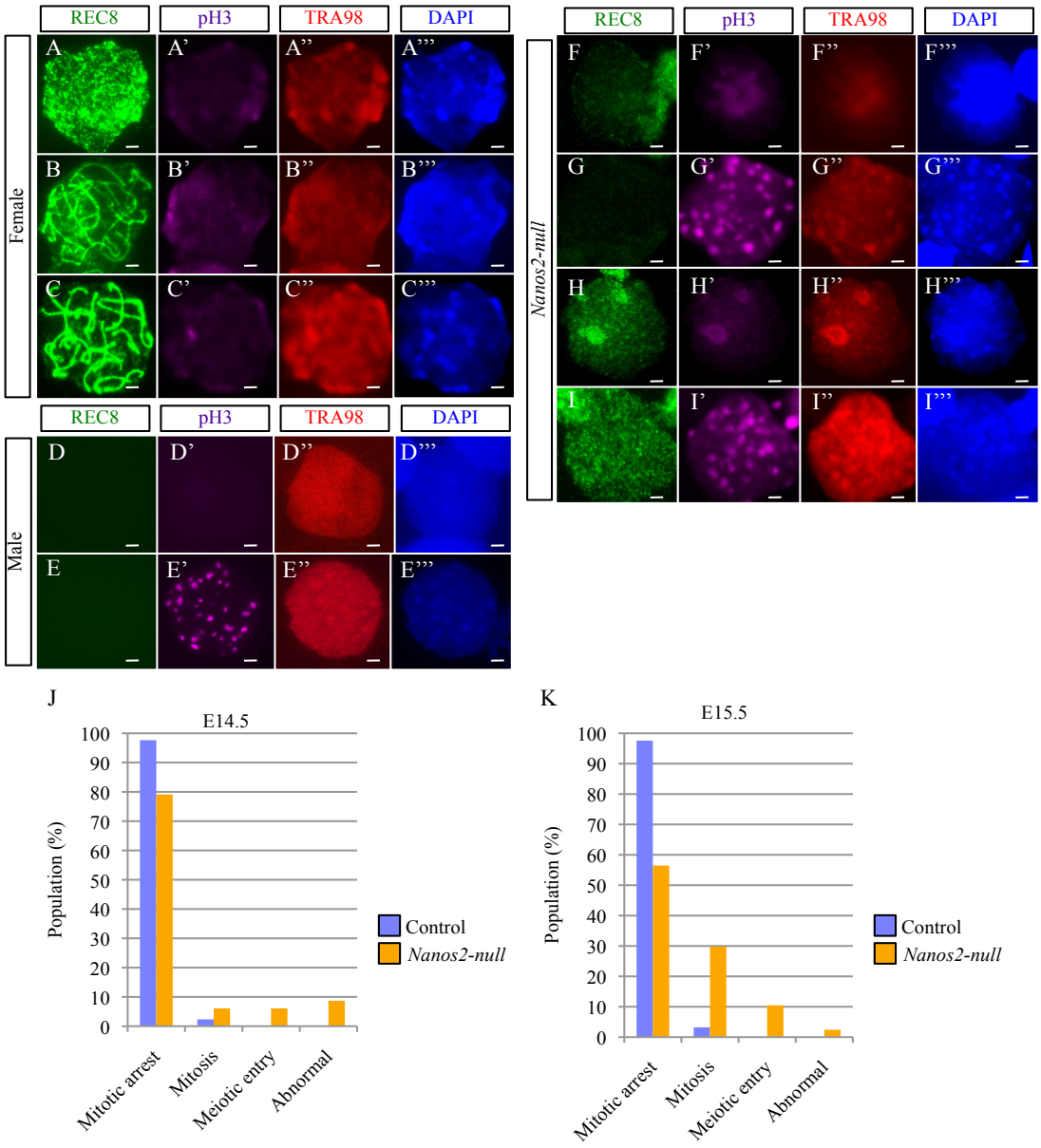
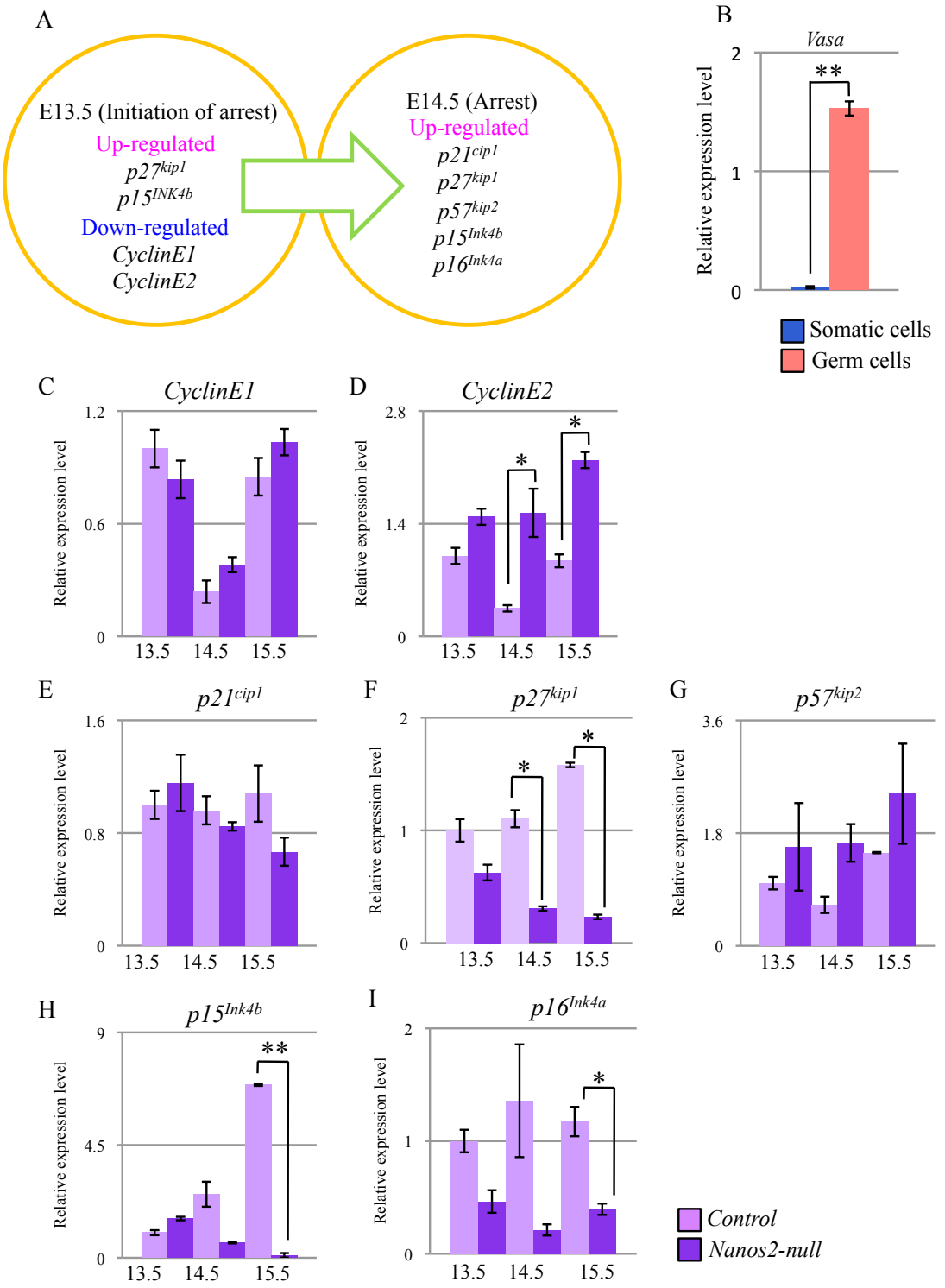


Figure I-3. Many of *Nanos2-null* male germ cells resume mitosis rather than entering meiosis.

(A-I) Immunofluorescence analysis of meiosis (REC8), mitosis (pH3), and germ cells (TRA98) markers in female germ cells (A-C), male germ cells (D-E), and *Nanos2-null* male germ cells (F-I) at E15.5.

(J-K) Quantification of $REC8^-/pH3^-$ (mitotic arrest), $REC8^+/pH3^-$ (meiosis), $REC8^-/pH3^+$ (mitosis) and $REC8^+/pH3^+$ (abnormal) germ cell numbers in control male germ cells and *Nanos2-null* male germ cells at E14.5 (J) and E15.5 (K).

Scale bar:1μm


A
E13.5 (Initiation of arrest)
Up-regulated
p27kip1
p15INK4b
Down-regulated
CyclinE1
CyclinE2
E14.5 (Arrest)
Up-regulated
p21cip1
p27kip1
p57kip2
p15Ink4b
p16Ink4a
B
Vasa
Relative expression level
Somatic cells
Germ cells
C
CyclinE1
D
CyclinE2
E
p21cip1
F
p27kip1
G
p57kip2
H
p15Ink4b
I
p16Ink4a
13.5
14.5
15.5
Control
Nanos2-null

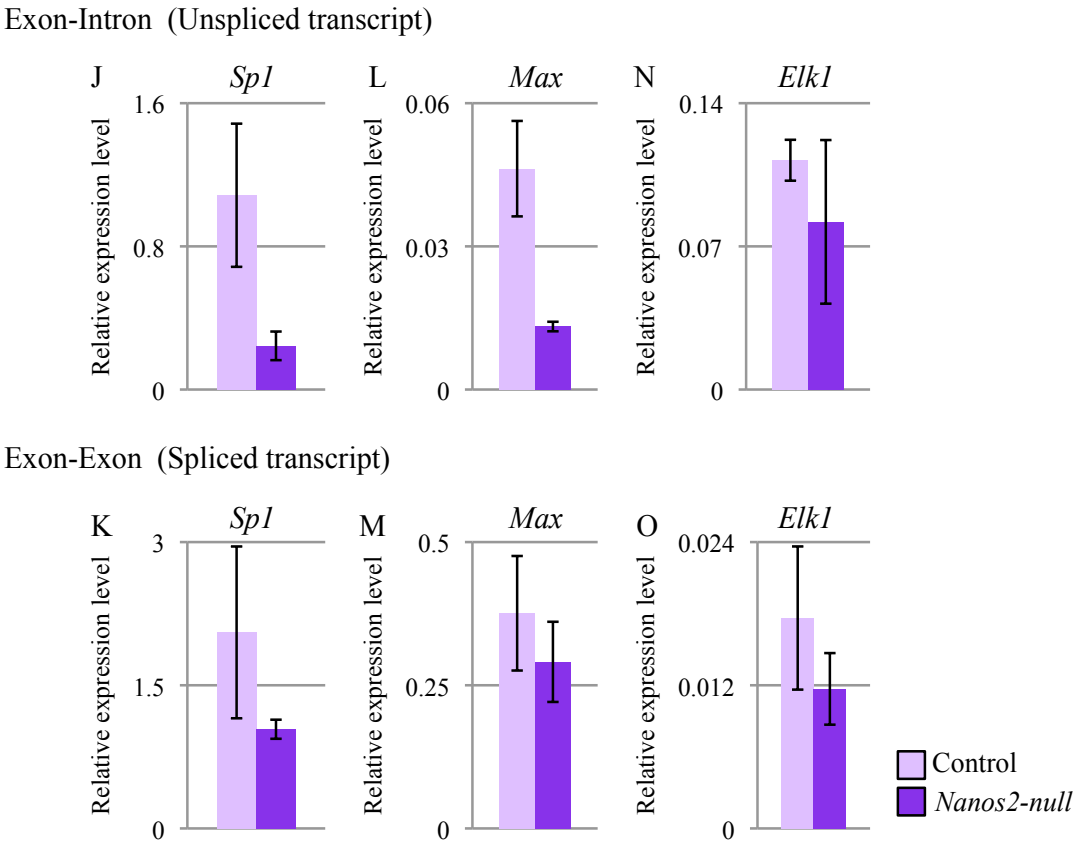


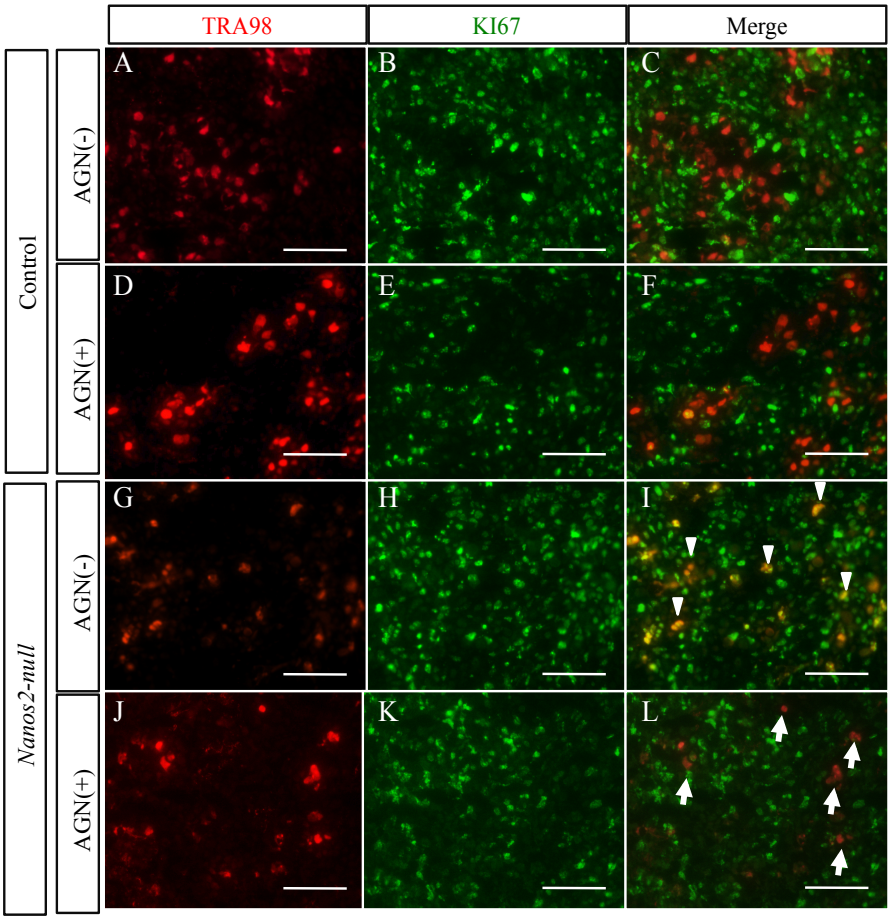
Figure I-4. Resumption of mitosis in *Nanos2-null* male germ cells.

(A) Key regulators involved in the mitotic arrest in male germ cells (Western et al., 2008).

(B) qRT-PCR analyses showed that expression of *Vasa* was observed from RNA derived from *Nanos2*$^{+/-}$ (n=4) or *Nanos2-null* (n=4) male germ cells but not from somatic cells at E14.5.

(C-I) Comparative qRT-PCR analyses of cell cycle regulators. RNAs were extracted from sorted germ cells from control and *Nanos2-null* male gonads from E13.5 to E15.5. (E13.5 control n=3, *Nanos2-null* n=3; E14.5 control n=5, *Nanos2-null* n=6; E15.5 control n=5, *Nanos2-null* n=4)

(J-O) qRT-PCR analyses showed that expression of unspliced and spliced transcripts of candidate genes for Nanos2 from *Nanos2*$^{+/-}$ (n=4) or *Nanos2-null* (n=4) male germ cells at E14.5.Data are shown as mean ± SD. *P<0.05. **P<0.001 (Student's *t* test).


TRA98
KI67
Merge
Control
Nanos2-null
AGN(-)
AGN(+)
A
B
C
D
E
F
G
H
I
J
K
L

M

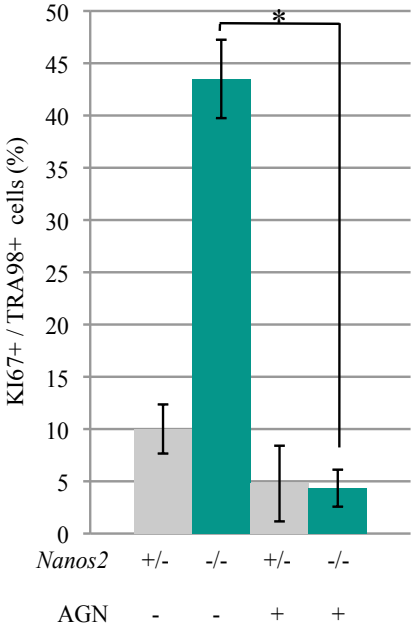
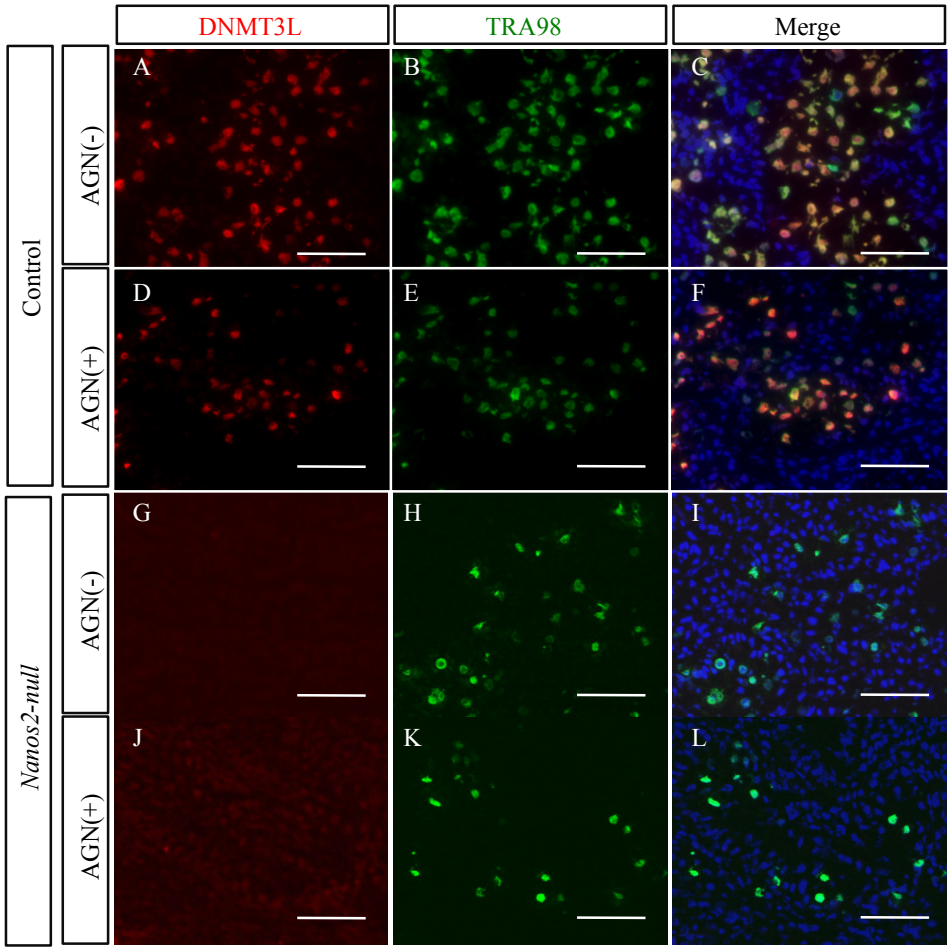

KI67+ / TRA98+ cells (%)
50
45
40
35
30
25
20
15
10
5
0
*
Nanos2 +/- -/- +/- -/-
AGN - - + +

Figure I-5. Nanos2 maintains mitotic arrest by suppressing retinoic acid signaling.
(A-I) Immunofluorescence analyses of male gonads from control (*Nanos2*$^{+/-}$) and *Nanos2-null* embryos using mitosis marker (KI67) and germ cell marker (TRA98). The gonads were obtained at E12.5 and cultured for 3 days with DMSO (vehicle control) (A-C, G-I) or with retinoic acid receptor antagonist, AGN193190 (D-F, J-L). KI67 signals were observed in *Nanos2-null* male germ cells cultured without AGN190390 (G-I), but the signal was not observed with AGN1903190 (J-I). White arrowheads indicate KI67-positive mitotic cells. White arrows indicate mitotic quiescent cell.
(M) Quantification of mitotic cells in control and *Nanos2-null* male germ cells cultured with DMSO or AGN1903190. Data are shown as mean ± SD. *P<0.05 (Student's *t* test).


DNMT3L
TRA98
Merge
Control
Nanos2-null
AGN(-)
AGN(+)
A
B
C
D
E
F
G
H
I
J
K
L

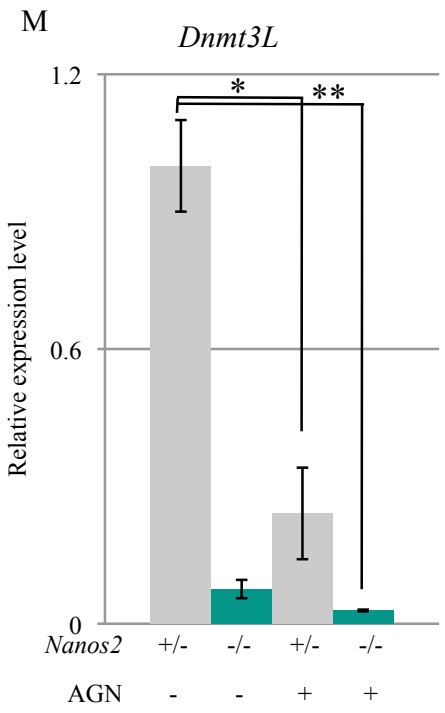

M
Dnmt3L
1.2
0.6
0
Relative expression level
*
**
Nanos2 +/- -/- +/- -/-
AGN - - + +

Figure I-6. DNMT3L expression was not recovered in spite of suppression of abnormal cell cycle resumption in *Nanos2-null* male germ cells.

(A-L) Immunofluorescence analyses of male gonads from control (*Nanos2*$^{+/-}$) and *Nanos2-null* embryos using male differentiation marker (DNMT3L) and germ cell marker (TRA98). The gonads were obtained at E12.5 and cultured for 3 days with DMSO (A-C, G-I) or AGN1903190 (D-F, J-L). In *Nanos2-null* male germ cells, DNMT3L-positive cells were never observed in either with (J-L) or without AGN193190(G-I).

(M) qRT-PCR analysis of *Dnmt3L* expression in the control and *Nanos2-null* male gonads cultured without and with AGN193190. Data are shown as mean ± SD. *P<0.05 (Student's *t* test).

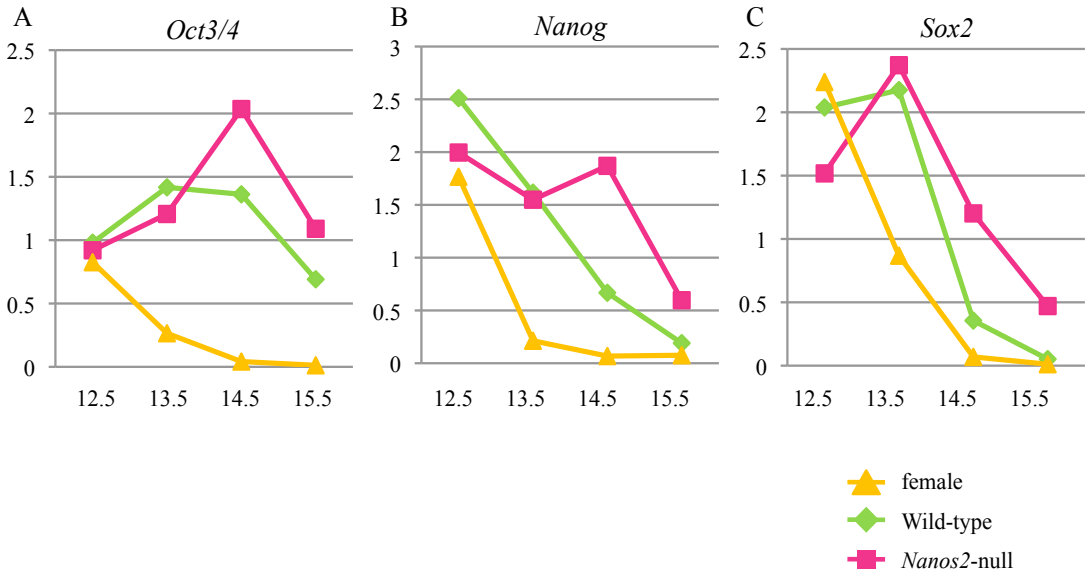
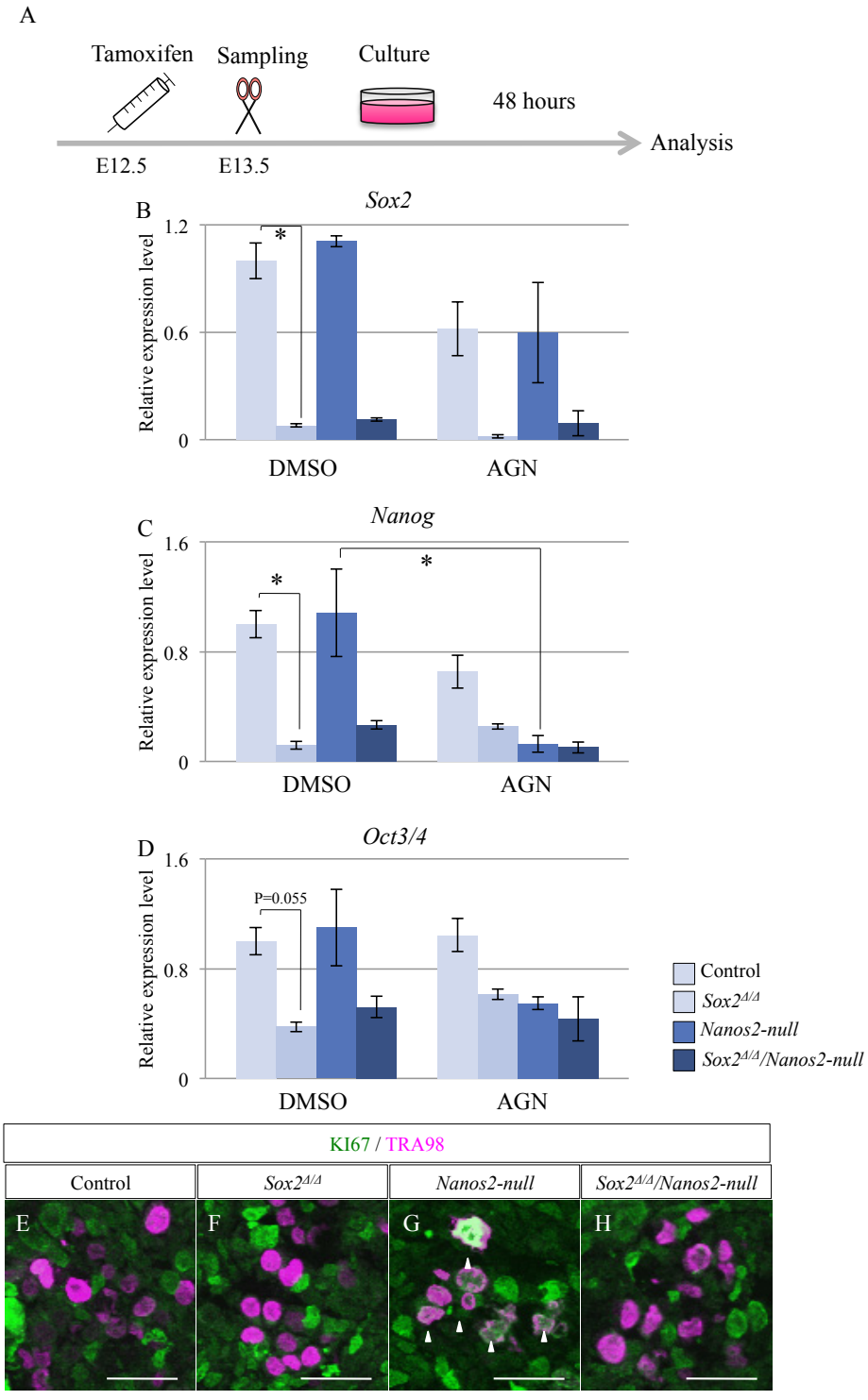


Figure I-7. Maintenance of pluripotency gene expression in *Nanos2-null* male germ cells.

Microarray data for *Oct3/4* (A), *Nanog* (B), *Sox2* (C) were plotted to show the expression changes during germ cell development in female gonad (yellow), wild-type male gonad (green) and *Nanos2-null* male gonad (pink) from E12.5 to E15.5. The original data were obtained by Dr. Rie Saba (Saba et al., 2013).


A
Tamoxifen
Sampling
Culture
48 hours
Analysis
E12.5
E13.5
B
Sox2
Relative expression level
1.2
0.6
0
DMSO
AGN
C
Nanog
1.6
0.8
D
Oct3/4
P=0.055
Control
Sox2Δ/Δ
Nanos2-null
Sox2Δ/Δ/Nanos2-null
KI67 / TRA98
E
F
G
H

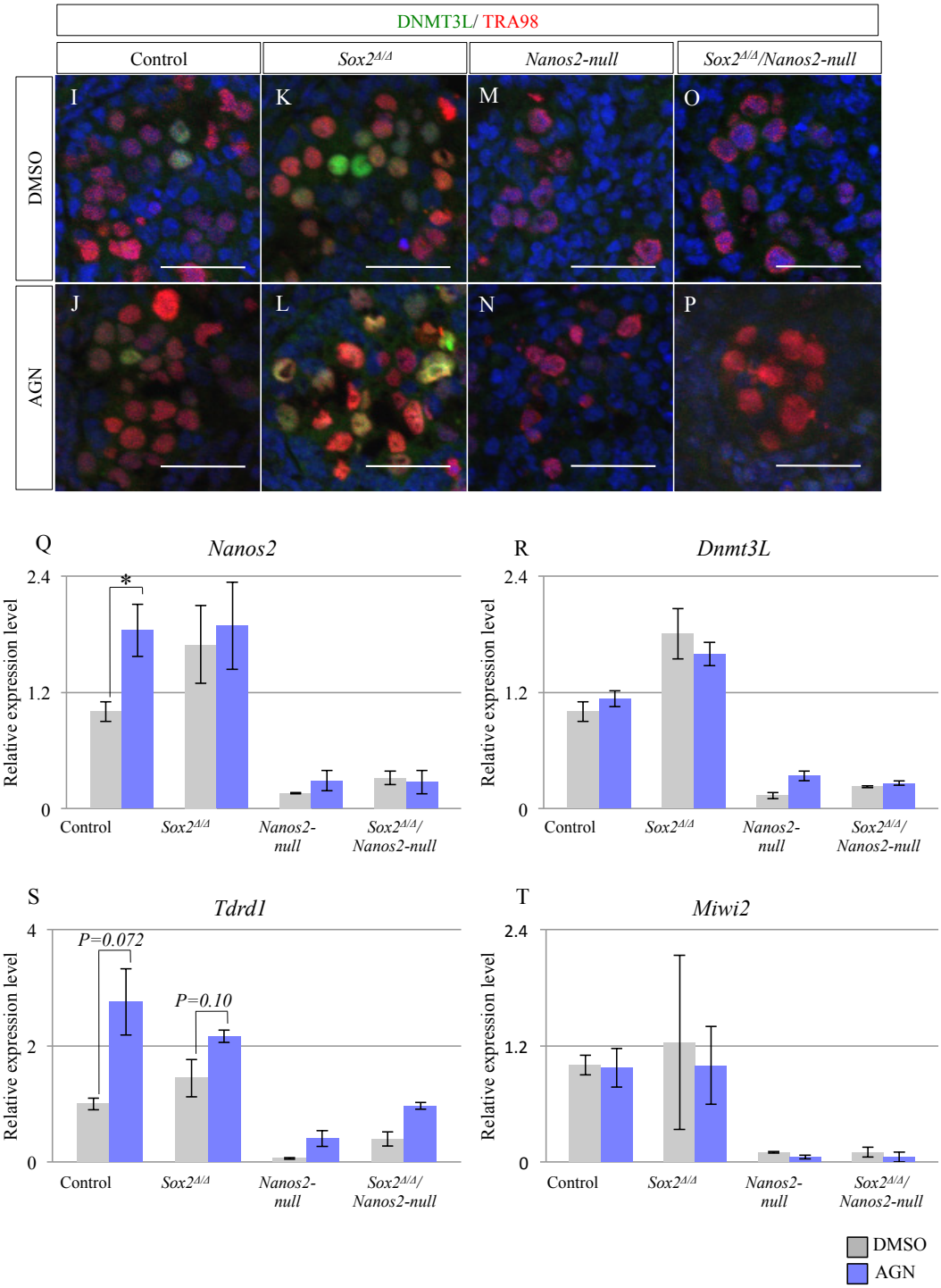

DNMT3L/ TRA98
Control
Sox2Δ/Δ
Nanos2-null
Sox2Δ/Δ/Nanos2-null
DMSO
AGN
I
K
M
O
J
L
N
P
Q
Nanos2
R
Dnmt3L
S
Tdrd1
T
Miwi2
Relative expression level
2.4
1.2
0
4
2
*
P=0.072
P=0.10
Control
Sox2Δ/Δ
Nanos2-null
Sox2Δ/Δ/Nanos2-null
DMSO
AGN

Figure I-8. Male-type gene expression was not rescued in *Nanos2/Sox2* dKO male germ cells cultured with retinoic acid receptor antagonist.

(A) The experimental scheme. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E12.5. After 1 day, male gonads for each genotype were harvested and cultured for 48 hours with DMSO (control) or retinoic acid receptor antagonist AGN1903190.

(B-D) qRT-PCR data of *Sox2* (B), *Nanog* (C) and *Oct3/4* (D) in control (n=4), $Sox2^{\Delta/\Delta}$ (n=5), *Nanos2-null* (n=4) and *Nanos2/Sox2* dKO (n=4) gonads cultured with DMSO or AGN1903190.

(E-H) Immunofluorescence of KI67 in control (E), $Sox2^{\Delta/\Delta}$ (F), *Nanos2-null* (G) and *Nanos2/Sox2* dKO (H) gonads cultured with DMSO. While arrows indicate KI-67-positive mitotic cells.

(I-P) Immunofluorescence of DNMT3L in control (I,J), $Sox2^{\Delta/\Delta}$ (K,L), *Nanos2-null* (M,N) and *Nanos2/Sox2* dKO (O,P) gonads cultured with DMSO or AGN193190.

(Q-T) qRT-PCR analyses of *Nanos2* (Q), *Dnmt3L* (R), *Tdrd1* (S) and *Miwi2*(T) in control (n=4), $Sox2^{\Delta/\Delta}$ (n=5), *Nanos2-null* (n=4) and *Nanos2/Sox2* dKO (n=4) gonads cultured with DMSO or AGN193190. Data are shown as means ± SD. *P<0.05 (Student's *t* test). Scale bars: 50μm

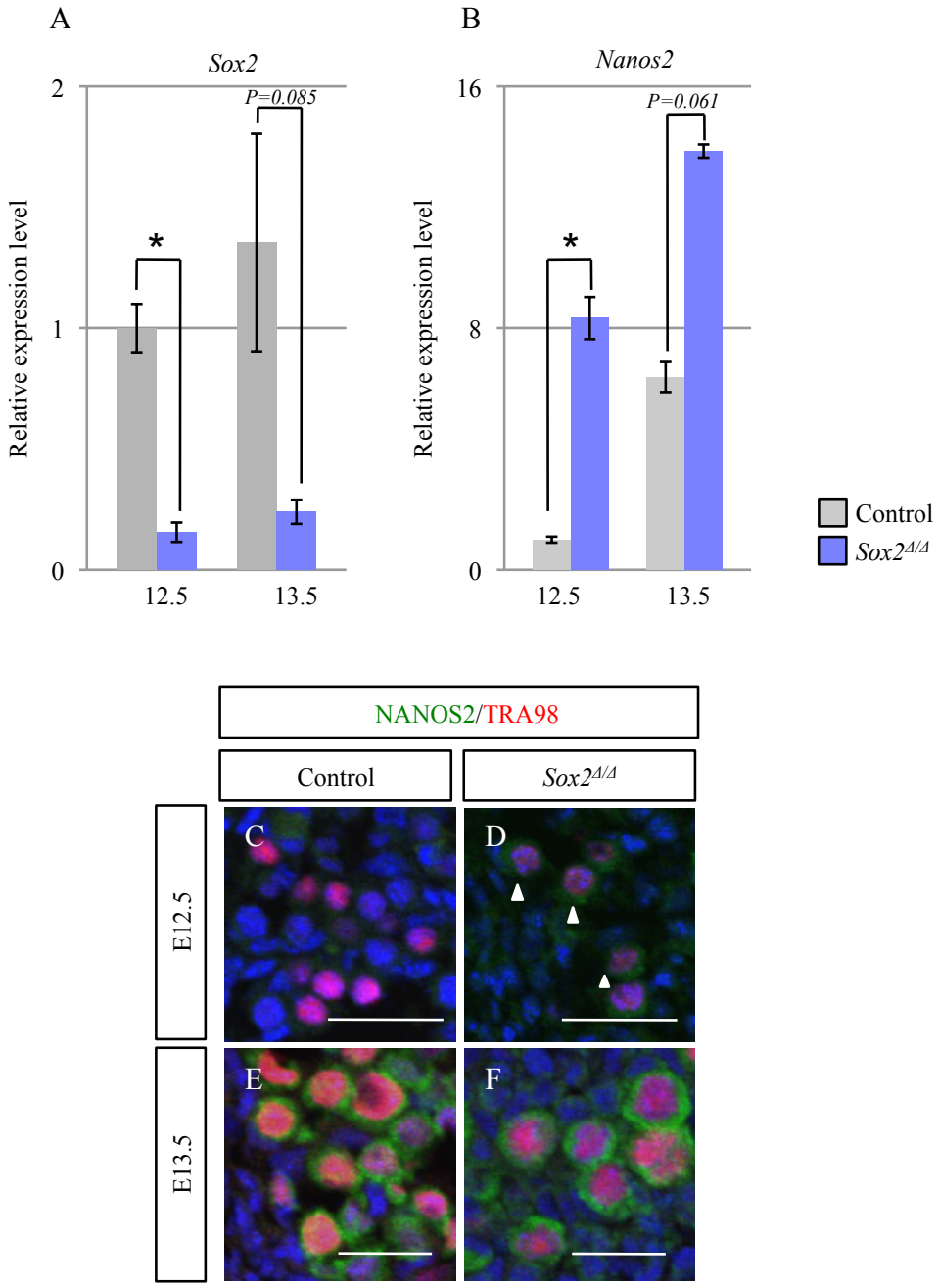


Figure II-1. Sox2 negatively controls *Nanos2* expression in male germ cells.

(A,B) qRT-PCR analysis showed expression of *Sox2* (A) and *Nanos2* (B) in control (n=4), *Sox2*$^{\Delta/\Delta}$(n=4) male gonads at E12.5 and control (n=6), *Sox2*$^{\Delta/\Delta}$(n=6) male gonads at E13.5. Results were normalized to *Vasa*. Data are shown as means ± SD. *P<0.05 (Student's *t* test).

(C-F) Immunofluorescence of NANOS2 in control and *Sox2*$^{\Delta/\Delta}$ male gonads at E12.5 and E13.5. While arrow indicates NANOS2-positive cells. Scale bars: 30μm

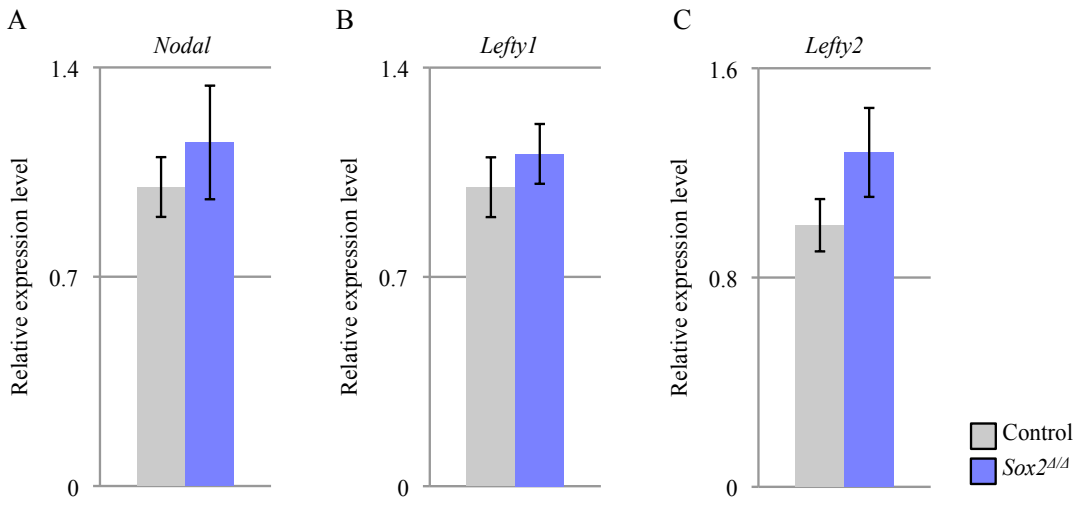


Figure II-2. Sox2 controls *Nanos2* expression independently of Nodal/activin signal.

(A-C) qRT-PCR analysis showing expressions of *Nodal* (A), *Lefty1* (B), *Lefty2* (C) in control (n=4) and $Sox2^{\Delta/\Delta}$ male gonads (n=4) at E12.5. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E10.5. Results were normalized to *Vasa*. Data are shown as means ± SD. *P<0.05 (Student's *t* test).

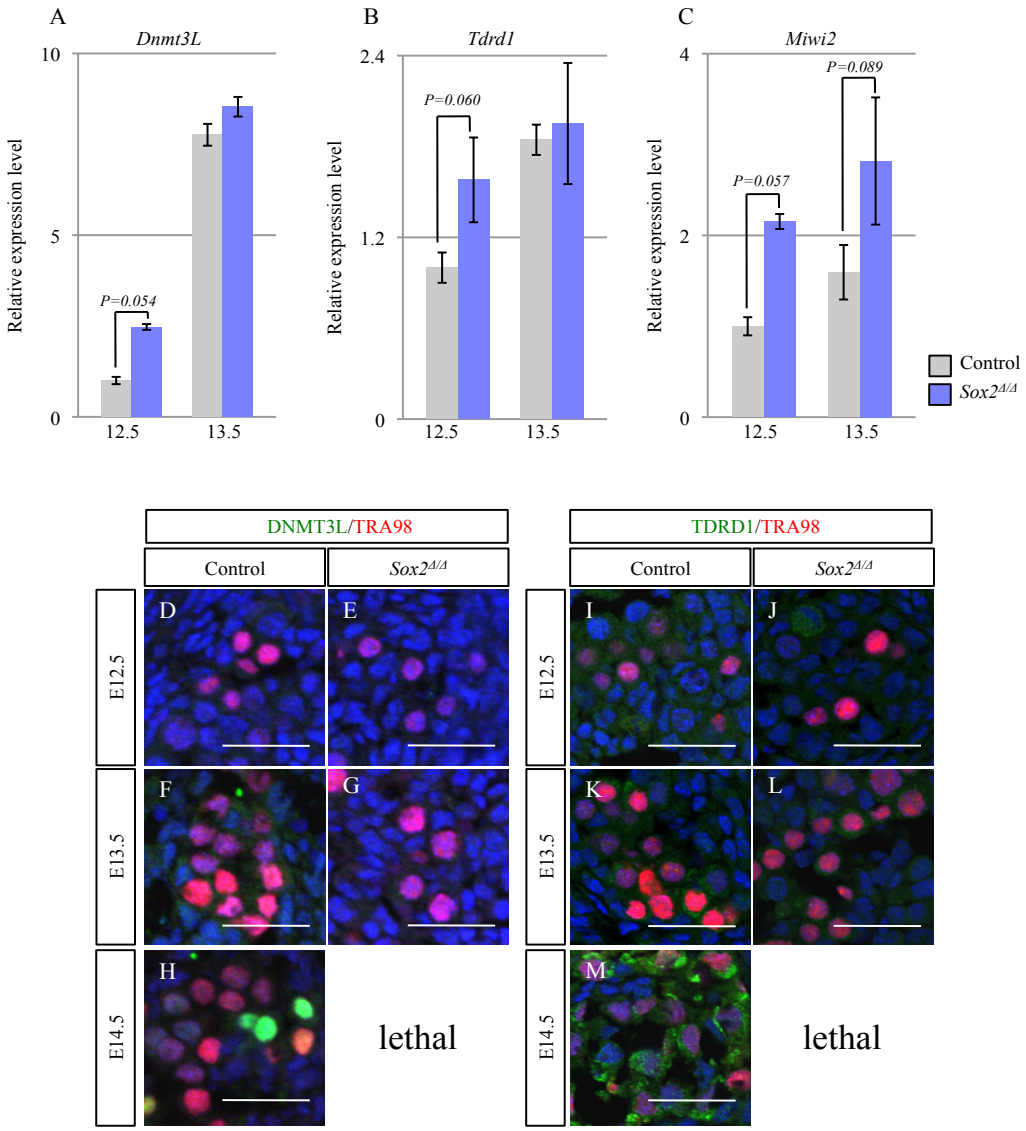
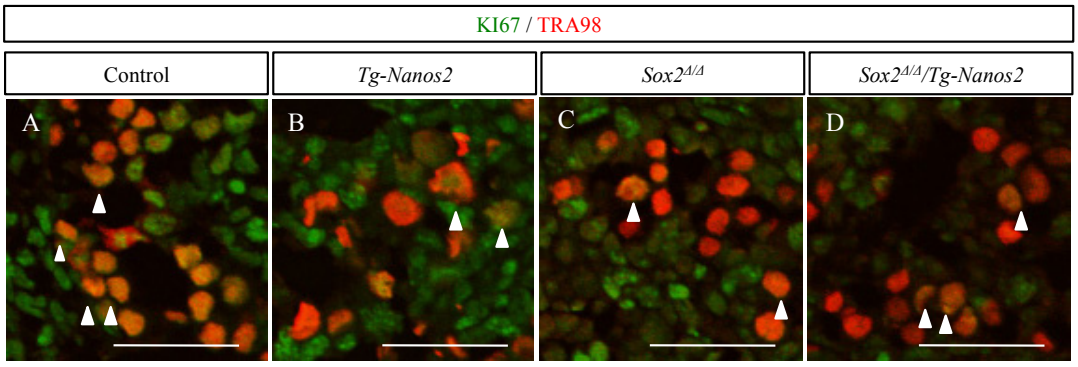
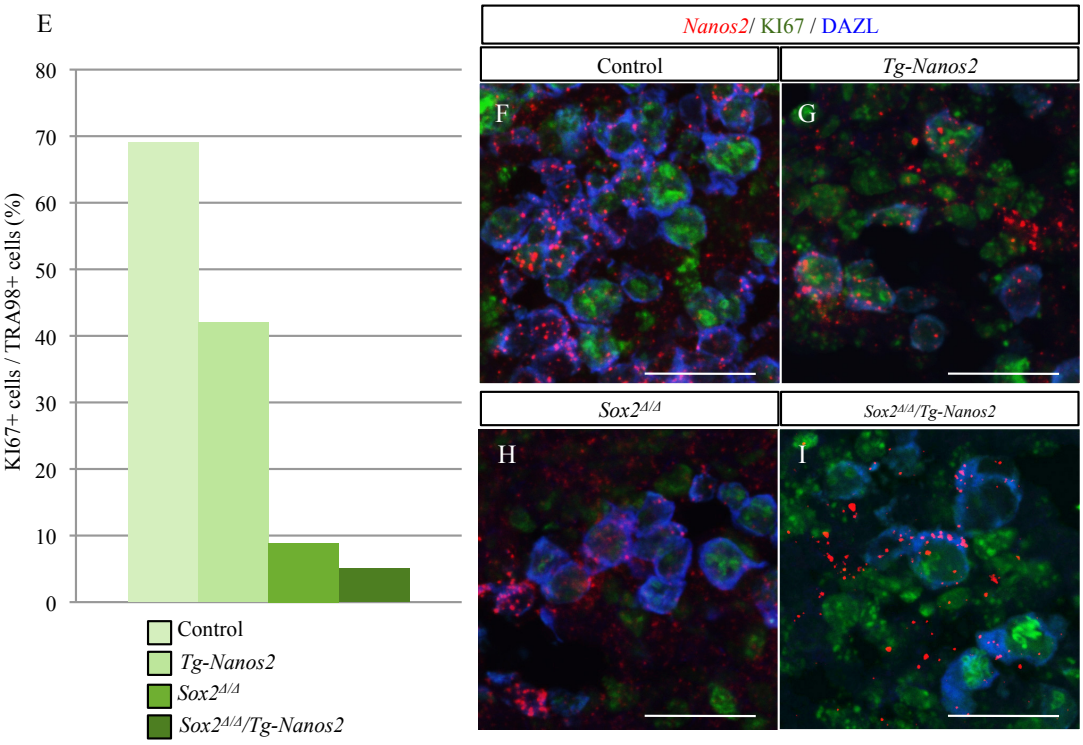


Figure II-3. Marginal effects of *Sox2*-defeciency in male-type gene expression.

(A-C) qRT-PCR analysis showing expression of *Dnmt3L* (A), *Tdrd1* (B), and *Miwi2* (C) in control (n=4), $Sox2^{\Delta/\Delta}$(n=4) male gonads at E12.5 and control (n=6), $Sox2^{\Delta/\Delta}$ (n=6) male gonads at E13.5. Results were normalized to *Vasa*. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E10.5 and gonads were harvested at E12.5 or E13.5. Data are shown as means ± SD. P values are indicated in graphs. There is no significant difference between control and *Sox2-null* gonads.

(D-M) Immunofluorescence analyses using male differentiation marker (DNMT3L (D-H), TDRD1 (I-M)) and germ cell marker (TRA98) for male gonads prepared from control and $Sox2^{\Delta/\Delta}$ male embryos at E12.5, 13.5 and E14.5 (control only). Scale bars: 30μm





J

Proportion (%)	KI67^{+}/ *Nanos2*$^{+}$	KI67^{+}/ *Nanos2*$^{-}$	KI67^{-}/ *Nanos2*$^{+}$	KI67^{-}/ *Nanos2*$^{-}$
Control	12.5	58.4	27.6	1.5
Tg-Nanos2	34.5	8.4	54.6	2.5
Sox2$^{Δ/Δ}$	3.5	5.3	88.8	2.4
Sox2$^{Δ/Δ}$*/Tg-Nanos2*	1.2	2.4	93.6	2.8

Figure II-4. Deletion of Sox2 prematurely induces mitotic arrest

(A-D) Immunofluorescence analyses of male gonads prepared from control (A), *Tg-Nanos2* (B), *Sox2*$^{\Delta/\Delta}$ (C), *Tg-Nanos2/Sox2*$^{\Delta/\Delta}$ (D) male embryos at E13.5 using mitosis marker (KI67) and germ cell marker (TRA98). White arrows indicate KI67-positive mitotic germ cells. Scale bar: 50μm

(E) Quantification of mitotic germ cells in control (n=234), *Tg-Nanos2* (n=215), *Sox2*$^{\Delta/\Delta}$ (n=201), *Tg-Nanos2/Sox2*$^{\Delta/\Delta}$ (n=208) at E13.5.

(F-I) Detection of *Nanos2* transcripts by in situ hybridization together with Immunofluorescence (KI67) in control (F,G), *Tg-Nanos2* (H,I), *Sox2*$^{\Delta/\Delta}$(J,K), *Sox2*$^{\Delta/\Delta}$*/Tg-Nanos2* (L,M). Scale bar: 100μm

(J) Quantification of results obtained in (F-M). I counted control (n=229), *Tg-Nanos2* (n=204), *Sox2*$^{\Delta/\Delta}$ (n=198), *Tg-Nanos2/Sox2*$^{\Delta/\Delta}$ (n=216) male germ cells at E13.5.

Tamoxifen was injected via intraperitoneal administration to pregnant female mice of *Sox2*$^{flox/+}$*/CAG-floxed-CAT-Flag-Nanos2* crossed with *Sox2*$^{flox/+}$*/CAG-floxed-CAT-Flag-Nanos2/Rosa-CreER*^{T2} male mouse at E10.5.

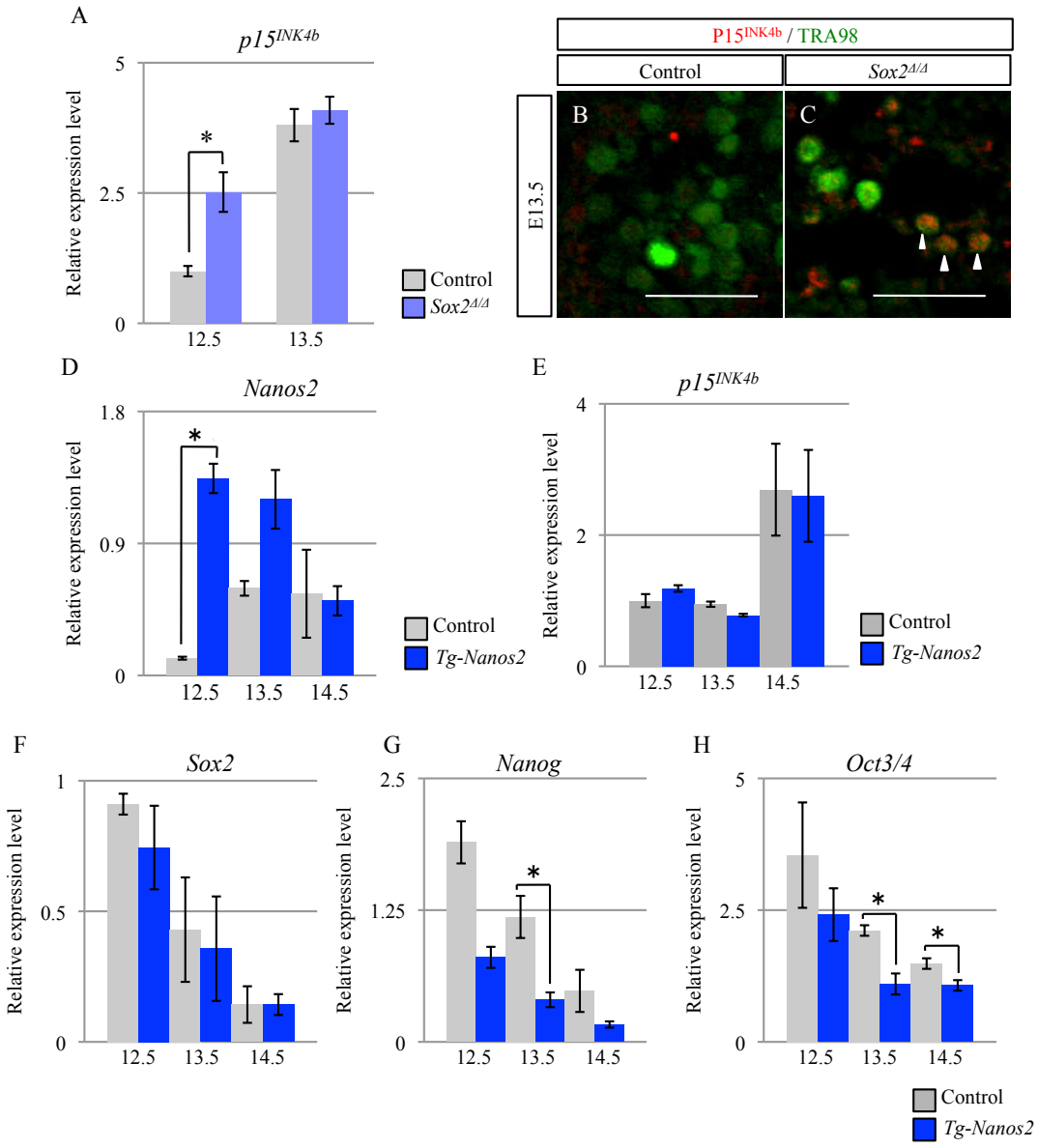

A
p15INK4b
Relative expression level
5
2.5
0
12.5
13.5
*
Control
Sox2Δ/Δ
P15INK4b / TRA98
Control
Sox2Δ/Δ
E13.5
B
C
D
Nanos2
1.8
0.9
0
12.5
13.5
14.5
*
Control
Tg-Nanos2
E
p15INK4b
4
2
0
12.5
13.5
14.5
Control
Tg-Nanos2
F
Sox2
1
0.5
0
12.5
13.5
14.5
G
Nanog
2.5
1.25
0
12.5
13.5
14.5
*
H
Oct3/4
5
2.5
0
12.5
13.5
14.5
*
*
Control
Tg-Nanos2

Figure II-5. Sox2 controls mitosis via suppressing *p15^{INK4b}* independently of Nanos2.

(A) qRT-PCR analysis showing expression of *p15^{INK4b}* in control (n=3), *Sox2$^{\Delta/\Delta}$*(n=3) male gonads at E12.5 and control (n=5), *Sox2$^{\Delta/\Delta}$*(n=6) male gonads at E13.5.

(B-C) Immunofluorescence analyses of P15^{INK4b} and TRA98 in male gonads from control (B) and *Sox2$^{\Delta/\Delta}$* (C) embryos. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E10.5 and samples were prepared at E13.5. White arrows indicate P15^{INK4b}-positive cells. Scale bar: 30μm.

(D-H) qRT-PCR analysis showing expression of *Nanos2* (D), *p15^{INK4b}*(E) *Sox2* (F), *Nanog* (G), *Oct3/4* (H) in control (n=3), *Nanos2*-over expressing (n=5) male gonads at E12.5 and control (n=4), *Nanos2*-over expressing (n=4) male gonads at E13.5 and control (n=4), *Nanos2*-over expressing (n=4) male gonads at E14.5. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E10.5. Results of qRT-PCR were normalized to *Vasa*. Data are shown as means ± SD. *P<0.05 (Student's *t* test).

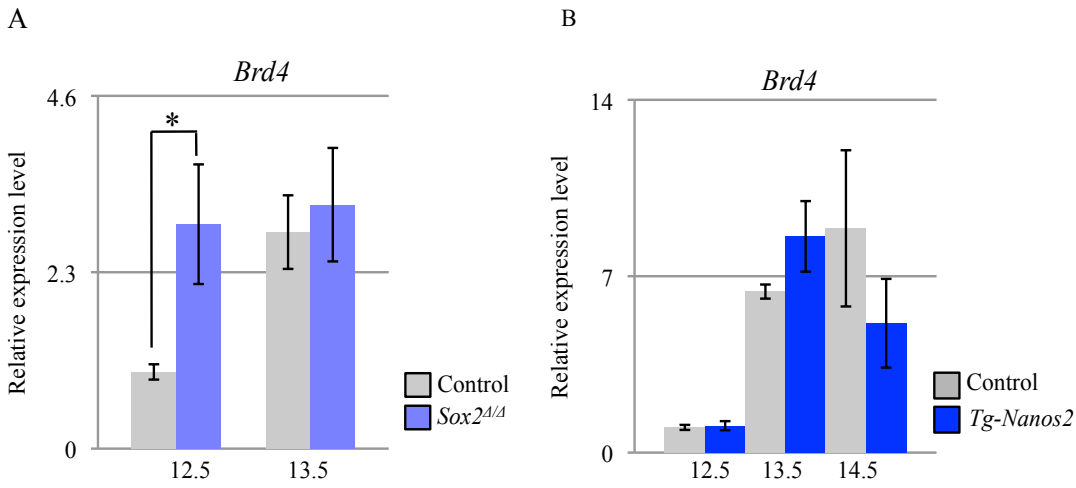
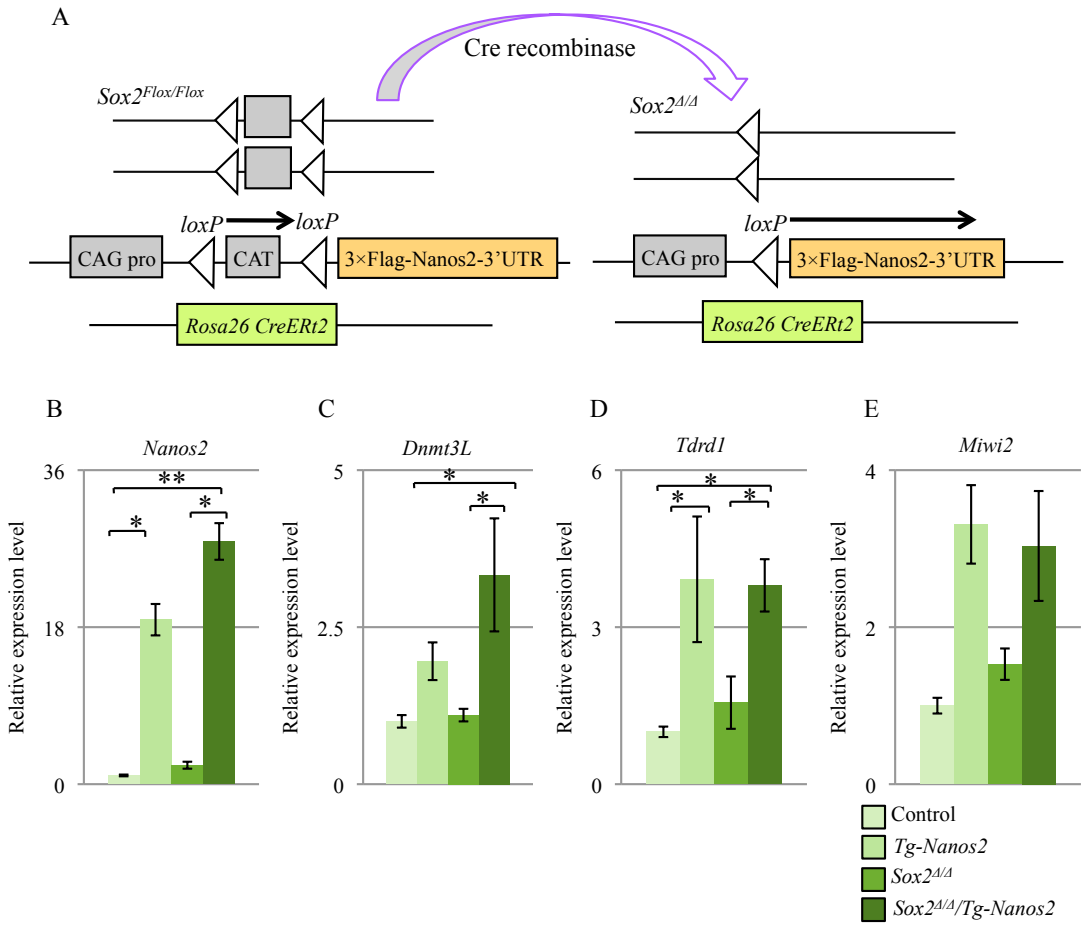


Figure II-6. Sox2 controls expression of *Brd4* in male germ cells.

(A) qRT-PCR results showing expression of *Brd4* in control (n=3), *Sox2*$^{\Delta/\Delta}$ (n=3) male gonads at E12.5 and control (n=5), *Sox2*$^{\Delta/\Delta}$(n=6) male gonads at E13.5.

(B) qRT-PCR results showing expression of *Brd4* in control (n=3), *Nanos2*-over expressing (n=5) male gonads at E12.5 and control (n=4), *Nanos2*-over expressing (n=4) male gonads at E13.5 and control (n=4), *Nanos2*-over expressing (n=4) male gonads at E14.5. Tamoxifen was injected via intraperitoneal administration to pregnant female mice at E10.5. Data are shown as means ± SD. *P<0.05 (Student's *t* test).


A
Cre recombinase
Sox2Flox/Flox
Sox2Δ/Δ
loxP
loxP
loxP
CAG pro
CAT
3×Flag-Nanos2-3'UTR
CAG pro
3×Flag-Nanos2-3'UTR
Rosa26 CreERt2
Rosa26 CreERt2
B
Nanos2
C
Dnmt3L
D
Tdrd1
E
Miwi2
Relative expression level
Control
Tg-Nanos2
Sox2Δ/Δ
Sox2Δ/Δ/Tg-Nanos2

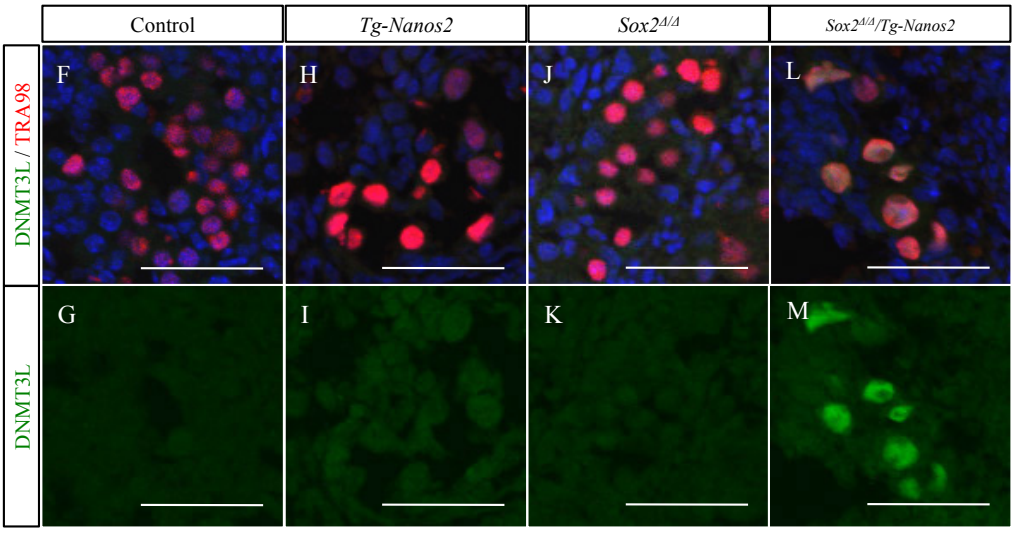

Control
Tg-Nanos2
Sox2Δ/Δ
Sox2Δ/Δ/Tg-Nanos2
DNMT3L / TRA98
DNMT3L
F
G
H
I
J
K
L
M

Figure II-7. Male differentiation pathway is successfully promoted in *Sox2*$^{\Delta/\Delta}$ */Tg-Nanos2* male germ cells.

(A) A schematic view of *Sox2*$^{\Delta/\Delta}$ with *Tg-Nanos2* mice. Cre activity was induced by intraperitoneal injection of tamoxifen. Tamoxifen was injected at E10.5 and gonads were harvested at E13.5.

(B-E) qRT-PCR results showing expression of *Nanos2* (B), *Dnmt3L* (C), *Tdrd1*(D) and *Miwi2* (E) in control (n=4), *Sox2*$^{\Delta/\Delta}$ (n=3), *Nanos2-null* (n=3) and *Sox2*$^{\Delta/\Delta}$ with Tg-Nanos2 (n=4) male gonads. Results were normalized to *Vasa*. Data are shown as means ± SD. *P<0.05 (Student's *t* test).

(F-M) Immunofluorescence analyses detecting DNMT3L and TRA98 in male gonads from control (F-G), Tg-Nanos2 (H-I), *Sox2*$^{\Delta/\Delta}$ (J-K), *Sox2*$^{\Delta/\Delta}$/Tg-Nanos2 (L-M) embryos. White arrows indicate DNMT3L^{+} cells.

Scale bars: 30μm

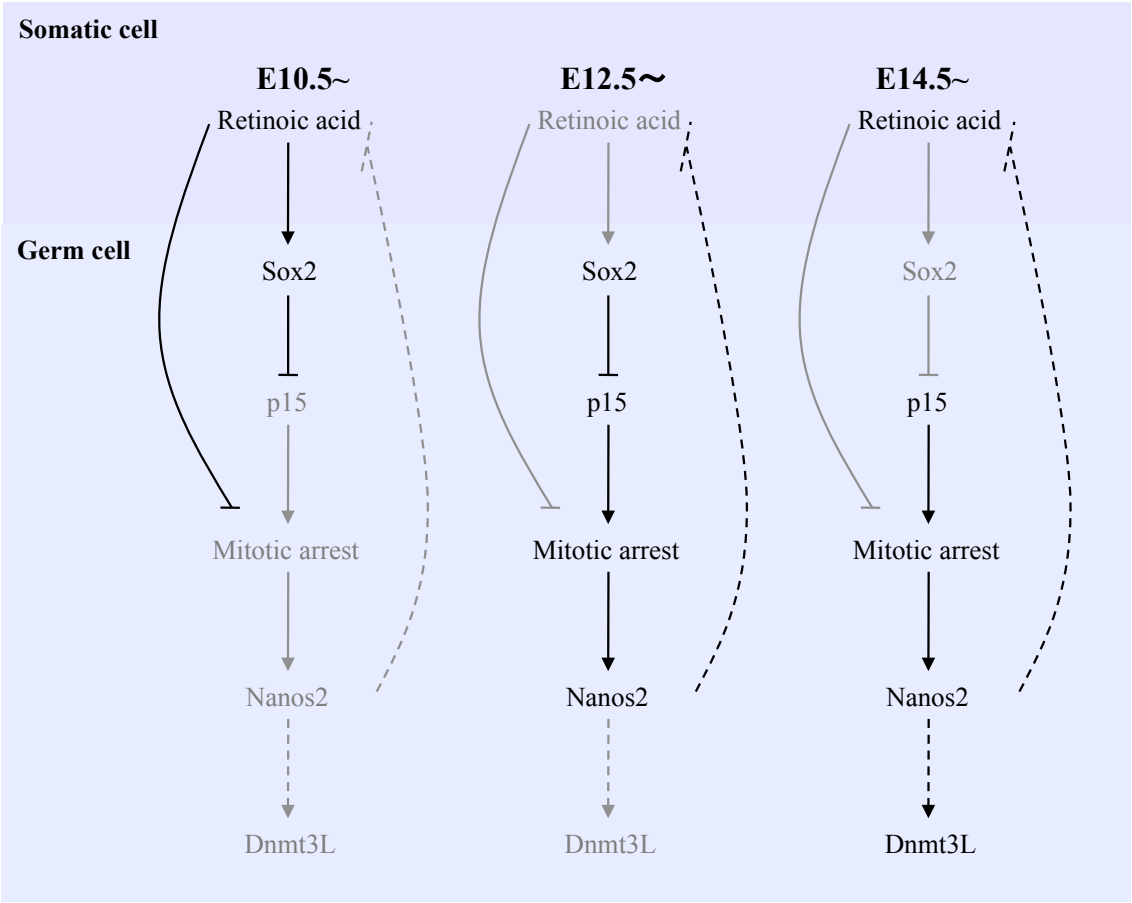


Figure II-8. Schematic summary indicating temporal changes in reciprocal relatioship between Nanos2 and Sox2 along with germ cell differentiation.

At the early stage of germ cell development, RA is secreted from mesonephros which may promote up-regulation of *Sox2* expression from E10.5. In addition, RA prevents mitotic arrest (Trautmann et al., 2008). After E12.5, *Sox2* expression is dramatically down-regulated because retinoic acid is degraded by Cyp26b1. Down-regulation of *Sox2* may induce up-regulation of $p15^{INK4b}$ via repressing *Brd4* an activator of $P15^{INK4b}$, which induces mitotic quiescence in germ cells. *Nanos2* is predominantly expressed in these quiescent cells. Once Nanos2 is expressed, it suppresses RA signaling and Sox2 expression is maintained lower level, which contribute to the maintenance of quiescent status of germ cells. Finally, Nanos2 promotes male differentiation pathway via pup-regulating Dnmt3L expression from E14.5.

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