Nanos3 is required for proper expansion of spermatogonial progenitors in mice

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【Introduction】

Germ cells are the only cell type that can transmit genetic information to the next generations. During the germ cell development, many complex processes should be precisely regulated by germline-specific genes for germ cells to acquire the unique function. Our laboratory identified important germ cell-specific genes *Nanos2* and *Nanos3*, both of which are indispensable for the mouse germ development.

The NANOS is an RNA-binding protein that is evolutionarily conserved among many organisms and plays crucial roles during the germ cell development. Three *Nanos* genes (*Nanos1*, *Nanos2* and *Nanos3*) were identified in the mouse, among which *Nanos2* and *Nanos3* are expressed specifically in germ cells. Our previous studies have revealed that *Nanos2* is expressed in a male-specific manner and plays important roles in leading germ cells to male type differentiation in embryonic stage (Suzuki and Saga, 2008), and in the maintenance of germline stem cells in postnatal stage. On the other hand, *Nanos3* is expressed in both male and female germ cells in embryonic stage and male germ cells in the postnatal stage. We have shown that *Nanos3* is required for the survival of germ cells in embryonic stage (Tsuda, 2003). However, the function of postnatal Nanos3 remains elusive. In this thesis, I analyzed the functions of *Nanos3* in spermatogenesis.

In the mouse, all germ cells are originated from primordial germ cells (PGCs) which are specified from proximal epiblast at around embryonic day (E) 6 (McLaren, 2003; Saitou et al., 2002). The PGCs can be identified by the expression of *Blimp1* that is the earliest PGC marker gene and is required for their specification (Ohinata et al., 2005). At E7.25, PGCs start to proliferate and migrate along the hindgut to the genital ridge that is the precursor of the testis and ovary. During the migration, PGCs express *Nanos3*. Genetic analysis showed that *Nanos3*-deficient mice are sterile in both male and

female because they lose PGCs by E9.5 (Tsuda, 2003). This indicates that *Nanos3* plays an important role in survival of PGCs. Colonized PGCs in the genital ridge start gender-specific differentiation at E13.5: in female, they immediately enter meiosis in response to retinoic acid (RA) produced in the mesonephros that is the neighboring tissue of the genital ridge (Bowles et al., 2006; Bowles and Koopman, 2007), whereas in male, they enter mitotic arrest and start to express *Nanos2*, a master regulator of male germ cell differentiation, to become gonocytes (Suzuki et al., 2010a; Suzuki and Saga, 2008).

In the postnatal testis, gonocytes resume proliferation and migrate toward the periphery of the seminiferous tubules to become spermatogonia within 7 days after birth and start spermatogenesis from spermatogonial stem cells (SSCs) established in the early days (Bellve et al., 1977; de Rooij, 1998; de Rooij and Russell, 2000; McLean et al., 2003). SSCs give rise to “undifferentiated” and “differentiating” spermatogonial pools with incomplete cytokinesis. Therefore, the mitotic divisions produce spermatogonial clusters with each cell interconnected. According to the number of connected cells in a cluster, undifferentiated spermatogonia are called as A_{single} (A_s, single cell), A_{paired} (A_{pr}, interconnected two cells) and $A_{aligned}$ (A_{al}, more than three connected spermatogonia). The expansion of undifferentiated spermatogonia in this process is essential to constitutively produce millions of sperms. The expanded undifferentiated spermatogonia differentiate into A_1 differentiating spermaotognia. A_1 spermaotognia further differentiate into A_2, A_3, A_4 intermediate, and B spermatogonia. These type-A (A_s, A_{pr}, A_{al} and A_1 - A_4) and type-B spermatogonia are distinguished by the cellular shapes and nuclear morphology. Then, type-B spermatogonia enter meiosis to become spermatocytes, eventually giving rise to the haploid spermatozoa. The process from type-A spermatogonia to spermatozoa is coordinated by seminiferous epithelial cycle containing

of 12 stages (I-XII) in mice, and one cycle spends 8.6 days (Leblond and Clermont, 1952). The initiation of spermatogonial differentiation and meiosis occurs at VII-VIII stages of this cycle. At these stages, Sertoli cells which are the only somatic cells in the seminiferous tubule secrete RA (Endo et al., 2015), and spermatogonia expressing *Rarg*, a receptor of RA, respond to it to enter differentiation stage and undergo meiosis (Ikami et al., 2015).

SSCs are maintained by glial cell line-derived neurotrophic factor (GDNF) secreted by Sertoli cells (Meng et al., 2000), and its receptor *Gfra1* is expressed mainly in the A_s and A_{pr} spermatogonia. Thus, GFRA1-expressing A_s and A_{pr} are thought as the stem cell population.

Our laboratory previously showed that *Nanos2* is required to maintain SSCs as a downstream factor of *Gfra1* (Sada et al., 2012). Postnatal *Nanos2*-deficiency results in the failure of stem cell maintenance. Moreover, artificial induction of NANOS2 in spermatogonia inhibits their differentiation (Sada et al., 2009). Further mechanistic analyses demonstrated that NANOS2 binds and degrades the target mRNAs, such as *Sohlh1/2* which are involved in the spermatogonial differentiation to maintain SSCs (Zhou et al., 2015).

While NANOS2 is expressed in A_s and A_{pr} clusters, NANOS3 is predominantly detected in A_{al} undifferentiated spermatogonia, and a weak expression can be detected in A_s, A_{pr} and type-A differentiating spermatogonia (Suzuki et al., 2009). In addition to the difference of expression profile between *Nanos2* and *Nanos3*, as I mentioned above, postnatal *Nanos2*-deficient mice cannot maintain the stem cell population even NANOS3 is still expressed in spermatogonia (Sada et al., 2009). These results suggest that NANOS3 is functionally distinct from NANOS2 in spermatogenesis.

In this study, I established the conditional knockout mice to assess the role of NANOS3 in spermatogenesis. Postnatal *Nanos3*-depletion resulted in the reduction of undifferentiated spermatogonia, and it influenced the number of fully matured sperm. Conversely, overexpression of *Nanos3* showed the increase of spermatogonia. However, loss or gain of NANOS3 did not impact the stem cell population. I therefore propose that NANOS3 is a positive regulator of spermatogonial progenitor expansion in murine spermatogenesis.

【Results】

Generation of *Nanos3* conditional knockout mice

Since *Nanos3*-deficient mice completely lose germ cells before birth due to apoptosis of primordial germ cells (PGCs) (Suzuki et al., 2008; Tsuda, 2003), we needed to establish a conditional knockout mouse to examine the function of *Nanos3* during spermatogenesis. As one of the strategies, we generated a bacterial artificial chromosome transgenic (BAC-Tg) mouse line which expresses a floxed red fluorescent protein (*Rfp)-tagged Nanos3* (*Nanos3-Rfp*) under the control of *Nanos3* regulatory elements. First I examined whether the transgene could rescue the germ cell-loss phenotype in *Nanos3*-deficient mice. By intercrossing with *Nanos3*$^{+/-}$*;BAC-Tg*, I obtained adult ovary and testis from *Nanos3*$^{-/-}$*;BAC-Tg*. The external observation and histological examination confirmed that both ovary and testis showed normal morphology and contained mature oocytes and sperms, indicating that the *Nanos3-Rfp* rescued the sterile phenotype of *Nanos3*-deficient mice in both male and female and that the NANOS3-RFP is functional. Next I analyzed whether *Nanos3-Rfp* reproduce the endogenous *Nanos3* expression profile during spermatogenesis. Endogenous NANOS3 marks most of undifferentiated spermatogonia, but its expression levels are different among the cell-types: in the most primitive undifferentiated spermatogonia (A_s and A_{pr}) identified by GFRA1 expression, NANOS3 is weakly expressed, whereas its expression is up-regulated in A_{al} undifferentiated spermatogonia which expresses *Ngn3*. Then finally it is gradually down-regulated concomitant with an up-regulation of KIT during the spermatogonial differentiation (Suzuki et al., 2009). I performed whole-mount immunostaining of seminiferous tubule for RFP with several marker genes. Consistent with the previous observation, the RFP signals were hardly observed in GFRA1-positive A_s and A_{pr} spermatogonia (Suzuki et

al., 2009). I used *Ngn3-GFP* transgenic mice to monitor the expression of *Ngn3* and found that NGN3-GFP-positive A_{al} spermatogonia were also positive for NANOS3-RFP. Finally I confirmed that NANOS3-RFP was down-regulated in KIT-positive cells. These observations indicate that NANOS3-RFP reproduces the endogenous NANOS3 expression.

To eliminate *Nanos3-Rfp* during spermatogenesis, I used *Nanos3-Cre* mice, in which the *Cre* recombinase was knocked-in into the *Nanos3* locus (Suzuki et al., 2008). Although *Nanos3* is expressed in PGCs from embryonic day (E) 7.25 to E13.5, our previous study showed that the Nanos3-Cre activity was very low in PGCs but was elevated after E14.5, suggesting that this Cre line is useful to knock-out *Nanos3-Rfp* at the later stages with avoiding *Nanos3*-deficient phenotype in PGCs. I also confirmed the lack of the Nanos3-Cre activity in PGCs by crossing *Nanos3-Cre* mice with *CAG-CAT-EGFP* reporter mice which expresses *EGFP* after Cre mediated recombination. The results showed that EGFP-positive PGCs were hardly observed in E13.5 gonads. This result was in contrast to a PGC-specific Cre line, *Blimp1-Cre*, in which Cre recombination occurs efficiently in migrating PGCs. Thus, I decided to use *Nanos3-Cre* mice in the following analyses.

Testicular degeneration and reduction of sperm count in *Nanos3*-cKO mice

To obtain *Nanos3* conditional knockout (cKO) mice, I crossed $Nanos3^{-/-}$;*BAC-Tg* female with $Nanos3^{Cre/+}$ male. Whole-mount immunostaining for RFP and CDH1, a marker of undifferentiated spermatogonia, at 8 week-old (W) of age showed that signals of NANOS3-RFP were not observed in almost all CDH1 positive cells in *Nanos3*-cKO testes, while strong RFP signals were observed in CDH1 positive cells in control testes. This

indicates that *Nanos3-Rfp* was successfully deleted in undifferentiated spermatogonia.

Next, I examined temporal changes in the testicular size of *Nanos3*-cKO mice. I found that the testis size became smaller in cKO than control starting from 2W and the difference was much greater in later stages. Quantification analysis showed that testis weight was significantly reduced in *Nanos3*-cKO mice starting from 2W compared with those of control. Histological analyses showed that seminiferous tubules in *Nanos3*-cKO testes were smaller than those in control testes at 8W and 12W. In addition, counting the spermatozoa in a cauda epididymis, the storage organ of functional sperm, showed the significant reduction of spermatozoa in *Nanos3*-cKO mice. These results indicate that *Nanos3* has a crucial role in spermatogenesis.

Spermatogonia may differentiate into functional spermatozoa without *Nanos3*

Despite the severe defects in *Nanos3*-cKO testis, many spermatogenic cells and spermatozoa were observed in mutant testis and epididymis, respectively. I therefore examined whether spermatogonia which were escaped from the *Nanos3-Rfp* elimination differentiated into spermatozoa or they completed spermatogenesis without *Nanos3*. To this end, I crossed *Nanos3*-cKO males with wild-type females and determined whether *Nanos3-Rfp* deleted transgene was transmitted to the next generation. Genotyping of the offspring showed the existence of offspring carried only the deleted transgenic allele.

Undifferentiated spermatogonia are reduced in *Nanos3*-cKO testis

Even though functional sperms were produced in cKO testes, the testis size was obviously reduced in cKO mice, indicating that *Nanos3* plays crucial roles during spermatogenesis. Because *Nanos3* is predominantly expressed in undifferentiated spermatogonia (Suzuki

et al., 2009), it is likely that *Nanos3* depletion causes some defects in the population. To examine the abnormalities in cKO testis in more detail, I performed immunostaining for a marker of undifferentiated spermatogonia. Since testicular tubule size was different between control and cKO testes, I used the number of Sertoli cells to normalize germ cell number because Sertoli cells are sole somatic cells in the seminiferous tubule and their number is unlikely to be affected by *Nanos3*-KO. I found that the relative number of undifferentiated spermatogonia in the *Nanos3*-cKO testis was significantly fewer than that in the control testis.

Precocious differentiation of undifferentiated spermatogonia occurs in the *Nanos3*-cKO testes

Given that the reduction of undifferentiated spermatogonia in *Nanos3*-cKO testis, I expected that it could be caused by cell proliferation defect and/or precocious differentiation of spermatogonia. However, I found that the loss of *Nanos3* does not affect the proliferation of spermatogonia.

Next, I examined whether precocious differentiation of undifferentiated spermatogonia causes the reduction of them. The results suggest that precocious differentiation of undifferentiated spermatogonia occurs in *Nanos3*-cKO testes, and this may be the main cause of the reduction of spermatogonia.

Generation of *Nanos3*-overexpression mouse line

Our previous study showed that *Nanos2*, a paralog of *Nanos3*, predominantly expressed in A_s and A_{pr} undifferentiated spermatogonia, plays a crucial role to maintain the primitive state of SSC through suppressing the genes involved in spermatogonial

differentiation (Sada et al., 2009; Zhou et al., 2015). Because NANOS2 and NANOS3 have conserved amino acid sequences in the N-terminus and the zinc-finger domain and bind to CNOT family proteins involved in the RNA degradation (Suzuki et al., 2010a; Suzuki et al., 2012), it is possible that NANOS3 also acts as a suppressor of differentiation in A_{al} spermatogonia as NANOS2 does in A_s and A_{pr}. To test this possibility, I generated a *Nanos3*-overexpression (OE) mouse line that carries *CAG-floxed-mRFP-3xFlag-tagged Nanos3* transgene, and examined whether or not the differentiation of spermatogonia was suppressed by artificial *Nanos3* induction. To induce *3xFlag-tagged Nanos3* (*3xFlag-Nanos3*) expression in male germ line, I used *Nanos3-Cre*. I confirmed the up-regulation of *Nanos3* mRNA and the expression of 3xFLAG-NANOS3 protein in *Nanos3*-OE testis.

Undifferentiated spermatogonia were increased by *Nanos3* overexpression

Immunofluorescence analyses revealed that 3xFLAG-NANOS3 was detected only in type-A spermatogonia, but was not detected in more differentiated spermatogenic cells. This observation raised the possibility that *Nanos3*-overexpressing type-A spermatogonia stopped their differentiation. If so, the number of PLZF-positive cells should be increased and KIT-positive cell counts should be decreased. To assess the possibility, I counted PLZF or KIT-positive cells of testis cross-sections by immunostaining. I found that the number of PLZF-positive spermatogonia was increased in the *Nanos3*-OE testes. However, unexpectedly, the number of KIT-positive cells, not only type-A but also type-B spermatogonia in which *Nanos3* is negative, was also increased in the *Nanos3*-OE testes. These observations suggest that the forced expression of *Nanos3* promotes the expansion of undifferentiated spermatogonial population. Then, the expanded undifferentiated spermatogonia did not stop their spermatogenesis but they initiated

differentiation. I think that it might be caused by the repression of 3xFLAG-NANOS3 expression during the spermatogonial differentiation in the *Nanos3*-OE testes even it is driven by the synthetic ubiquitous promoter (*CAG*). This repression may be caused by post-transcriptional regulation of *3xFlag-Nanos3* because RFP signals are observed in whole body, and it is reported that NANOS3 expression is regulated post-transcriptionally (Suzuki et al., 2010b). In addition, the number of GFRA1-positive SSC population showed no difference between control and *Nanos3*-OE testes, indicating that the SSC population was not affected in *Nanos3*-OE mice. Finally, I counted undifferentiated spermatogonial clusters and found that A_{al} spermatogonia were increased in *Nanos3*-OE mice, opposite to the *Nanos3*-cKO phenotype, whereas A_{pr} was not significantly changed. These results indicate that *Nanos3* positively regulates the expansion of spermatogonial progenitor population but not stem cells to maintain an appropriate pool size of undifferentiated spermatogonia.

【Discussion】

Our laboratory has reported that *Nanos3* is expressed in PGCs at embryonic stage and in undifferentiated and differentiating type-A spermatogonia (Suzuki et al., 2009). Although the importance of *Nanos3* for the survival of PGCs has been elucidated, its role in spermatogenesis has not been addressed. In this study, I addressed this issue by conducting loss and gain of function analyses of *Nanos3* in spermatogenic cells, and demonstrated that *Nanos3* plays crucial roles in the maintenance of an appropriate pool size of undifferentiated spermatogonia by regulating the expansion of them. There were two possibilities that *Nanos3* is required for the expansion of the undifferentiated spermatogonia through 1) suppressing differentiation until they expand to the appropriate amount or 2) promoting proliferation.

Proliferation of undifferentiated spermatogonia

Although *Nanos3* promotes the expansion of spermatogonia, cell proliferation defect was not observed in *Nanos3*-cKO mice. However, I could not definitely conclude that *Nanos3* plays no role in proliferation of spermatogonia because proliferation activity of undifferentiated spermatogonia at the steady-state is shown to be very low. Proliferation of undifferentiated spermatogonia is activated when the number of total spermatogonia including differentiating spermatogonia was reduced by some drug treatment (de Rooij, 2001; De Rooij and Lok, 1987; Nakagawa et al., 2010). Thus, to clarify the effect of *Nanos3* on the proliferation of spermatogonia, it is required to conduct the experiment under the high proliferative condition after the elimination of spermatogonia.

Spermatogonial differentiation

Retinoic acid (RA) exposure is required for initiation of spermatogonial differentiation (Endo et al., 2015; Raverdeau et al., 2012). One study has suggested a role of *Nanos3* in

suppression of RA signaling: in the NANOS2/NANOS3-double depletion situation in gonocytes, an RA responsible gene, *Stra8* expression level was much higher than the *Nanos2* single mutant (Suzuki et al., 2014). It is reported that the expression level of RA receptors influence the sensitivity for RA in spermatogonia (Ikami et al., 2015). Thus, more detail expression analysis of RA receptors in *Nanos3*-cKO and OE mice may clarify the contribution of NANOS3 to the RA signaling.

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