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NANOS2 promotes male germ cell development independent of meiosis suppression

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ABSTRACT

NANOS2 is an RNA-binding protein essential for fetal male germ cell development. While we have shown that the function of NANOS2 is vital for suppressing meiosis in embryonic XY germ cells, it is still unknown whether NANOS2 plays other roles in the sexual differentiation of male germ cells. In this study, we addressed the issue by generating *Nanos2*/*Stra8* double knockout (dKO) mice, whereby meiosis was prohibited in the double-mutant male germ cells. We found that the expression of male-specific genes, which was decreased in the *Nanos2* mutant, was hardly recovered in the dKO embryo, suggesting that NANOS2 plays a role in male gene expression other than suppression of meiosis. To investigate the molecular events that may be controlled by NANOS2, we conducted a series of microarray analyses to search putative targets of NANOS2 that fulfilled 2 criteria: (1) increased expression in the *Nanos2* mutant and (2) the mRNA associated with NANOS2. Interestingly, the genes predominantly expressed in undifferentiated primordial germ cells (PGCs) were significantly selected, implying the involvement of NANOS2 in the termination of the characteristics of PGCs. Furthermore, we showed that NANOS2 is required for the maintenance of mitotic quiescence, but not for the initiation of the quiescence in fetal male germ cells. These results suggest that NANOS2 is not merely a suppressor of meiosis, but instead plays pivotal roles in the sexual differentiation of male germ cells.

Introduction

The germline is established as a singular cell lineage that transmits genetic information to the next generation during early embryonic stages in both vertebrates and invertebrates. In the mouse embryo, primordial germ cells (PGCs) are formed at the base of the allantois by embryonic day (E) 7.25 (Saitou and Yamaji, 2010; Kurimoto et al., 2008). They then migrate through the hindgut toward the genital ridge. PGCs become competent to initiate meiosis after colonizing the gonad, regardless of their sex chromosome constitution (Lin et al., 2008). However, once gonadal sex is determined, the PGCs commence sex-specific differentiation by responding to factors derived from somatic cells in the developing gonads. In the female gonads, PGCs immediately initiate meiosis and enter meiotic prophase I. By contrast, in male gonads, the PGCs cease cell division, enter quiescent G0/G1 mitotic arrest and do not enter meiosis during the embryonic stage (Spiller and Koopman, 2011).

In the female gonad, retinoic acid (RA) produced and secreted by the mesonephros acts as an inducer of meiotic initiation via activation of the RA responsive gene *Stimulated by retinoic acid gene 8* (*Stra8*) in PGCs (Koubova et al., 2006; Bowles et al., 2006; Anderson et al., 2008). STRA8 is required for premeiotic DNA replication and the subsequent events of meiotic prophase I (Baltus et al., 2006). In male development, fibroblast growth factor 9 (FGF9) secreted from Sertoli cells induces PGCs to progress through the male pathway, thereby leading to the production of spermatogonial precursors (Bowles et al., 2010). In addition, cytochrome P26B1 (CYP26B1) is also implicated as an important somatic factor required for suppression of meiosis in XY PGCs via the degradation of meiosis-inducing substances, including RA (Koubova et al., 2006; Bowles et al., 2006; Maclean et al., 2007). Once germ cells receive the appropriate signals in the male gonad, they start to express NANOS2, which is an intrinsic factor that is essential for the promotion of the male pathway (Tsuda et al., 2003; Suzuki and Saga, 2008).

Abbreviations: 5-bromo-2′-deoxyuridine, BrdU; double knockout, dKO; embryonic day, E; immunoprecipitation, IP; retinoic acid, RA; reverse transcription, RT; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; primordial germ cell, PGC; RNA immunoprecipitation, RIP

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Nanos is an evolutionarily conserved RNA-binding protein containing 2 CCHC-type zinc finger motives. Nanos proteins are involved in post-transcriptional RNA metabolism via their binding to target mRNAs in germ cells (Curtis et al., 1997; Kadyrova et al., 2007; Lai et al., 2010). *Nanos2* is one of 3 Nanos family genes (*Nanos1*, *2*, and *3*) in mice that play essential roles in the survival of embryonic male germ cells and the maintenance of spermatogonial stem cells after birth (Tsuda et al., 2003; Sada et al., 2009). In the embryo, NANOS2 is required for the sexual differentiation of XY PGCs because *Nanos2*-deficient XY germ cells fail to express male-type genes and abnormally initiate meiosis (Suzuki and Saga, 2008). In addition, the ectopic expression of NANOS2 in female gonads causes germ cells to express male-type genes and suppresses the meiotic program (Suzuki and Saga, 2008). Thus, NANOS2 directs both the suppression of meiosis and promotion of male gene expression. Recently, we showed that NANOS2 associates with the deadenylation complex, thereby suggesting that NANOS2 induces PGCs to differentiate into male gonocytes via the RNA-degradation pathway (Suzuki et al., 2010). However, it is still unknown whether the suppression of meiotic genes is crucially required for male-type gene expression or whether NANOS2 independently regulates these gene expressions.

To clarify the roles of NANOS2, we adopted a genetic approach whereby meiosis was prevented in *Nanos2*-deficient male germ cells via the deletion of the *Stra8* gene together with *Nanos2*. The results indicate that NANOS2 is required for male-type gene expression independent of the suppression of meiosis. The gene expression and immunoprecipitation analyses predicted putative NANOS2 targets, which implied the possible involvement of NANOS2 in the termination of the characteristics of PGCs. Lastly, we showed that NANOS2 is required for the maintenance of mitotic quiescence, but not for entry into this state. These results suggest that NANOS2 is essential for male germ cell differentiation irrespective of its ability to suppress meiosis.

Materials and methods

Mice

The *Nanos2*-knockout mouse line used in this study has been previously described (Tsuda et al., 2003). The *Stra8*-knockout mouse line was established in our laboratory using the strategy shown in the Supplementary Material, Fig. S1A. The targeting vector was constructed to knock in the *EGFP* cDNA in-frame at the translational initiation site, with a long 6.6-kb homology arm and a short 1.7-kb arm just upstream and downstream of the site, respectively. The vector (25 μg) was electroporated into TT2 embryonic stem cells (Yagi et al., 1993) and correct homologous recombinants were aggregated with MCH (a closed ICR colony established at CLEA Japan Inc., Tokyo, Japan) 8-cell-stage embryos and then transferred into pseudopregnant female recipients. The resulting chimeric mice were bred with MCH females to obtain the *Stra8*$^{+/-}$ mice.

RNA isolation

XX and XY gonads from E12.5 to E15.5 were dissected in ice-cold phosphate-buffered saline (PBS) and deposited in RNAlater (Ambion, Life Technologies, Carlsbad, USA) at −80 °C until required for total RNA extraction. Samples of 6–20 gonads were used for each RNA analysis. Total RNA was purified using an RNeasy mini kit (Qiagen, Venlo, Netherlands) and TURBO DNA-free (Ambion). Quantification and qualitative analysis of purified RNAs were performed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Bioanalyzer 2000 (Agilent, SantaClara, CA, USA), respectively.

Microarray analysis

For each hybridization assay, 500 ng of total RNA was labeled with Cy3 and hybridized to a Whole Mouse Genome Oligo Microarray (G4122F; Agilent) in accordance with the manufacturer's protocol for the Low RNA Input Linear Amplification Kit, One Color (Agilent), and Gene Expression Hybridization Kit (Agilent), respectively. Arrays were scanned using a Microarray Scanner System (G2565BA; Agilent) and the generated images were processed using Feature Extraction, version 9.1 (Agilent) software to generate signal values for each probe set. Two independent datasets were obtained for each collation. The processed data were analyzed using Genespring GX software, version 7.3.1 (Agilent). Each data set was normalized. Specifically, measurements of less than 5 were set to 5 for data transformation, per chip normalization to the 50th percentile, and per gene normalization to the median. The microarray data reported in this study were registered in the Genome Network Platform (http://genomenetwork.nig.ac.jp/download/experimental_data/MicroArray/Micro-Array_mouse_111102_e.html) and in the Gene Expression Omnibus (GEO) database (GSE37720).

RT-qPCR

cDNAs were synthesized from the total RNA of mouse fetal gonads using Superscript III (Invitrogen, Life Technologies, Carlsbad, CA, USA) and oligo dT or random primers. Quantitative PCR analysis was then performed using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA) and a Thermal Cycler Dice (Takara, Otsu, Japan). The primer sets are listed in the Supplementary Material, Table S1.

Purification of NANOS2-associated RNAs

NANOS2-associated RNAs were obtained as previously described (Suzuki et al., 2010) with some modifications. The E14.5 XY gonads of the transgenic mice, which expressed FLAG-tagged NANOS2, were dissected in ice-cold PBS and stored at −80 °C until required for RNA-immunoprecipitation (IP). Samples of 80–100 gonads were used per one precipitation reaction. The tissues were homogenized in 5 volumes of ice-cold extraction buffer, and the lysates were pre-cleared using protein A-agarose (Sigma, St. Louis, USA) for 30 min at 4 °C. The pre-cleared lysates were then divided into 2 tubes and incubated with either anti-FLAG IgG-agarose or IgG-agarose (Sigma) for 3 h at 4 °C. Inputs and immunoprecipitated mRNAs were isolated using an RNeasy mini kit (Qiagen) and were then used as templates to generate Cy3-labeled RNA probes to hybridize to the microarrays.

Immunohistochemistry

XY gonads were dissected in ice-cold PBS, fixed with 4% paraformaldehyde in PBS at 4 °C for 2 h. 5-Bromo-2′-deoxyuridine (BrdU) was administered to pregnant mice at 100 μg/g body weight 2 h before dissection. Fixed embryos were embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), frozen and sectioned at 8 μm using a cryostat. Frozen sections were incubated with the primary and secondary antibodies: rat TRA98 (a gift from Y. Nishimune, Osaka University); rabbit anti-DNMT3L (a gift from S. Yamanaka, Kyoto University); rabbit anti-SYCP3 (a gift from S. Chuma, Kyoto University); rabbit anti-KI67 (Neomarkers, Thermo Scientific, Waltham, MA, USA); rabbit anti-PRB1 (Cell Signaling Technologies, Danvers, MA, USA); mouse anti-BrdU (BD Biosciences,