Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development

Atsushi Suzuki1,*, Masayuki Tsuda2,3,*,† and Yumiko Saga1,2,‡

The mouse Nanos proteins, Nanos2 and Nanos3, are required for germ cell development and share a highly conserved zinc-finger domain. The expression patterns of these factors during development, however, differ from each other. Nanos3 expression in the mouse embryo commences in the primordial germ cells (PGCs) just after their formation, and a loss of this protein results in the germ cell-less phenotype in both sexes. By contrast, Nanos2 expression begins only in male PGCs after their entry into the genital ridge and a loss of this protein results in a male germ cell deficiency, irrespective of the co-expression of Nanos3 in these cells. These results indicate that these two Nanos proteins have distinct functions, which depend on the time and place of their expression. To further elucidate this, we have generated transgenic mouse lines that express Nanos2 under the control of the Oct4PE promoter and examined Nanos2 function in a Nanos3-null genetic background. We find that ectopically produced Nanos2 protein rescues the Nanos3-null defects, because the germ cells fully develop in both sexes in the transgenic mice. This result indicates that Nanos2 can substitute for Nanos3 during early PGC development. By contrast, our current data show that Nanos3 does not rescue the defects in Nanos2-null mice. Our present findings thus indicate that there are redundant functions of the Nanos proteins in early PGC development, but that Nanos2 has a distinct function during male germ cell development.

KEY WORDS: Primordial germ cell, Transgenic mouse, Oct4 enhancer, Antibody, Genetic rescue, Spermatogenesis

INTRODUCTION

Nanos is a putative RNA-binding protein that has now been implicated in germ cell development in both invertebrates (Asaoka et al., 1998; Fujii et al., 2006; Lehmann and Nusslein-Volhard, 1991; Pilos and Weisblat, 1997; Subramaniam and Seydoux, 1999) and vertebrates (Koprumer et al., 2001; MacArthur et al., 1999; Tsuda et al., 2003). Although the expression of the Nanos genes is confined to the germ cell lineage in most instances, it has also been shown that Nanos mRNA and protein are supplied as germ-plasm components in Drosophila oocytes. In mouse, three Nanos genes, Nanos1-3, have now been identified (Haraguchi et al., 2003), among which Nanos2 and Nanos3 are expressed in the embryonic germ cells, and a deficiency in these genes results in the loss of germ cells (Tsuda et al., 2003). The initial expression of Nanos3 is detectable in the early PGCs that have emerged from the base of the allantois (our unpublished observation), and this expression continues during the migration stage. After entering the genital ridge, the expression of Nanos3 in male PGCs is retained for a while, but disappears by embryonic day 15.5 (E15.5), whereas, in PGCs entering into the female gonad, this expression disappears prior to E13.5. Nanos3 expression then restarts in a small population of spermatogonia after birth (our unpublished data). In contrast to the Nanos3 expression profile that initiates in early PGCs prior to sexual specification, Nanos2 expression is male-PGC specific and commences only in PGCs that have entered the male genital ridge. All male PGCs express Nanos2, and this pattern continues during the embryonic stage when these cells are arrested in the G0-G1 phase. However, the expression of Nanos2 decreases just before birth and only a small number of spermatogonia express this gene after birth, similar to Nanos3 (Tsuda et al., 2006).

We have previously reported the phenotypes of Nanos2-null and Nanos3-null mice (Tsuda et al., 2003), and our findings indicate that both genes are essential for the maintenance of PGCs. Nanos3-null PGCs are lost during the migration stage, irrespective of the sex of the animal, whereas Nanos2-null PGCs die in only the male gonads and show no evident defects in females. Because Nanos2 and Nanos3 share a conserved zinc-finger domain that is implicated in their functional roles (Curtis et al., 1997), we speculated that these proteins have equivalent functions and that any differences in their associated null-phenotypes may depend on their expression pattern only. However, PGCs are lost in Nanos2-null mice, although both Nanos2 and Nanos3 transcripts can be observed in the wild-type embryos, which may suggest that a non-redundant role exists for Nanos2 during the maintenance of male PGCs. Moreover, because Nanos3-null PGCs are lost before entry into the gonads, analysis of the compound knockout mice provides no information concerning the impact upon PGC development at later stages. To overcome this problem, we adopted a transgenic strategy in our current study that will enable us to determine whether these two proteins share any functional similarities. For this purpose, we employed Oct4 enhancer elements, because they have been fully characterized in previous transgenic strategies. In particular, Oct4PE shows both broad and low levels of activity in the epiblasts of egg-cylinder-stage mouse embryos, but the expression of genes under its control becomes restricted in the PGCs that form at the base of the allantois at E7.25, and is continuously observed in the PGCs during the migration stage and in the male gonads (Yeom et al., 1996; Yoshimizu et al., 1999). In addition, the ectopic expression of Oct4PE-controlled genes in the PGCs upon entry into the female gonads is repressed. Significantly, these expression profiles are very similar to
endogenous Nanos3, and Oct4ΔPE thus serves as an ideal enhancer to drive the Nanos2 gene in a Nanos3-expressing lineage, as we have not yet identified the Nanos3 enhancer elements. Permanent transgenic mouse lines were therefore established using Oct4/H9004PE to drive a Nanos2 transgene in a Nanos3-null background to determine whether Nanos2 can functionally substitute for the loss of Nanos3. We further generated transgenic mice in a Nanos2-null background that drive Nanos3 expression in a Nanos2-expressing PGC lineage. Our results indicate that Nanos2 can replace Nanos3 function, but that Nanos3 does not rescue Nanos2-null defects.

MATERIALS AND METHODS

Production of polyclonal antibodies against Nanos2 and Nanos3
Recombinant proteins were used as antigens to generate antibodies against both Nanos2 and Nanos3. DNA fragments encoding the full-length Nanos2 and Nanos3 proteins were cloned into both the pET21d (Novagen) and pGEX-4T1 (Amersham Biosciences) vectors. 6His-tagged Nanos2 and Nanos3 fusion proteins were then expressed in E. coli BL21(DE3), purified with TALON Metal Affinity Resin (BD Biosciences) and used to immunize rabbits. GST-tagged Nanos2 and Nanos3 fusion proteins were expressed in BL21, purified with glutathione-sepharose beads (Amersham Biosciences) and cross-linked with NHS-activated Sepharose (Amersham Biosciences) to generate GST-Nanos2 and GST-Nanos3 beads. Specific antibodies were affinity-purified with these GST beads from the obtained antisera and stored in 0.1% BSA containing 0.05% NaN3 at 4°C.

Western blotting
Embryonic gonads were sonicated with an ultrasonic processor (Vibra Cell, Sonics and Material Inc.) in 2× SDS-PAGE sample buffer (200 mM Tris-HCl, pH 8.3, 4% SDS, 400 mM DTT, 20% glycerol, 2 mM EDTA, 0.05% bromophenol blue). Extracts were then resolved on 15% SDS-PAGE gels and electroblotted onto nitrocellulose membrane (BioTrace NT, Pall Corporation). Western blotting was performed using the primary antibodies anti-Nanos2 (1:200), anti-Nanos3 (1:200) and anti-actin (1:2000, Sigma). This was followed by incubation with swine anti-rabbit IgG conjugated with AP (1:2000, DAKO) as the secondary antibody for anti-Nanos2 and anti-Nanos3, and goat anti-mouse IgG conjugated with AP (1:2000, Novagen) for the anti-actin primary antibody. The detection of immunoreactivity was performed using a BCIP/NBT Phosphatase Substrate kit (KPL) according to the manufacturer’s instructions.

Generation of transgenic mouse lines
The Oct4ΔPE-FLAG-Nanos2 construct was generated by ligating the Oct4ΔPE promoter-enhancer (Yoshimizu et al., 1999) with a 3′-FLAG-tagged Nanos2 cDNA insert containing a 3′-UTR. To construct Nanos2E-FLAG-Nanos3, a BamHI fragment (9.2 kb) containing the upstream region of the mouse Nanos2 gene was ligated with a 3′-UTR of the Nanos3 gene followed by the 3′-UTR of the Nanos2 gene. These transgenes (schematically shown in Figs 2 and 5) were injected into fertilized eggs and two independent transgenic lines were subsequently established for each construct. The specific expression of Nanos2 and Nanos3 in the embryonic male germ cells was confirmed by western blot analysis using polyclonal antibodies. Genotyping was performed using the primers FLAG-F (5′-
CTACAAGACCATGACGTTG-3') and Nanos2-3'UTR-R (5'-CCCGAGAATGACACCCACCG-3') for Oct4-ΔPE-FLAG-Nanos2 and Nanos2E-FLAG-Nanos3.

Reverse transcription-PCR
Total RNA isolates from male gonads were prepared using an RNaseasy Mini kit (Qiagen). Total RNA (1 μg) was then used for cDNA synthesis. The following PCR primer pairs were used for amplification of Nanos2 and Nanos3:
Nanos2-F: 5'-AACTTCTGCAAGCACAATGG-3';
Nanos2-R: 5'-CCGGAATGACACCCACCG-3';
Nanos3-F: 5'-TCCCGTGCCATCTATCAG-3';
Nanos3-R: 5'-GCCATGCCATCAC-3'.

Control PCR-amplification reactions were performed using primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH): G3PDH-F: 5'-ACCACAGTCCGCACTGACAC-3';
G3PDH-R: 5'-TCCACCACCTGGTGTGACAT-3'.

Histological methods
For histological analysis, samples (testes, ovaries and male gonads) were fixed in Bouin’s solution and embedded in paraffin. Sections (6 μm) were stained with hematoxylin and Eosin. For immunohistochemical detection of male germ cells, sections were incubated with TRA104 (1:10) or TRA98 (1:9000) followed by Alexa-488 conjugated goat anti-rabbit IgG (Molecular Probes) and observed using a fluorescent microscope (Olympus BX61). The method for whole-mount detection of PGCs by alkaline phosphatase staining has been described previously (Tsuda et al., 2003).

RESULTS
Expression profile of Nanos proteins during germ cell development
Our previous study has shown that Nanos3 plays an essential role during the early stages of PGC development and that Nanos2 is required for male germ cell development in the mouse (Tsuda et al., 2003). However, our reverse transcription (RT)-PCR analysis indicated that Nanos3 was also expressed during male germ cell development (Tsuda et al., 2003). To investigate the functional relationship between these proteins in our current study, we first attempted to generate polyclonal antibodies and successfully obtained anti-Nanos2 and anti-Nanos3 antibodies that specifically recognize the endogenous proteins (Nanos2, approximately 18 kDa; Nanos3, approximately 27 kDa; Fig. 1A and see Fig. S1 in the supplementary material). We then examined the expression profiles of these two factors in embryonic male gonads by western blot analyses using extracts derived from one-half of each single testis. In the wild-type mouse embryo, Nanos2 expression was undetectable at E12.5 but was found to increase at E13.5 and continue until E17.5 (Fig. 1B). Because male germ cells are arrested in the G0-G1 stage at E12.5 but was found to increase at E13.5 and continue until E17.5

speculate that this may be due to transcriptional regulation, because RT-PCR analysis revealed that Nanos3 mRNA is also up-regulated in the Nanos2-/- mouse (Fig. 1C).

Transgenic strategy
Our protein-expression data indicated that Nanos2 and Nanos3 may not have redundant functions, because Nanos3 was found to be abundantly expressed in the Nanos2-null embryonic gonad, in which
germ cells are lost by programmed cell death. However, during the early stages of PGC development, only \textit{Nanos3} is expressed. To examine whether Nanos2 could substitute for the loss of functional Nanos3 in early-stage PGCs, we adopted a transgenic strategy that used an Oct4 enhancer. Because the \textit{Nanos3}-promoter region has not yet been identified, we employed the Oct4-PE enhancer because the expression profiles of the transgenes driven by this element resemble the \textit{Nanos3} expression pattern. It has been shown previously that this Oct4 enhancer is active in early PGCs and maintains its activity during the migration stage, and also in the male gonads (Yoshimizu et al., 1999). Moreover, this expression continues after birth in the small population of spermatogonia that contribute to spermatogenesis. We expressed FLAG-tagged Nanos2 under the control of Oct4-PE in PGCs. Among the three transgenic lines that we established in these experiments, we confirmed the expression of the transgene in embryonic gonads in two lines. Western blot analysis also revealed that both of the transgenic mice that we generated produced an appreciable quantity of FLAG-tagged Nanos2, in addition to endogenous Nanos2 (Fig. 2A). However, it is noteworthy that the endogenous levels of Nanos2 were decreased in these transgenic mice, which may indicate the presence of some regulatory mechanism that controls the Nanos2-protein levels. Further analysis was conducted using a transgenic line (line #5), which showed a higher expression of the transgene. The transgenic mice showed no apparent defects as a result of the ectopic expression of FLAG-tagged Nanos2 in the early-stage embryos and the transgene was successfully transmitted via both males and females.

**The FLAG-tagged Nanos2 protein is functional**

We introduced our FLAG-tagged Nanos2 transgene into a Nanos2-null testis to determine whether this exogenously tagged protein would be functional in vivo (Fig. 2). As shown previously, Nanos2-null male mice have no germ cells from about 4 weeks after birth (Tsuda et al., 2003) (Fig. 2D). However, we observed an increased testis size in these knockout mice following the expression of the Nanos2 transgene (Fig. 2B,F), and a subsequent histological study of the transgenic tissues revealed the presence of normal spermatogonia, spermatocytes and spermatids in the mature seminiferous tubules (Fig. 2E). We also confirmed that these mice are fertile, indicating that the FLAG-tagged Nanos2 protein is functional. However, we did observe the presence of morphologically abnormal tubules in the Nanos2-null testis that expressed the transgene (Fig. 2E, asterisk), and that the relative size of the testis in the transgenic mouse remained smaller than the Nanos2+/– mouse. This may be due to the lower expression of the Nanos2 transgene, or might reflect the differences between the activities of the Oct4-PE and endogenous Nanos2 enhancers in the testis of adult mice.

**Nanos2 rescues the Nanos3 deficiency in both male and female mice**

We next introduced our FLAG-tagged Nanos2 transgene into a Nanos3-null genetic background to test whether this would rescue the Nanos3-null defect. Consistent with earlier reports (Tsuda et al., 2003), Nanos3-null male mice showed no detectable germ cells at
Immunohistological examination of these tissues further revealed testes as a result of transgene expression (Fig. 3A,E,I). However, we could detect significant increases in the sizes of the any of the developmental stages examined after birth (Fig. 3C,G). We further examined the phenotype of Nanos3-null females expressing the Nanos2 transgene. Ovaries at weeks 6–7 after birth were prepared and histological observations revealed the presence of a large number of oocytes at several stages, including some that had fully developed (Fig. 3J-L). In addition, these transgenic females were fertile and could deliver normal pups. Because Nanos3 is not expressed in female germ cells after birth, even in the wild-type mice, we conclude from these findings that Nanos3 is not involved in oocyte maturation and that normal female germ cells are already present at birth. These data indicate that Nanos3 is not required for spermatogenesis and that any role it may play in this process can be substituted for by Nanos2.

Nanos2 partially rescues the loss of Nanos3 function in early-stage embryos

To investigate the precise extent to which Nanos3-null germ cells are rescued by Nanos2-transgene expression, including the timing, we examined germ cell development at different embryonic stages. In the Nanos3-null embryo, the germ cells are gradually lost during the migration stage and only a few PGCs reach the genital ridge at E11.5 (Fig. 4B). However, many PGCs were found to have reached the genital ridge in the Nanos3-null embryos expressing the Nanos2 transgene (Fig. 4C,D). Although their number was still lower than in wild type (Fig. 4A), these PGCs also appeared to be maintained in the developing gonads in both the male and female transgenic embryos (Fig. 4E-J). We could also detect an appreciable number of TRA104- or TRA98-positive PGCs in both the E14.5 male (Fig. 4E) and E16.5 female (Fig. 4J) gonads in Nanos3-null mouse embryos harboring the Nanos2 transgene.

Nanos3 does not rescue the defects in Nanos2-null PGCs

The finding that Nanos2 rescues the function of Nanos3 in early-stage mouse embryos indicates that functional redundancy exists between these two proteins. However, we speculated as to whether this was really the case for Nanos3; defects could still be observed in Nanos2-null PGCs, in which we observed an increase in Nanos3 expression. To examine this further, we generated transgenic lines expressing a FLAG-tagged Nanos3 transgene under the control of the Nanos2 enhancer. We had already confirmed that the 8.6 kb upstream region of the Nanos2 gene is sufficient to facilitate Nanos2 expression in both embryonic and postnatal germ cells (unpublished data). Two transgenic lines were subsequently established and one of these expressed a good level of exogenous Nanos3 in the embryonic male gonads (Fig. 5A). This transgene was then introduced into a Nanos2-null background and the development of germ cells was examined in both the embryonic (Fig. 5B-D) and adult (Fig. 5E-G) testes. In the Nanos2-null testes, PGCs begin to undergo apoptosis from E15.5 and most of the gonocytes disappear at birth. The small population of gonocytes remaining after birth is eventually lost within 4 weeks (Fig. 5F). At first, we examined E16.5 gonads, in which Nanos2-null PGCs begin to migrate out from the testis cords (Fig. 5C). We did not find any differences in the dynamics of the TRA104-positive PGCs irrespective of the presence or absence of the transgene in the Nanos2-null background (compare Fig. 5C with 5D). Apoptotic cell death was detected in E18.5 testes from both Nanos2–/– and Nanos2–/– containing the Nanos3 transgene.
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**DISCUSSION**

**Functional relationship between Nanos2 and Nanos3**

Our current study using a specific transgenic strategy provides valuable information that further clarifies the disparate functions of the closely related Nanos proteins during germ cell development in the mouse. Nanos proteins are essential for the development of germ cells after the initial formation of these cells at E7.25. Nanos3 has been implicated in the maintenance of PGCs during the migration stage at E8.5-E11.5, whereas Nanos2 has been shown to play a role in the maintenance of male PGCs at E13.5-E17.5. Moreover, in either Nanos3- or Nanos2-null mice, PGCs are most likely to be eliminated by apoptosis (unpublished data) (Tsuda et al., 2003). If the suppression of this apoptotic response was the primary function of the Nanos proteins, we would expect that one could rescue the defects caused by a deficiency in another. The function of Nanos3 in early PGCs that were null for this gene was indeed found to be substituted for by Nanos2, emphasizing that functional redundancy does exist between these two proteins. However, we show that Nanos3 does not rescue the Nanos2 defect in Nanos2-null male PGCs, indicating that there are distinct functions of Nanos2 that cannot be complemented for by Nanos3. Hence, we conclude that Nanos3 does not have a similar role to Nanos2 in male PGCs in the mouse. The function of Nanos in the suppression of apoptosis has been shown previously in Drosophila development, and the direct target is indicated to be involved in this apoptotic response pathway (Hayashi et al., 2004) (S. Kobayashi, personal communication). However, neither the target RNA molecules nor the mechanisms underlying Nanos function during mouse PGC development are known. Our present study suggests the presence of different targets that may be specifically recognized by Nanos2 or that a distinct mechanism may be mediated via Nanos2 in male PGCs. However, we cannot exclude the possibility that the phenotype of the PGCs in Nanos2-null embryos reflects the activity of a functional Nanos3 in these cells and may in fact be a rescued state. This possibility is suggested by the fact that the male germ cell-less phenotype became severe after birth when the Nanos3 gene dosage was reduced in the Nanos2-null genetic background (see Fig. S3 in the supplementary material). It will be necessary to generate and analyze a Nanos2-Nanos3 double knockout in male PGCs to elucidate this fully, and conditional knockout mice will be available for future experiments to address this crucial issue.

One of remaining questions regarding the function of the Nanos proteins is their precise roles during spermatogenesis. Because both Nanos2 and Nanos3 are required during the embryonic stages of development in the mouse, we cannot yet address this issue, but will employ conditional knockout strategies in future studies to examine this important issue.

**Regulation of Nanos expression**

Our current western blot analyses have revealed the presence of two interesting regulatory mechanisms that are involved in the Nanos pathways. In the absence of Nanos2 in embryonic male PGCs, expression of Nanos3 is up-regulated, and this is accompanied by an increase in its transcript levels also. This is unlikely to be the result of a direct function of the loss of Nanos2, however, because the...
Nanos2 protein is most likely to be present in the cytoplasm and not in the nucleus. One possible mechanism could be the negative regulation of transcriptional activators by Nanos2. Our preliminary GeneChip analysis indicates that the levels of other mRNA species are increased in the absence of Nanos2. We thus speculate that one of major the functions of Nanos2 might well be translational repression of specific transcriptional activators.

Another noteworthy observation that has emerged from our current data is the possible auto-regulation of Nanos2. In our transgenic mice harboring enhanced exogenous Nanos2 expression, the endogenous levels of the Nanos2 protein were found to be decreased. This observation indicates the presence of a mechanism that maintains Nanos2-protein expression at constant levels. This contention is further supported by our analysis of Nanos2-heterozygous embryos, in which the levels of Nanos2 protein are equivalent to the wild-type embryos, although the transcript levels are decreased to about 50% of that of wild type. One possible explanation for this is translational repression of Nanos2 by Nanos2 itself. We expect that excess Nanos2 protein may negatively affect its own translational efficiency, but we have shown that the Nanos2–3′-UTR might be required for efficient translation also (Tsuda et al., 2006). Because the 3′UTR elements within the Nanos family of mRNAs have also been shown to be regulated by several proteins (Dahanukar and Wharton, 1996; de Moor et al., 2005; Duchow et al., 2005; Nelson et al., 2004), these factors may also affect the translational efficiency of Nanos2 in combination with other mechanisms.

Taken together, it is most likely that the Nanos proteins function as a cellular component of RNA metabolism. Further studies in our laboratory will therefore focus upon the mechanisms that regulate Nanos2 and Nanos3 expression, as well as the functions of these two proteins themselves.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02697/DC1

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