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

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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 Oct4ΔPE 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 in the mouse.

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; Pilon and Weisblat, 1997; Subramaniam and Seydoux, 1999) and vertebrates (Koprunner 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 of these cells. 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, Oct4ΔPE 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 Oct4ΔPE-controlled genes in the PGCs upon entry into the female gonads is repressed. Significantly, these expression profiles are very similar to

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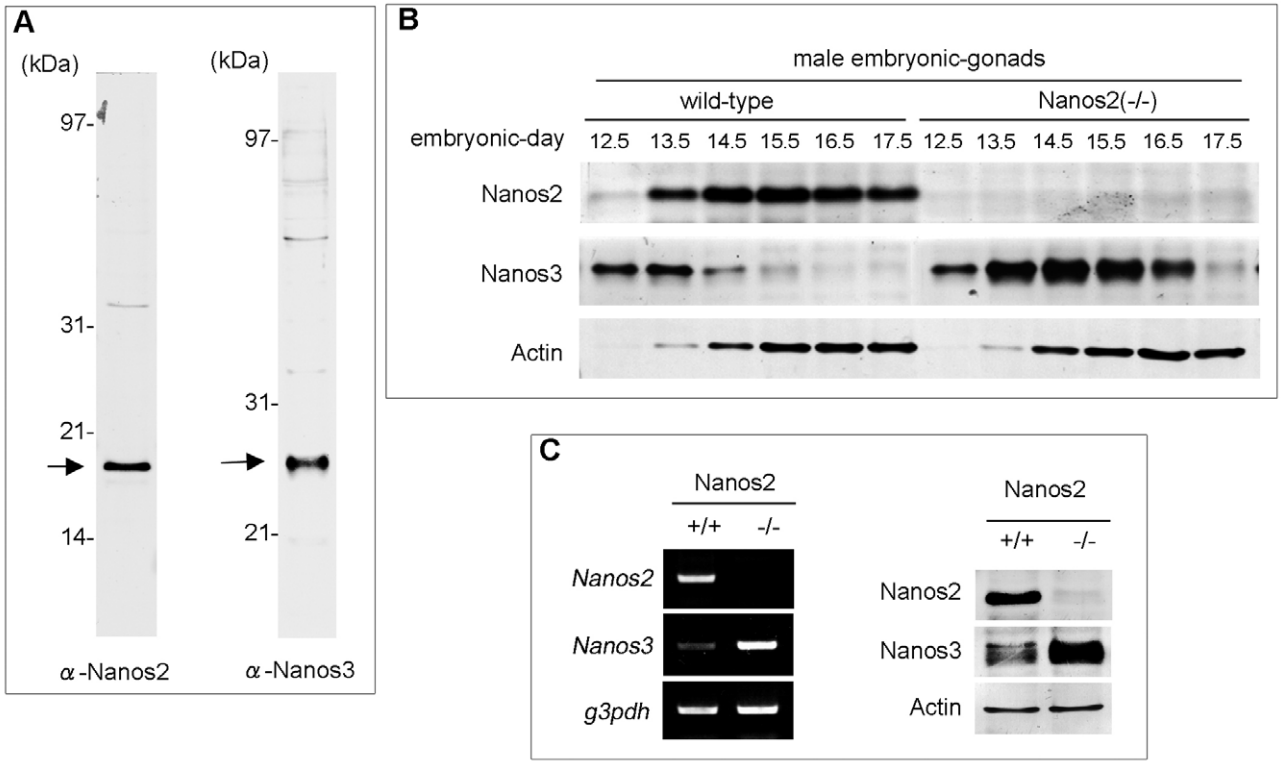

Fig. 1. Expression profile of Nanos proteins. (**A**) Characterization of Nanos2 and Nanos3 antibodies. Cell extracts were prepared from E14.5 and E12.5 male gonads, resolved by SDS-PAGE and subjected to western blot analysis with Nanos2 and Nanos3 antibodies, respectively. Arrows indicate the endogenous proteins. (**B**) Expression profile of Nanos proteins in embryonic gonads. Extracts were prepared from each single male embryonic gonad of wild-type and Nanos2$^{-/-}$ littermates. One-half of these extracts were resolved by SDS-PAGE and subjected to western blot analysis with Nanos2 and Nanos3 antibodies. Actin was used as a reference for protein amounts. (**C**) Analysis of Nanos mRNA and protein expression. Total RNA and protein extracts were prepared from E14.5 male gonads of wild-type and Nanos2$^{-/-}$ littermates. Expression of *Nanos2* and *Nanos3* mRNA was analyzed by RT-PCR. G3PDH was used as the internal standard for normalization. The expression levels of the Nanos2 and Nanos3 proteins were analyzed by western blotting as in B.

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 Oct4ΔPE 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. 6×His-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% NaN_3 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 *Bam*HI fragment (9.2 kb) containing the upstream region of the mouse *Nanos2* gene was ligated with 3×FLAG-tagged *Nanos3* cDNA 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′-

CTACAAAGACCATGACGGTG-3′) and *Nanos2-3′UTR*-R (5′-CCCG-AGAAGTCATCACCAG-3′) for Oct4-ΔPE-FLAG-Nanos2 and Nanos2E-FLAG-Nanos3.

Reverse transcription-PCR

Total RNA isolates from male gonads were prepared using an RNeasy 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′-CCGAGAAGTCATCACCAG-3′;
Nanos3-F: 5′-TCCCGTGCCATCTATCAG-3′;
Nanos3-R: 5′-GGATGTTGAGGCAACACC-3′.

Control PCR-amplification reactions were performed using primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH):

G3PDH-F: 5′-ACCACAGTCCATGCCATCAC-3′;
G3PDH-R: 5′-TCCACCACCCTGTTGCTGTA-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) (Tanaka et al., 1997) followed by subsequent detections using the Vectastain ABC kit (Vector Laboratories). Such sections were counterstained with hematoxylin prior to microscopic analysis. Both TRA antibodies specifically recognize germ cells, but TRA98 detects only a part of spermatogonia after birth. For embryonic female gonads, samples were directly embedded into OCT compound (Tissue-Tek, SAKURA) and sectioned by cryostat. The frozen sections were incubated with TRA98 antibody (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 during E13.5-E17.5, the change in the amount of the protein reflects the expression level in germ cells, whereas the change in the amount of actin is mainly due to the increase in somatic cell number. By comparison, Nanos3 was already detectable by E12.5 but became undetectable by E15.5. We additionally examined *Nanos3* expression in a *Nanos2*-null embryonic gonad, as we expected that Nanos3 may be downregulated in *Nanos2*-null mice and thus account for the PGC defects in the gonad. Interestingly, however, Nanos3 expression appeared to be up-regulated in the *Nanos2*-null germ cells, in which large amounts of Nanos3 could be detected until E16.5 (Fig. 1B). We

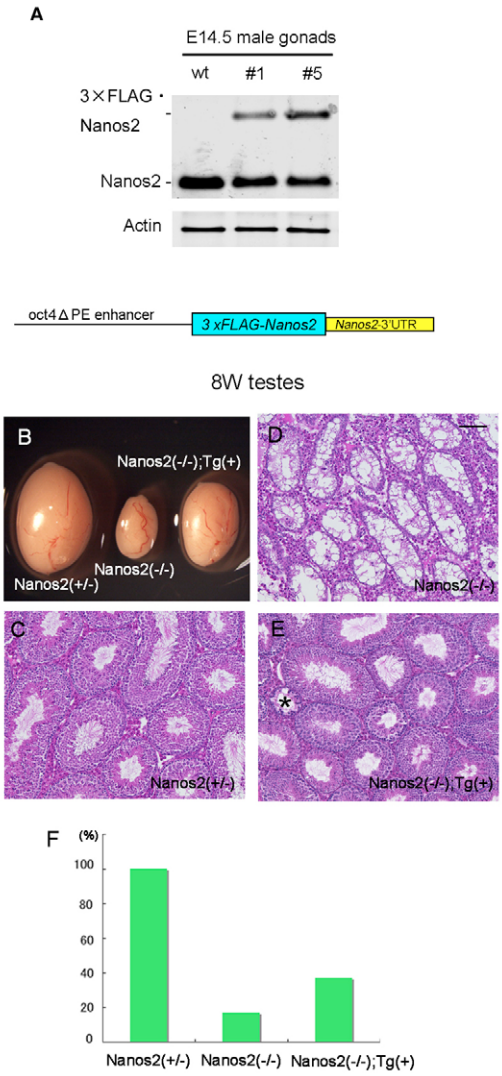

Fig. 2. Ectopic expression of a FLAG-tagged Nanos2 protein driven by the *Oct4ΔPE* enhancer can rescue the defects in *Nanos2*-null testes. (**A**) Western blot analysis of Nanos2 from both wild-type and transgenic mouse lines expressing a FLAG-tagged Nanos2 under the control of the *oct4ΔPE* enhancer. Extracts were prepared from E14.5 male gonads and actin was used as the loading control. The level of endogenous Nanos2 was decreased in the transgenic mice. The transgene construct is schematically depicted. (**B**) Comparison of testis size among littermates of Nanos2$^{+/-}$, Nanos2$^{-/-}$ and Nanos2$^{-/-}$ mice expressing the FLAG-tagged *Nanos2* transgene background (line #5). (**C-E**) Sections were prepared from the testes shown in B and stained with hematoxylin and Eosin. The genotypes are indicated in each panel. (E) Some empty (asterisk) or smaller tubules were observed in the rescued testis. (**F**) Comparison of testis weights among different genotypes at 8 weeks. The testis weight of Nanos2$^{+/-}$ was designated as 100%. Scale bar: 100 μm in D for C-E.

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

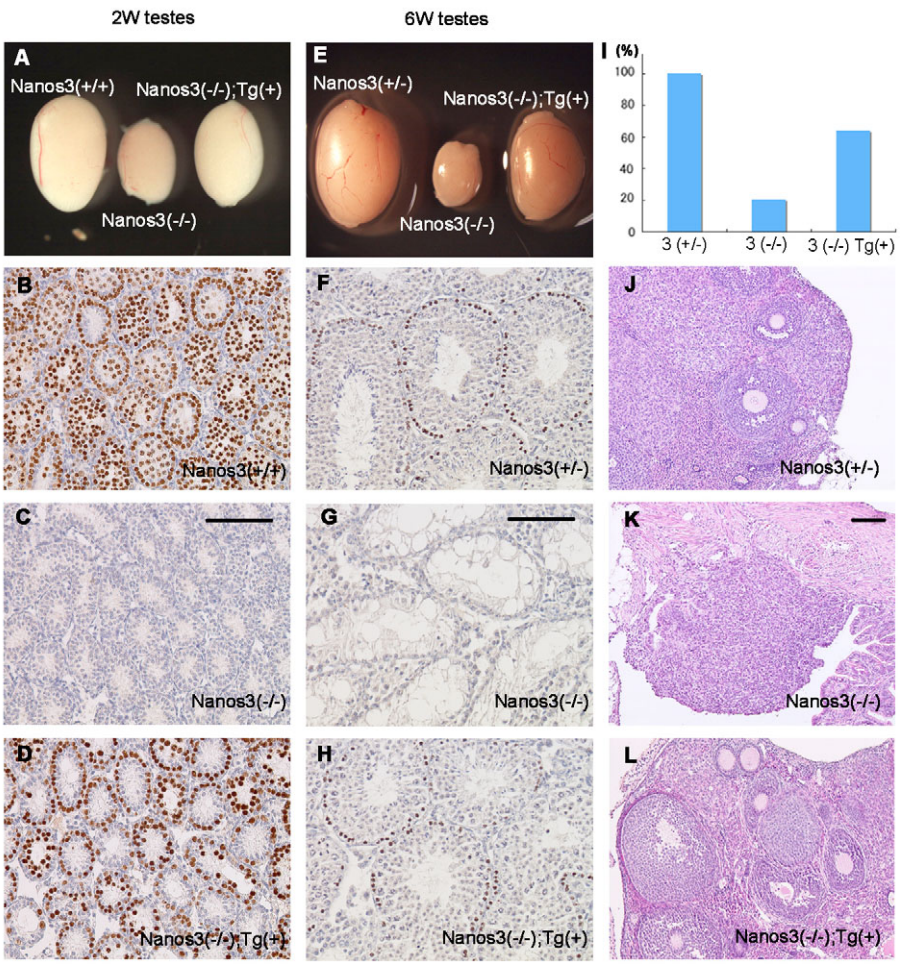

Fig. 3. Nanos2 can rescue the postnatel defects in Nanos3$^{-/-}$ testes and ovaries. Testis samples were prepared from littermates of Nanos3$^{+/+}$, Nanos3$^{+/-}$ or Nanos3$^{-/-}$, or from littermates of Nanos3$^{-/-}$ expressing the FLAG-tagged *Nanos2* transgene, at 2 weeks (**A-D**) and 6 weeks (**E-H**) after birth. The testis weights of different genotypes were compared at 6 weeks (**I**). The testis weight of Nanos3$^{+/-}$ was designated as 100%. Ovary samples were prepared from Nanos3$^{+/-}$ (**J**), Nanos3$^{-/-}$ (**K**) and Nanos3$^{-/-}$ harboring the Nanos2 transgene (**L**) at 6-7 weeks after birth. The genotypes are indicated in each panel. Germ cells were detected by immunostaining with TRA104 (B-D) and TRA98 (F-H) antibodies. Scale bars: 100 μm in C for B-D; 100 μm in G for F-H; and 100 μm in K for J-L.

germ cells are lost by programmed cell death. However, during the early stages of PGC development, only *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 *Nanos3*-promoter region has not yet been identified, we employed the Oct4-ΔPE enhancer because the expression profiles of the transgenes driven by this element resemble the *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-ΔPE 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-ΔPE 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

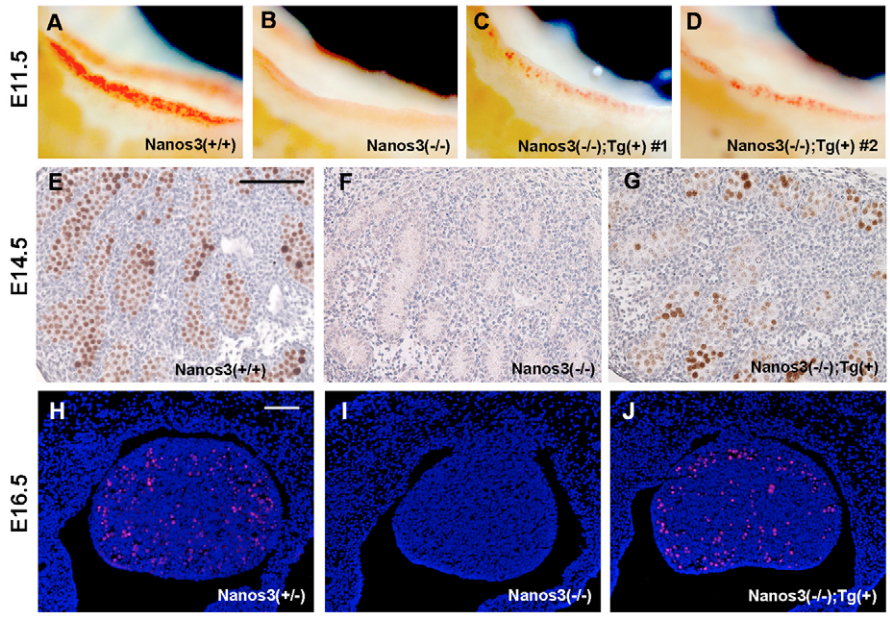

Fig. 4. Nanos2 can partially rescue the Nanos3$^{-/-}$ defects in the embryonic gonad. (**A-D**) Detection of migrating PGCs by ALP staining in E11.5 gonads from littermates of the Nanos3$^{+/+}$ (A), Nanos3$^{-/-}$ (B) and Nanos3$^{-/-}$ expressing the Nanos2 transgene (C,D) backgrounds. (**E-G**) Sections were prepared from the male gonads of E14.5 Nanos3$^{+/+}$ (E), Nanos3$^{-/-}$ (F) and Nanos3$^{-/-}$ expressing the Nanos2 transgene (G) backgrounds. Each section was then stained with TRA104 antibody. (**H-J**) Sections were prepared from the gonads of E16.5 females of Nanos3$^{+/-}$ (H), Nanos3$^{-/-}$ (I) and Nanos3$^{-/-}$ expressing the Nanos2 transgene (J) and stained with anti-TRA98 antibody. Signals are observed in the nuclei of germ cells. Blue, DAPI staining. Scale bars: 100 μm in E for E-G; and 100 μm in H for H-J.

any of the developmental stages examined after birth (Fig. 3C,G). However, we could detect significant increases in the sizes of the testes as a result of transgene expression (Fig. 3A,E,I). Immunohistological examination of these tissues further revealed that TRA104- and TRA98-positive germ cells were present in the Nanos3$^{-/-}$ testes harboring the *Nanos2* transgene (Fig. 3D,H). In 2-week-old Nanos3$^{-/-}$ testes expressing exogenous Nanos2, the number of germ cells was lower, reflecting their smaller size (compare Fig. 3B with 3D). However, normal maturation of the spermatocytes could be observed in 6-week-old testes from both the normal and transgenic animals (compare Fig. 3F with 3H), although the relative sizes of these rescued Nanos3$^{-/-}$ testes were still less than wild type (Fig. 3I). This defect could be due to the incomplete rescue during embryonic development (see below) or, alternatively, could result from the difference in expression caused by the heterologous Oct4 promoter and enhancer. Nevertheless, we confirmed that the transgenic mice were fertile and could transmit their germ lines to the next generation. 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.

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 thus indicate that *Nanos3*-null germ cells are rescued by Nanos2 during the embryonic stages of development and that Nanos3 is not required for the further development of the germ cells.

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. 4G) 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

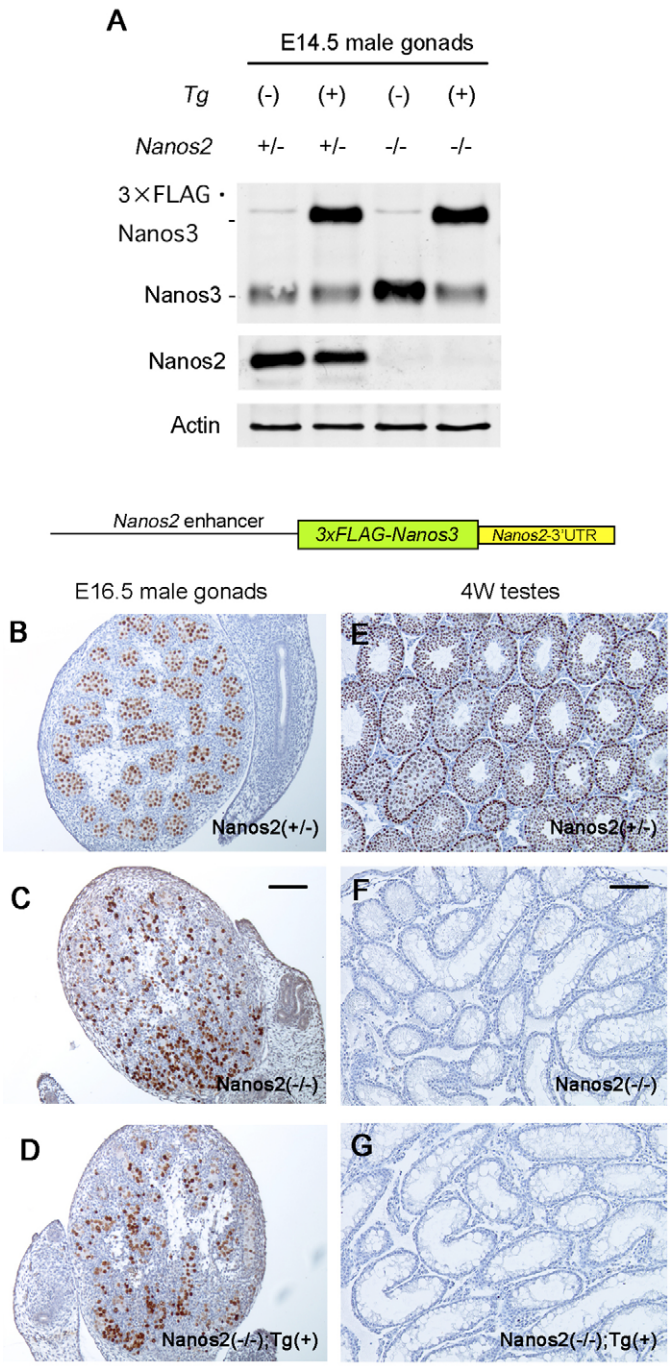

Fig. 5. Nanos3 cannot rescue the Nanos2$^{-/-}$ defects. (**A**) Western blot analysis of Nanos3 from Nanos2$^{+/-}$ and from Nanos2$^{+/-}$ mouse lines with or without a FLAG-tagged Nanos3 transgene under the control of the Nanos2 enhancer. Extracts were prepared from E14.5 male gonads. The transgene construct is schematically depicted. (**B-G**) Immunostaining and detection of germ cells with TRA104 antibody. Sections were prepared from E16.5 gonads (B-D) and testes at 4 weeks after birth (E-G) from Nanos2$^{+/-}$ (B,E), Nanos2$^{-/-}$ (C,F) and Nanos2$^{-/-}$ expressing the FLAG-tagged Nanos3 transgene (D,G) male littermates. Scale bars: 100 μm in C for B-D; and 100 μm in F for E-G.

(see Fig. S2 in the supplementary material). Furthermore, no germ cells were evident in *Nanos2*-null testes expressing the *Nanos3* transgene in 4-week-old mice (Fig. 5F,G). These results indicate that Nanos3 can not compensate for the loss of Nanos2 function in male mouse PGCs.

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*-knockout 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 can not 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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