Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells

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The pluripotency factor Nanog is expressed in peri-implantation embryos and primordial germ cells (PGCs). Nanog-deficient mouse embryos die soon after implantation. To explore the function of Nanog in germ cells, Nanog RNA was conditionally knocked down in vivo by shRNA. Nanog shRNA transgenic (Nri-Tg) mice were generated through the formation of germline chimeras with Nri-Tg embryonic stem cells. In E12.5 Cre-induced ER-Cre/Nri-Tg and TNAP-Cre/Nri-Tg double-transgenic embryos, the number of alkaline phosphatase-positive and SSEA1-positive PGCs decreased significantly. In the E9.5 and E10.5 migrating Nanog-knockdown PGCs, TUNEL-positive apoptotic cell death became prominent in vivo and in vitro, despite Oct4 expression. Single-cell microarray analysis of E10.5 Nanog-knockdown PGCs revealed significant up- and downregulation of a substantial number of genes, including Tial1, Id1 and Suz12. These data suggest that Nanog plays a key role in the proliferation and survival of migrating PGCs as a safeguard of the PGC-specific molecular network.

KEY WORDS: Nanog, Knockdown, Primordial germ cell, Apoptosis, Mouse

INTRODUCTION

Core regulators, including Oct4 (Pou5f1 – Mouse Genome Informatics), Sox2 and Nanog, play key roles in the transcriptional network that maintains the pluripotent state of human and mouse embryonic stem cells (ESCs). The homeodomain transcription factor Nanog is expressed in the nuclei of ESCs in vitro and of morulae, in the inner cell mass (ICM) cells of blastocysts, in the epiblast of E6.5 and E7.5 embryos (Chambers et al., 2003; Mitsui et al., 2003; Hatano et al., 2005), and in the primordial germ cells (PGCs) of E8.5-13.5 embryos (Hart et al., 2004; Yamaguchi et al., 2005) in vivo. Nanog plays an essential role in the maintenance of the pluripotency of the epiblast shortly after implantation (Mitsui et al., 2003). Overexpression of Nanog promotes the clonal expansion of mouse ESCs (Chambers et al., 2003) and of ES-somatic hybrid cells (Silva et al., 2006) and enhances the stable propagation of human and monkey ESCs (Darr et al., 2006; Yasuda et al., 2006).

Nanog is cis-regulated via Octamer and Sox elements in its promoter region by a synergistic action induced by the binding of Oct4 and Sox2 (Kuroda et al., 2005; Rodda et al., 2005). Furthermore, Sall4 and FoxD3 activate Nanog transcription, whereas p53 (Trp53) and Tcf3 are implicated in repressing Nanog transcription (Wu et al., 2006; Pan and Thomson, 2007). It has been shown that dimer formation by self-association of Nanog through its C-terminal domain is functionally important (Mullin et al., 2008; Wang et al., 2008), and Nanog-Oct4 protein complexes are associated with several repressive protein complexes, including the NuRD, Sin3A and Pm1 complexes in mouse ESCs (Liang et al., 2008). Thus, it has been hypothesized that certain key regulators control Nanog transcription through several independent pathways. However, the molecular mechanism of transcriptional regulation of Nanog in germ cells is not fully understood.

PGCs are first observed in E7.25 embryos at the base of the allantois and in the caudal end of the primitive streak as a group of 20-25 alkaline phosphatase (ALP)-positive cells. On subsequent days, PGCs proliferate and migrate into the hindgut of developing embryos and finally reach, and enter, the genital ridge of E11.5 embryos. After a few further mitotic divisions in the genital ridge, the developmental pathways of male and female germ cells diverge. Thus, the developmental stages of mitotic germ cells are roughly classified into germ cell specification, migration in developing embryos, and sexual divergence of germ cell behavior in gonads. In germ cell specification prior to the initiation of high-level Nanog expression, Dppa3 (Stella), Fragilis (Ifitm3) and Prdm1 (Blimp1) are key players in the mechanism involved in the acquisition of germ cell competence (Hayashi et al., 2007). In post-mitotic spermatogenesis and oogenesis, when dramatic morphological changes occur, a large number of differentiation-specific molecules are involved, and loss-of-function mutations is through conditionally targeted disruption by knockout or knockin of these genes often result in impaired fertility (O’Bryan and de Kretser, 2006; Roy and Matzuk, 2006). However, in migrating PGCs, only a few genes have been identified as key regulators, including Nanos3 and Dnd1 (Tsuda et al., 2003; Youngren et al., 2005). Nanog protein, which is first detected in male and female PGCs of E7.75-8.0 embryos, is expressed throughout the migration stages and is subsequently downregulated in the gonads in male and female mitotic arrest and meiotic germ cells, respectively (Yamaguchi et al., 2004).
The predominance of Nanog expression suggests that it plays an important role in early germ cell development. Recently, a role for Nanog in gonadal germ cells has been suggested by the contribution of Nanog-null germ cells of E11.5, but not of E12.5, chimeric embryos (Chambers et al., 2007). However, it remains unclear whether the lack of Nanog-null mice in the gonads of E12.5 chimeric embryos indicates Nanog function in gonadal PGCs. It also remains unclear whether these results reflect the role of wild-type ES and embryonic cells in germ cell development. Therefore, it is necessary to investigate the molecular function of Nanog in germ cells by other approaches.

Post-transcriptional gene silencing through RNA interference (RNAi), which is mediated by degradation of RNA complementary to ~20-nt small interfering RNAs (siRNAs) after incorporation into an RNA-induced silencing complex, is widely used to investigate the molecular function of a target gene in cells cultured in vitro (Filipowicz, 2005). Cre/loxP-regulated conditional RNAi of CD8 and p53 mediated by lentiviral vectors has been successfully demonstrated by mating with tissue-specific Cre-expressing transgenic mice in vivo (Ventura et al., 2004). The crucial roles of sprouty2 (Spry2) and CREB (cAMP-responsive element binding protein; Creb1) have been revealed by in vivo lentiviral short hairpin shRNA-mediated knockdown (KD) in mice (Shaw et al., 2007; Cheng et al., 2008). Although it is desirable to avoid genetic manipulation of the endogenous gene when analyzing the biological role of genes expressed in mouse germ cells, in vivo lentivirus-mediated conditional KD of a germ cell-specific gene has yet to be reported.

Here, we made Nanog shRNA transgenic mice (NRi-Tg) for Cre/loxP-mediated conditional KD. These mice were crossed with mouse receptor (ER; Esr1)-Cre or tissue non-specific alkaline phosphatase (TNAP; Alp)-Cre transgenic mice. Cre expression is induced by the administration of tamoxifen (TM) to pregnant mice and upregulated in E9.0 TNAP-Cre/NRi-Tg embryos (Ventura et al., 2004). The crucial roles of sprouty2 (Spry2) and CREB (cAMP-responsive element binding protein; Creb1) have been revealed by in vivo lentiviral short hairpin shRNA-mediated knockdown (KD) in mice (Shaw et al., 2007; Cheng et al., 2008).

The silent mutant form of Nanog was constructed with pCAG-Nanog (Hatano et al., 2004) by introduction of the mutation into the shRNA target region through PCR-mediated nucleotide replacement. pCAG-Mut-Nanog was co-transfected with pPigk-Neo into NRi ES cells using Lipofectamine 2000 (Invitrogen). G418 (250 μg/ml)-resistant colonies were cloned and expanded.

**Lentiviral infections**

The supernatant, which was collected 48 hours after co-transfection of the SIN vector and each packaging vector into HEK 293T cells, was centrifuged at 6000 g for 16-24 hours (Miyoshi et al., 1998). The pellet was dissolved in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) and stored at ~80°C. Lentiviral infectivity was estimated by counting GFP-positive cells after infection of the titrated supernatants into the 293T cells. Following overnight culture of ESCs at 1 × 10^6 cells per well of a 12-well culture plate (BD Falcon), the cells were infected with the supernatant at MOI=15 and cultured overnight. After washing out the virus several times with PBS, the ESCs were plated on an inactivated mouse embryonic fibroblast (MEF) feeder layer. GFP-positive cells were cloned and expanded. Expression of shRNA was induced in vitro by treatment with adenovirus expressing Cre recombinase (AdCre; AxCanCre) (Kanegae et al., 1995).

**Culture of ESCs and MEFs**

Mouse R1 ESCs were maintained in DMEM F-12 HAM (Sigma) supplemented with 15% fetal bovine serum (FBS; BioWest), 0.1 mM 2-mercaptoethanol and 400 units/ml recombinant LIF (Chemicon) (ES medium) on MEF feeder cells inactivated with mitomycin C.

**Mice**

TNAP-Cre and ER-Cre mouse lines were maintained by mating them with C57BL/6J mice. The PCR primer sets for genotyping are summarized in Table 1. C57BL/6J blastocysts, into which the NRi-shRNA-infected ESCs were microinjected, were transferred into the uteruses of pseudo-pregnant ICR females. Chimeras were mated with C57BL/6J females, and germline transmission to the next generation was checked by coat color and GFP fluorescence. Mice homogenous for the NRi-Tg were detected by genomic PCR with a specific primer set (Table 1). For Cre induction in embryos, 3.0

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
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<tr>
<td>TNAP-Cre</td>
<td>GGCCTCCTCAGGAACGTATCTCAAC</td>
<td>CAAACGGAGCAAGACATTTCCAG</td>
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<tr>
<td>CR-Cre</td>
<td>CTCTAGAGCTTGTCAAGCC</td>
<td>CTCGGGCATCTTGGAAACTAGCC</td>
</tr>
<tr>
<td>pSico excision</td>
<td>CAACACAGCTGACACACGG</td>
<td>GCACACAGCTTGGAGAAG</td>
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<tr>
<td>Inverse PCR 1st</td>
<td>GCAATGGGGCCAGGCATTACCG</td>
<td>GCCGTGCTTGCTGTTGCCC</td>
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<tr>
<td>Inverse PCR 2nd</td>
<td>AATGGGGCCAGGCATTACCG</td>
<td>CACCGCCAGCTTCTCTCCGC</td>
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<tr>
<td>NRi-Wt allele</td>
<td>GTTAAAGAGGCTCCTGTGCTTCCC</td>
<td>GGGAAGCTCAACAGCAAC</td>
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<td>Same as Wt allele</td>
<td>GGCTGCTGCTTGGCTGCCC</td>
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<tr>
<td>Nanog</td>
<td>CTTCACACCTTAAGGGCATTGCC</td>
<td>TGGCATGCGGTACATCGTGC</td>
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<td>Id1</td>
<td>CAACAGACCTGCCCCCTC</td>
<td>AAGAATCGAGAAAAACGAGA</td>
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<tr>
<td>Tial1</td>
<td>GGGCAATGGCCAAGAAATGCTC</td>
<td>TGGTGGTTAGTGGTCC</td>
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<tr>
<td>Suz12</td>
<td>AAGGCTAGACATTGCTGGC</td>
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<tr>
<td>Gapdh</td>
<td>ATGAACTGCAGACATCGAAACAG</td>
<td>CTCCAAGCAGAAGACATTTCCAG</td>
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<td>GTTAAAGACGGCTGTTTCCAAA</td>
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<tr>
<td>shNC probe</td>
<td>GGCCTCCTCAGGAACGTATCTCAAC</td>
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Signals were visualized using the ECL Western Blotting Detection Kit antibodies at 4°C overnight. The membrane was incubated with a HRP-linked probe with anti-Nanog (1:1500 dilution) and anti-histone H3 (1:3000) transferred onto a nitrocellulose membrane (Millipore). The membrane was and twice in 0.1 M HEPES at 4°C. Room temperature were used for further analyses.

PGCs fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at containing 5 µM 4-hydroxytamoxifen (4OH-TM; Sigma) for 6 days. Each expanded blastocyst that was morphologically analyzed was genotyped by genomic PCR with specific primer sets (Table 1).

**RESULTS**

**Generation of Nanog-knockdown ESCs and transgenic mice**

A temporally and spatially controlled in vivo Nanog KD system was constructed with a lentiviral vector for conditional Cre/loxP-regulated RNAi (Ventura et al., 2004). Before Cre recombinase expression, the GFP reporter driven by the CMV promoter is widely expressed and Nanog shRNA is repressed, whereas after Cre-mediated recombinase, GFP is flipped out and Nanog shRNA expression is then driven by the U6 promoter (Fig. 1A).

The most effective shRNA was introduced by viral infection of R1 ESCs for establishment of Nanog KD ESCs (NRi-ESCs). Transcription of the shRNA after infection with adenovirus Cre (AdCre) was confirmed by northern blot analysis in NRi-ESCs (Fig. 1B). Western blot analyses showed efficient reduction of Nanog expression to a relative value of 0.14 [compared with AdCre(–) NRi-ESCs] 96 hours after AdCre infection (Fig. 1C). Cre-dependent Nanog repression was verified by GFP expression and immunostaining of Nanog 48 hours after AdCre infection (Fig. 1D). Downregulation of Nanog was detected within the first 24 hours (data not shown). Differentiation of ESCs was detected 96 hours after AdCre infection with NRi shRNA, indicating that cell differentiation was induced 72 hours after Nanog KD (Fig. 1E). Clonal expansion of ESCs was disturbed by transcription of NRi shRNA (see Fig. S1A,B in the supplementary material).

shRNA silences a target gene with a completely homologous sequence through a post-transcriptional cleavage mechanism. It has been noted that siRNA often triggers off-target effects, which could be caused by unintended RNAi-specific toxic events or cleavage of an unintended RNA target (Ui-Tei et al., 2008). Cre-dependent expression of the non-specific negative control shRNA resulted in a normal ESC phenotype (Fig. 1C-E). Disappearance of Cre-dependent NRi shRNA-mediated repression of Nanog and promotion of differentiation by co-transfection with the silent-mutation form of Nanog (Mut-Nanog) again showed that the NRi shRNA was highly specific to Nanog (see Fig. S1B-D in the supplementary material). Inverse PCR and DNA sequence analyses demonstrated that the NRi lentiviral vector was integrated between Cdh2 and Dsc3, near to the proximal region of chromosome 18 (see Fig. S2A in the supplementary material). No known gene was disrupted by the lentiviral integration. Therefore, the NRi shRNA-infected ESCs were used for further in vivo experiments.

**Nanog** shRNA transgenic mice were made by mating a male germline chimera carrying NRi shRNA-infected ESCs with C57BL/6 females. NRi-Tg founder mice were detected by expression of GFP (see Fig. S2B in the supplementary material). By crossing mice heterozygous for NRi-Tg, homozygous NRi-Tg mice were generated and stably maintained as a transgenic line.

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**REFERENCES**

Li, L., et al. (1999). ...
Effect of Nanog knockdown on somatic cells

To further examine possible off-target effects in vivo, we analyzed peri-implantation development of ER-Cre/NRi-Tg double-transgenic embryos generated by mating females homozygous for NRi-Tg with males heterozygous for ER-Cre driven by the CAG promoter [C57BL/6.Cg-Tg(cre-Esr1)5Amc/J]. In the ER-Cre transgenic embryos and mice, Cre activity is induced by the synthetic estrogen-like agonist tamoxifen (TM), but not by endogenous estrogens (Hayashi and McMahon, 2002). Zona pellucida-free blastocysts were cultured in the presence of TM for 7 days. Each blastocyst that expanded on the bottom of a culture well was genotyped by genomic PCR. The ICM cells were poorly developed in the ER-Cre/NRi-Tg double-transgenic embryos, which Nanog shRNA was transcribed in all tissues of the body (Fig. 2A). In single-transgenic gonads, 43 SSEA1+ PGCs were detected on average per section, versus only 14 in the double-transgenic gonads (Fig. 2B). Nanog KD resulted in a ~70% reduction in PGCs in male and female ER-Cre/NRi-Tg embryos. The majority of SSEA1+ PGCs were negative for Nanog (Fig. 2E). Similarly, in E11.5 ER-Cre/NRi-Tg embryos, the number of ALP+ gonadal PGCs was markedly reduced (see Fig. S3A-C in the supplementary material).

A striking decrease in the number of ALP+ PGCs was evident by the sparsity of red-stained cells in the gonads of E12.5 ER-Cre/NRi-Tg double-transgenic embryos, in contrast to the high number of red-stained cells in the gonads of single-transgenic NRi-Tg embryos collected from the same littersmates (Fig. 2A). Cre activity (excision as assessed by genomic PCR) was nearly 100% in the liver and ~75% in the gonads, although in PGCs it was difficult to determine the Cre activity precisely.

To calculate the number of PGCs, cells positive for SSEA1 (stage-specific embryonic antigen 1; Fut4 – Mouse Genome Informatics) were counted in transverse sections of E12.5 gonads. In single-transgenic gonads, 43 SSEA1+ PGCs were detected on average per section, versus only 14 in the double-transgenic gonads (Fig. 2B). Nanog KD resulted in a ~70% reduction in PGCs in male and female ER-Cre/NRi-Tg embryos. The majority of SSEA1+ PGCs were negative for Nanog (Fig. 2E). Similarly, in E11.5 ER-Cre/NRi-Tg embryos, the number of ALP+ gonadal PGCs was markedly reduced (see Fig. S3A-C in the supplementary material).

Next, to determine whether Nanog KD induces detrimental effects on early gonadal PGCs, TM was administered at 9.5 dpc to pregnant NRi-Tg mice that had been mated with ER-Cre Tg males. Interestingly, no decrease in ER-Cre/NRi-Tg PGCs was observed at E12.5 by ALP staining (Fig. 2C,D), irrespective of the repression of Nanog in SSEA1+ PGCs (Fig. 2E). These data indicate that Nanog mainly plays a role in migrating PGCs, but not in gonadal PGCs.

Decrease in PGCs in TNAP-Cre/NRi-Tg embryos

To verify the function of Nanog in migrating PGCs, NRi-Tg mice were mated with the PGC-specific Cre recombinase transgenic mouse line TNAP-Cre, which was generated by knockin of Cre into the TNAP (Alpl) locus. Cre excision was first detected in early PGCs at E9.0, and Cre activity in PGCs was detected in ~50% of E13.5 PGCs (Lomeli et al., 2000). E12.5 TNAP-Cre/NRi-Tg, NRi-Tg, TNAP-Cre and wild-type embryos developed normally (Fig. 2F). When E12.5 TNAP-Cre/NRi-Tg double- and single-transgenic embryos were compared, the intensity of staining of ALP+ cells was drastically reduced in the TNAP-Cre/NRi-Tg gonads (Fig. 2F). To estimate the number of PGCs, SSEA1+ cells were counted in each transverse section of the E12.5 gonads. The number of PGCs in the TNAP-Cre/NRi-Tg gonads was half that in the control gonads (Fig. 2G).

Immunohistochemical analysis demonstrated that SSEA1+ PGCs were frequently Nanog+ in the TNAP-Cre/NRi-Tg gonads, whereas the majority of SSEA1+ PGCs were Nanog+ in the controls (Fig. 2H), indicating that Nanog KD resulted in a reduction in PGCs, as seen in E1-Cre/NR1-Tg transgenic mice.

Decrease in proliferation and increase in cell death in PGCs in vivo

To examine the sequential expression of PGC markers, cell proliferation and apoptosis during migration, immunohistochemical analyses and TUNEL assays were performed in E9.5 and E10.5 ER-
Cre/NRi-Tg double-transgenic embryos and NRi-Tg embryos. At E9.5 and E10.5, the gross morphology of the ER-Cre/NRi-Tg double-transgenic embryos was normal. No sex-specific differences were detected.

At E9.5 (2 days after TM administration), Nanog–/SSEA1+ migrating PGCs were detected in the ER-Cre/NRi-Tg double-transgenic but not NRi-Tg embryos (Fig. 3A). The number of Nanog+/SSEA1+ PGCs was significantly reduced (to ~70%; Fig. 3B). At E10.5 (3 days after TM administration), Nanog–/SSEA1+ PGCs were more abundant in the ER-Cre/NRi-Tg embryos as compared with the control embryos (Fig. 3C). Nanog+/SSEA1+ PGCs were markedly decreased (to ~40%; Fig. 3D). An important finding was that the number of TUNEL+ PGCs noticeably increased in the ER-Cre/NRi-Tg embryos. TUNEL+/SSEA1+ PGCs were first detected in E9.5 PGCs (Fig. 4A,B), and at E10.5 their abundance was about three times that in the control PGCs (Fig. 4C,D). The number of PGCs positive for the mitotic marker phosphorylated-histone H3 was significantly lower in ER-Cre/NRi-Tg than in control embryos (Fig. 4E). Notably, the majority of SSEA1+/Oct4+ PGCs were positive for Nanog in the control embryos, whereas almost half were negative for Nanog in the ER-Cre/NRi-Tg embryos (Fig. 4F). Thus, the majority of E7.5 TM-treated Nanog–/TUNEL+ apoptotic PGCs stopped migrating before entry into the genital ridges.

Cre-mediated recombination is detectable in embryos within 24 hours of TM administration to pregnant mice (Hayashi and McMahon, 2002). Taking this into consideration, following Cre-mediated recombination within the first day after TM injection at E7.5, Nanog+/Oct4+ SSEA1+/TUNEL+ PGCs had appeared by the second day. Within the next 24 hours, apoptosis occurred in PGCs marked as Nanog+/SSEA1+/TUNEL+ (Fig. 4A,B). Oct4 expression was observed in all Nanog+/SSEA1+ PGCs, but not TUNEL+ PGCs (data not shown). Following TM injection at E9.5, Nanog KD did not occur sufficiently in E10.5 migrating PGCs, while induced markedly with no significant reduction in the number of E12.5 gonadal PGCs (Fig. 5A-C), suggesting that Nanog function is dispensable for the survival of gonadal PGCs (Fig. 2C-E). The effects on PGC development of Nanog KD induced by TM injection at E7.5 and E9.5 are summarized in Fig. 5D.

Decrease in proliferation and increase in cell death in PGCs in vitro

To examine whether the reduction in PGCs was caused by the death or differentiation of PGCs, dissociated gonads of E10.5 embryos were cultured on inactivated Sl4-m220 cells expressing the membrane-associated form of steel factor (kit ligand) with culture medium containing leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and forskolin (Koshimizu et al., 1996). The proliferation of PGCs was clearly repressed in the ER-Cre/NRi-Tg double-transgenic PGCs as compared with control PGCs after 12 hours of culture in the presence of TM (Fig. 6A). Although a gradual decrease in the number of ALP+ PGCs was observed even in the control embryos from 12 to 60 hours after TM administration, ER-Cre/NRi-Tg PGCs were significantly less abundant than control embryos.
PGCs. Notably, immunocytochemical analyses revealed that Nanog expression was repressed in the ER-Cre/NRi-Tg, but not in the NRi-Tg PGCs 12 and 36 hours after TM administration (Fig. 6B). From 12 to 36 hours after TM administration, TUNEL+ cells were prominent in the ER-Cre/NRi-Tg embryos but not in the control embryos (Fig. 6C). The number of SSEA1+/TUNEL+ PGCs in the ER-Cre/NRi-Tg embryos was about twice that in the NRi-Tg control embryos (Fig. 6D). Nanog–/Oct4+ PGCs were often detected in the ER-Cre/NRi-Tg culture, but not in the control, 12 and 36 hours after TM administration (see Fig. S4A in the supplementary material). The number of PGCs positive for the mitotic marker phosphorylated histone H3 was significantly reduced in ER-Cre/NRi-Tg PGCs (see Fig. S4B,C in the supplementary material). Cre-mediated recombination was efficiently induced in more than 50% and nearly 100% of MEFs 12 and 24 hours after culture in the presence of TM, respectively (see Fig. S4D in the supplementary material). Differentiation of ESCs was induced 72 hours after Nanog repression (Fig. 1E), suggesting that apoptotic cell death prior to cell differentiation was induced within 24 hours of Nanog repression in PGCs.

Effect of Nanog knockdown on adult gonads
After injection of TM at 3 mg/40 g body weight to 7.5 dpc pregnant females, embryos developed normally until E13.5 but died in the second semester of pregnancy, although most embryos were viable and developed normally until E13.5. Thus, the testes or ovaries of 6-week-old TNAP-Cre × NRi-Tg F1 mice were analyzed morphologically and immunohistochemically. The testes, but not the ovaries, tended to be smaller in the TNAP-Cre/NRi-Tg double-transgenic embryos than in the control embryos, although both the testes and ovaries varied in size (see Fig. S5A,B in the supplementary material).

In two out of six testes examined from 6-week-old TNAP-Cre/NRi-Tg adults, spermatogonia, marked as TRA98+ germ cells, were dissociated from the tubule wall and scattered in the empty tubules (see Fig. S5C in the supplementary material). These features are observed in germ cells undergoing mitotic division in prepubescent newborn mice, demonstrating that developmental retardation of the seminiferous tubule in some regions of adult testes may be caused by the loss of Nanog– germ cells during the perinatal stage. Consistently, the number of TRA98+ germ cells in TNAP-Cre/NRi-Tg newborn (P1) testes was reduced (see Fig. S5D,E in the supplementary material).

No significant difference in the number of oocytes was detected in 6-week-old TNAP-Cre/NRi-Tg versus control mice by immunostaining of cryosections with anti-Oct4 antibody (data not shown).

![Fig. 3. Expression of Nanog in migrating PGCs in E9.5 and E10.5 ER-Cre/NRi-Tg embryos.](image)

**Fig. 3. Expression of Nanog in migrating PGCs in E9.5 and E10.5 ER-Cre/NRi-Tg embryos.** (A) Expression of Nanog in SSEA1+ PGCs of E9.5 ER-Cre/NRi-Tg mouse embryos. (B) The proportion of Nanog+ PGCs in SSEA1+ PGCs in E9.5 ER-Cre/NRi-Tg embryos. (C) Expression of Nanog in SSEA1+ PGCs of E10.5 ER-Cre/NRi-Tg embryos. (D) The proportion of Nanog+ PGCs in SSEA1+ PGCs in E10.5 ER-Cre/NRi-Tg embryos. The circles indicate SSEA1+/Nanog– PGCs. *P<0.01, **P<0.05. Error bars, s.e.m.

![Fig. 4. Apoptosis and proliferation of Nanog-negative migrating PGCs in E9.5 and E10.5 ER-Cre/NRi-Tg embryos after E7.5 tamoxifen administration.](image)

**Fig. 4. Apoptosis and proliferation of Nanog-negative migrating PGCs in E9.5 and E10.5 ER-Cre/NRi-Tg embryos after E7.5 tamoxifen administration.** (A) TUNEL assay in E9.5 ER-Cre/NRi-Tg mouse embryos. The arrowheads indicate SSEA1+/TUNEL+ PGCs. (B) The proportion of TUNEL+ cells in SSEA1+ PGCs in E9.5 ER-Cre/NRi-Tg embryos. (C) TUNEL assay in E10.5 ER-Cre/NRi-Tg embryos. The arrowheads indicate SSEA1+/TUNEL+ PGCs. (D) The proportion of TUNEL+ cells in SSEA1+ PGCs in E10.5 ER-Cre/NRi-Tg embryos. (E) The proportion of phosphorylated histone H3+ cells in SSEA1+ PGCs of E10.5 ER-Cre/NRi-Tg embryos. (F) Expression of Oct4 in Nanog– PGCs of E10.5 ER-Cre/NRi-Tg embryos. Arrowheads indicate Oct4+/SSEA1+ PGCs. Circles indicate Nanog– PGCs. *P<0.01, **P<0.05. Error bars, s.e.m.
Changes in gene expression profile upon Nanog knockdown in each PGC

To explore the molecular mechanism involved in apoptotic cell death by Nanog KD, the global gene expression profile of each E10.5 PGC was analyzed by a single-cell microarray assay (Kurimoto et al., 2007). A PGC-specific cDNA library was identified by RT-PCR of Oct4 and Dppa3 (Fig. 7A). The libraries were classified into Nanog low [Nanog (L)] and Nanog high [Nanog (H)] by qPCR. Hybridization with the amplified cDNAs to the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix) demonstrated that 759 out of 45,101 probes were significantly different between Nanog (L) and (H) in their relative expression level (P<0.05; greater than 2-fold change) (Fig. 7B).

No change was detected in the Oct4 expression level between Nanog (L) and (H), in agreement with immunostaining (Fig. 4F). Furthermore, Sox2, Dppa3, Slh4, Kit, Dnd1, Zfp42 (Rex1), Prdm1, Utf1 and Klf5 were highly transcribed even in Nanog (L) PGCs, similar to in control PGCs. The expression of a few genes in the development and lineage-annotated sequences in the gene ontology list (Affymetrix) had changed in the Nanog (L) PGCs (see Fig. S6A in the supplementary material). These data suggest that Nanog KD leads PGCs to apoptotic cell death and not to differentiation. Notably, the expression level of some genes was significantly up- or downregulated (Fig. 7C; see Table S1 in the supplementary material). For example, those encoding the RNA-binding protein Tial1 (Beck et al., 1998), helix-loop-helix (HLH) family protein Id1 (Norton et al., 1998) and Polycomb repressive complex 2 (PRC2) subunit Suz12 (Lee et al., 2006), were markedly repressed in Nanog (L) PGCs. Disruption of the PGC-specific molecular network, at least that due to downregulation of these key genes, might trigger prompt mitotic arrest and cell death (Fig. 7D).

Some genes downstream of Nanog (Kim et al., 2008) were up- or downregulated in Nanog (L) PGCs (see Fig. S6B in the supplementary material). The significant decrease in Id1 transcription was verified by qPCR with a single-cell cDNA library (see Fig. S7A in the supplementary material). Although the mechanism of transcriptional regulation of Id1, which bypasses the BMP/phosphorylated Smad pathway (Dudley et al., 2007), is unclear, Id1 might be directly downstream of Nanog in PGCs, as shown by the binding of Nanog to Id1 in ESCs (Kim et al., 2008).

DISCUSSION

Nanog is expressed not only in the pluripotential cells of peri-implantation embryos, but also in the migrating and early gonadal PGCs of post-implantation embryos (Yamaguchi et al., 2005). A key function of Nanog at the peri-implantation stage is to maintain the pluripotency of early embryonic cells, as revealed in Nanog-deficient embryos produced by genetic disruption of Nanog (Mitsui et al., 2003). Here, to investigate the function and mechanism of Nanog in PGCs, we constructed the NRi-Tg transgenic line, in which Nanog activity is controlled in vivo through inducible transcription of Nanog-specific shRNA with a pSico lentiviral vector (Ventura et al., 2004). In combination with two independent Cre-expressing transgenic lines, ER-Cre and TNAP-Cre, Cre expression beginning at ~E8.5-9.0 resulted in a significant reduction in the
gonadal PGCs of E12.5 male and female embryos. Immunohistochemical analyses of the migrating PGCs of E9.5 and E10.5 TM-administered ER-Cre/NRi-Tg embryos demonstrated that Nanog/Oct4 PGCs were first detected at E9.5, and then Nanog/TUNEL+ PGCs appeared at E10.5. The immediate induction of cell apoptosis following Nanog repression in PGCs cultured in vitro suggests that cell death, but not cell differentiation, is the key reason for the decrease in PGC numbers. The adult TNAP-Cre/NRi-Tg males and females were fertile. Notably, some male TNAP-Cre/NRi-Tg adult mice showed partially retarded development of the seminiferous tubule. A single-cell microarray analysis revealed that changes in gene expression, including downregulation of Tail1, Id1 and Suz12, were associated with apoptotic cell death of Nanog KD PGCs. Our data provide conclusive evidence that (1) Nanog is required for the survival of migrating PGCs, (2) a deficiency in Nanog triggers apoptosis but not cell differentiation in PGCs, and (3) Nanog is involved in safeguarding the PGC molecular network.

The use of an inducible KD system without genetic alteration of the endogenous gene is a powerful tool for analyzing the molecular function of a possibly heteroinsufficient germ cell-specific gene. This is the first report of a successful conditional KD of a germ cell-specific gene in vivo. A conditional KD system is quicker to build than a conventional conditional knockout system, although possible off-target effects have to be carefully examined in order to avoid an overestimation of gene function resulting from non-specific gene silencing.

In migrating germ cells, only a few genes have been identified as key regulators. Following germ cell specification, Nanog, Kit, Tail1, Nanos3 and Dnd1 are known to be highly transcribed in migrating PGCs (Beck et al., 1998; Tsuda et al., 2003; Youngren et al., 2005; Yamaguchi et al., 2005). Kit plays a crucial role in the survival of migrating PGCs (Loveland and Schlatt, 1997). Tail1-deficient mice are sterile owing to the loss of PGCs at ~E11.5 (Beck et al., 1998). Knockout of Nanos3 results in the complete loss of PGCs in both sexes in E12.5 embryos (Tsuda et al., 2003). A similar phenomenon was detected after germ cell-specific knockout of Oct4. Oct4-deficient PGCs undergo apoptosis, and a marked reduction in PGCs is detected in E10.5-12.5 embryos (Kehler et al., 2004). Importantly, a common consequence of the loss of gene expression is apoptotic cell death, not cell differentiation. Single-cell microarray analysis demonstrated that abnormal transcription of various types of core regulators, including the RNA-binding protein Tail1, differentiation inhibitor Id1, and PRC2 subunit Suz12, occurred within 24 hours of Nanog downregulation in E10.5 PGCs. Notably, the absence of any significant change in the expression level of genes downstream of Id1 and Suz12 suggests that the prompt cell death response might be induced by abrupt disruption of the PGC molecular network prior to the disordered expression of peripheral genes. Thus, we speculate that the apoptotic cell death of PGCs is triggered by ‘disharmony’ in the gene regulation network. The molecular mechanism involved in monitoring ‘harmony’ in the PGC molecular network is unclear. It is also unknown whether the apoptosis of Nanog (L) PGCs depends on the Bax pathway, as reported in Steel/Kitl-deficient (Runyan et al., 2006) and Nanos3-deficient (Suzuki et al., 2008) PGCs. Apoptotic cell death triggered by a deficiency in any core gene, including Nanog, might play an important role in preventing the transmission of abnormal genetic information to the next generation.

An interesting point is that Oct4 and Nanog exhibit similar dual physiological roles, which are essential for maintaining pluripotency in early embryonic cells and for supporting survival in migrating PGCs. It is still unclear why a deficiency in Nanog and Oct4 induces a distinctive phenotype in early embryonic cells and PGCs. A possible explanation is that the molecular network supporting the properties of pluripotent embryonic cells differs from that of unipotent PGCs (Kato et al., 1999). In pluripotent embryonic cells, differentiation-associated genes may be ready to be transcribed quickly following the downregulation of pluripotent guardian genes including Nanog and Oct4, whereas in unipotent PGCs, which are specialized toward generating germ cells through tight epigenetic regulation of gene activation and silencing, a defect in the PGC-specific molecular network triggered by a lack of Nanog or Oct4 may cause apoptosis without the alternative option of trans-lineage differentiation. We found no evidence that Nanog PGCs differentiated into another type of somatic cell instead of undergoing apoptosis, although apoptosis and differentiation are induced in the ICM cells of Nanog-deficient blastocysts (Silva et al., 2009). In this
context, the fate of migrating PGCs may be strictly determined by the fixed transcriptional circuitry regulated by dominant negative epigenetic modifications that is inappropriate for trans-differentiation to somatic cells.

Id1 is downstream of the BMP/phosphorylated Smad pathway and functions as a dominant-negative binding factor for HLH genes (Norton et al., 1998). However, phosphorylated Smad1, 5 and 8 are not detected in migrating PGCs (Dudley et al., 2007). Thus, Id1 has to be upregulated by another pathway. Considering that Nanog binds to the upstream sequence of Id1 in ESCs, as determined by ChIP-on-chip analysis (Kim et al., 2008), and that Id1 is downregulated in Nanog (L) PGCs, as revealed by single-cell microarray analysis (see Fig. S7B in the supplementary material), one may suggest that Nanog is involved in the regulation of Id1 in PGCs.

Oct4 and Sox2 activate Nanog expression through binding to the Octamer/Sox elements upstream of the transcription start site (Kuroda et al., 2005; Rodda et al., 2005), although full transcriptional regulation of Nanog is complicated by its association with many other regulatory factors. In Oct4-deficient PGCs, it is not evident whether Nanog and Sox2 are expressed appropriately (Kehler et al., 2004). It is possible that apoptosis of the Oct4-deficient PGCs might be detected as a consequence of the prompt repression of Nanog, which is downstream of Oct4. Apoptosis of Nanog /Oct4+ PGCs in our KD analyses clearly demonstrated that Oct4 expression is insufficient to prevent apoptosis in Nanog-deficient PGCs.

Interestingly, Nanog-null ESCs can self-renew indefinitely with an undifferentiated status, although they are prone to differentiation, suggesting that Nanog stabilizes ESCs in culture by resisting or reversing alternative gene expression programs (Chambers et al., 2007). Nanog-null ESCs have the potential to generate chimeric fetuses and adults through multi-lineage differentiation in somatic cells, indicating that Nanog expression is not required for the development and maturation of somatic tissues. In germ cells, the colonization of Nanog-null cells was detected in the PGCs of the genital ridges of E11.5, but not E12.5, chimeric embryos (Chambers et al., 2007). This finding differs from our present observation that Nanog-deficient PGCs start dying due to apoptosis within 48 hours of Nanog KD during the migrating stages. Survival of the Nanog-null PGCs in E11.5 chimeras could be a consequence of compensation by other transcriptional circuits acquired in ESC culture (Chambers et al., 2007). This would explain the discrepancy that Nanog KD in normal ESC lines induces cell differentiation (Fig. 1E) (Hough et al., 2006), whereas selected Nanog-null ESCs maintain a capability for self-renewal and pluripotency. Notably, Nanog is specifically required for the proliferation and survival of migrating PGCs of wild-type embryos.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/4011/DC1

References


