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Graphical Abstract

Highlights

- Germline deletion of Nanog reduces PGC numbers but does not abolish PGC development

- Without Nanog, PGCLCs form ineffectively with less proliferation and more apoptosis

- The Nanog target gene Esrrb can rescue PGCLC differentiation of Nanog\(^{-/-}\) ESCs

- Knockin of Esrrb at the Nanog locus restores PGC development efficiency

Authors

Man Zhang, Harry G. Leitch, Walfred W.C. Tang, ..., M. Azim Surani, Austin Smith, Ian Chambers

Correspondence

h.leitch@mrc.ac.uk (H.G.L.), ichambers@ed.ac.uk (I.C.)

In Brief

Although transcription factors functional in naive pluripotent cells are also expressed in primordial germ cells (PGCs), their PGC role remains unclear. Here, Zhang et al. show that, without Nanog, PGCs form ineffectively but that normal PGC development can be restored by induced expression of the NANOG target gene Esrrb.

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Esrrb Complementation Rescues Development of Nanog-Null Germ Cells

Man Zhang,1,7 Harry G. Leitch,2,4,6,7,9,10 Walfred W.C. Tang,3,4 Nicola Festuccia,1,10 Elisa Hall-Ponsele,1 Jennifer Nichols,2,4 M. Azim Surani,1,4,5 Austin Smith,2,5 and Ian Chambers1,8,11,*

1MRC Centre for Regenerative Medicine, Institute for Stem Cell Research, School of Biological Sciences, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, Scotland
2Wellcome Trust–Medical Research Council Stem Cell Institute, University of Cambridge, Cambridge CB2 1QR, United Kingdom
3Wellcome Trust/Cancer Research UK Gurdon Institute, Tennis Court Road, University of Cambridge, Cambridge CB2 1QG, United Kingdom
4Department of Physiology, Development and Neuroscience, Downing Street, University of Cambridge, Cambridge CB2 1EG, United Kingdom
5Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, United Kingdom
6Senior author
7These authors contributed equally
8Present address: MRC London Institute of Medical Sciences, Du Cane Road, London W12 0NN, England
9Present address: Institute of Clinical Sciences, Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, United Kingdom
10Present address: Department of Developmental and Stem Cell Biology, Institut Pasteur, CNRS UMR 3738, 25 Rue du Docteur Roux, 75015 Paris, France
11Lead Contact
*Correspondence: h.leitch@lms.mrc.ac.uk (H.G.L.), ichambers@ed.ac.uk (I.C.)
https://doi.org/10.1016/j.celrep.2017.12.060

SUMMARY

The transcription factors (TFs) Nanog and Esrrb play important roles in embryonic stem cells (ESCs) and during primordial germ-cell (PGC) development. Esrrb is a positively regulated direct target of NANOG in ESCs that can substitute qualitatively for Nanog function in ESCs. Whether this functional substitution extends to the germline is unknown. Here, we show that germline deletion of Nanog reduces PGC numbers 5-fold at midgestation. Despite this quantitative depletion, Nanog-null PGCs can complete germline development in contrast to previous findings. PGC-like cell (PGCLC) differentiation of Nanog-null ESCs is also impaired, with Nanog-null PGCLCs showing decreased proliferation and increased apoptosis. However, induced expression of Esrrb restores PGCLC numbers as efficiently as Nanog. These effects are recapitulated in vivo: knockin of Esrrb to Nanog restores PGC numbers to wild-type levels and results in fertile adult mice. These findings demonstrate that Esrrb can replace Nanog function in germ cells.

INTRODUCTION

Naive pluripotency is established in epiblast cells of the mouse blastocyst (Boroviak et al., 2014; Brook and Gardner, 1997). The transcription factors (TFs) Oct4, Sox2, and Nanog are required to establish epiblast identity and are fundamental pluripotency regulators in vivo and in vitro (Festuccia et al., 2013). Following implantation, the epiblast enters a transitional phase in which cells remain uncommitted and functionally pluripotent (Beddington, 1982; Osorno et al., 2012; Tam and Zhou, 1996). At this point, expression of OCT4 and SOX2, but not NANOG or other naive TFs, is maintained (Smith, 2017). Mouse primordial germ cells (PGCs) are induced from the pluripotent post-implantation epiblast early on embryonic day (E)6 (Ohinata et al., 2005) and upregulate expression of many naive pluripotency genes following specification (Kurimoto et al., 2006). PGCs do not contribute to chimeras when injected into blastocysts (Leitch et al., 2014), but possess a latent capacity to reacquire pluripotency, which can be revealed in vivo during teratocarcinogenesis (Stevens, 1983) or by the derivation in vitro of naive pluripotent stem cell lines called embryonic germ cells (Leitch et al., 2013; Matsui et al., 1992; Resnick et al., 1992). Furthermore, PGC development is dependent on the expression of pluripotency TFs. Conditional deletion of either Oct4 or Sox2 results in PGC death (Campolo et al., 2013; Kehler et al., 2004). Nanog-null embryonic stem cells (ESCs) exhibit broad differentiation potential, including to migratory PGCs, but contribution to germ cells at E12.5 was not observed in our previous study (Chambers et al., 2007). Induced knockdown of Nanog in PGCs results in significant alteration of their transcriptional program and subsequent apoptosis (Yamaguchi et al., 2009). Induction of PGC-like cells (PGCLCs) in vitro is impaired in the absence of Nanog, whereas exogenous Nanog improves PGCLC yield (Murakami et al., 2016), in keeping with in vivo findings.

Nanog is essential for the specification of pluripotency in vivo (Mitsui et al., 2003; Silva et al., 2009). However, Nanog-null ESCs can be maintained, albeit with a reduced self-renewal efficiency (Chambers et al., 2007). The orphan nuclear receptor Esrrb is a regulator of ESC self-renewal (Festuccia et al., 2012; Ivanova et al., 2006; Marcelli et al., 2012) and influences PGC numbers in vivo (Mitsunaga et al., 2004). Esrrb is also a direct NANOG target (Festuccia et al., 2012). Deletion of Esrrb abolishes the
ability of NANOG to confer leukemia inhibitory factor (LIF) independence in ESCs (Festuccia et al., 2012). Furthermore, ESRRB can compensate for NANOG function in epiblast stem cell (EpiSC) reprogramming and in induced pluripotent stem cell (iPSC) generation (Festuccia et al., 2012). Thus, Esrrb is a key downstream mediator of Nanog function in the maintenance and establishment of pluripotency in vitro. Here, we reassess the requirement for Nanog in PGCs and investigate whether ESRRB can compensate for NANOG function during PGC development.

RESULTS

Conditional Deletion of Nanog Reduces PGC Numbers
To assess whether Nanog is required cell autonomously in PGCs, a conditional knockout strategy was used. Mice homozygous for a Nanog conditional allele (Nanog<sup>fox/fox</sup>) (Chambers et al., 2007) were crossed with Nanog heterozygous mice (Nanog<sup>−/+</sup>) (Mitsui et al., 2003) harboring the Prdm1-Cre-BAC transgene (Ohinata et al., 2005) (Figure 1A). One in four offspring carried the Nanog null (−) and conditionally deleted (Δ) alleles in PGCs. As Prdm1-Cre-mediated excision has been reported to be incomplete until after E10.5 (Campolo et al., 2013; Kim et al., 2014), genital ridges in control and mutant embryos were dissected at E11.5 and analyzed by immunofluorescence. NANOG protein was not detected in mutant genital ridges (Figure 1B). However, GFP-positive cells were present, indicating successful deletion of Nanog (Figure 1B). Compared with littermate controls, PGC numbers in Nanog mutant embryos were reduced 80% (Figure 1B and 1C). Surprisingly, a small number of GFP-positive mutant PGCs expressing MVH were also detected at E12.5 (Figure 1D). To establish whether these surviving Nanog mutant PGCs were developmentally competent, subsequent litters were allowed to go to term, and adult mutant mice of both sexes were test-crossed (Figures S1A and S1B). Male and female mutant mice were fertile (Figure 1E), passing either the knockout or conditionally deleted allele to their offspring (Figure S1C). These findings indicate that Prdm1-Cre-mediated deletion of Nanog reduces the PGC number, but suggest that Nanog might not be strictly required for germline development.

Nanog Is Not Essential for Germline Development
The requirement for Nanog in germline development was next assessed using an alternative approach. First, Nanog<sup>fox/fox</sup>—ESC
lines were derived from Nanog<sup>flox/flox</sup> × Nanog<sup>fl/+</sup> intercrosses (Figures S2A and S2B). Two independent clones were expanded and exhibited normal ESC morphology (Figure S2C). Both lines gave high contribution chimeras and germline transmission (Figures S2D and S2E). Next, both Nanog<sup>flox/flox</sup> ESC lines were transiently transfected with Cre, and single GFP-positive cells that had deleted Nanog were isolated (Figure 2A). Two GFP-positive clones derived from each parental line were expanded (Figure 2B). All four Nanog<sup>fl/+</sup> clones showed a higher differentiation propensity than parental lines (Figure 2B), consistent with abrogated Nanog function. Successful recombination was confirmed by genomic PCR (Figure S3A). Nanog was undetectable by quantitative real-time PCR (Figure S3B) or immunostaining (Figure 2C). Nanog<sup>fl/+</sup> lines were injected into C57BL/6 blastocysts and three out of four clones produced high contribution coat color chimeras (Figure 2D and 2E). On test crossing, chimeras generated with two independent clones (derived from different parental lines) produced agouti pups, indicating successful germline transmission. This was confirmed by detection of either the null or the deleted band in agouti offspring (Figures 2E and S3D) and detection of GFP fluorescence from the recombined allele in inner cell masses (ICMs) from a further test cross (Figure S3C). These results demonstrate clearly that Nanog function is not absolutely required for germline development.

**Esrrb Can Compensate for Nanog Loss in PGCLCs In Vitro**

Early PGC development can be recapitulated in vitro by the induction of PGCLCs (Figure S4A) (Hayashi et al., 2011). Naive ESCs in 2 inhibitors (2i)/LIF acquire competence for PGCLC induction after 2 days of culture in basic fibroblast growth factor (bFGF), Activin A, and knockout serum replacement (KSR) (Figures S4A, SD, and SE). Expression of Prdm1 (also known as Blimp1) and Prdm14, accompanied by elevated levels of both Nanog and Esrrb (Figure S4E) indicates PGCLC induction. In keeping with recently published data (Murakami et al., 2016), Nanog-null ESCs produced fewer PGCLCs than wild-type controls, as measured by a decrease in CD61+/SSEA-1+ cells after day 4 (Figure 3A). Next, the doxycycline (Dox)-inducible system for gene expression in Nanog-null ESCs (Festuccia et al., 2012) was assessed for its ability to drive inducible transgene expression during PGCLC differentiation (Figure S4B). The addition of Dox on day 2 allowed robust expression of a tdTomato transgene (Figure S4C) without affecting PGCLC induction efficiency in either wild-type or Nanog mutant ESCs (Figures 3A and S4D). The same strategy induced expression of Nanog (Figures 3B and S5A) and rescued the deficit in PGCLCs on day 6 and 8 to wild-type levels (Figures 3A and 3B). ESRRB is a downstream mediator of NANOG function in ESCs and during reprogramming.
Deletion of Esrrb also reduces PGC numbers in vivo (Mitsunaga et al., 2004). Interestingly, therefore, Esrrb mRNA was detectable in E14Tg2a and Nanog null ESCs at day 2 of PGCLC differentiation (Figure S5B). This expression increased during subsequent days of differentiation in wild-type but not Nanog−/− cells (Figure S5B). However, induction of Nanog restored the increasing Esrrb mRNA levels during PGCLC differentiation of Nanog−/− (left) and Nanog−/−Esrrb−/− (right) ESCs in the presence (+) or absence (−) of Dox. The morphology and Nanog:GFP expression of aggregates are shown (left; scale bar, 200 μm) with SSEA1/CD61 analysis by fluorescence-activated cell sorting (FACS) (right). (D) Quantitative mRNA expression analysis during PGCLC differentiation of Nanog−/− (left) and Nanog−/−Esrrb−/− (right) ESCs in the presence (+) or absence (−) of Dox at the indicated number of days of PGCLC differentiation. Values are means ± SDs; n = 3 biological replicates. *p < 0.05; **p < 0.01; and ***p < 0.001 (unpaired Student’s t test).

Having established that Esrrb can compensate for Nanog loss in PGCLCs, we next devised a strategy to assess whether Esrrb might compensate for Nanog loss in PGCs in vivo. First, ESCs were generated by homologous recombination, in which Esrrb cDNA was expressed from the endogenous Nanog locus at the Nanog AUG start codon (designated Esrrb knockin [KI]) (Figure S7A). Correctly targeted Nanog+/EsrrbKI ESCs were identified (Figure S7B). To assess Esrrb mRNA expression in the Esrrb knockin model, we analyzed Nanog+/− ESCs carrying this Esrrb knockin allele. This showed that Nanog+/− ESCs express Esrrb mRNA at ~24% of the wild-type level and that Nanog+/−EsrrbKI ESCs express Esrrb mRNA at ~2-fold the level of wild-type ESCs (Figure S7C). Nanog+/−EsrrbKI ESCs were used to establish mouse lines by injection into blastocysts. Nanog+/−EsrrbKI mice were viable and fertile, with no obvious developmental defects (unpublished data). The Prdm1-Cre-BAC transgene was then
introduced and resulting mice crossed with the Nanog^{floxed} females (Figure 4A). In this case, one in four offspring would carry both an EsrrbKI and a Nanog conditional (floxed) allele in combination with a Prdm1-Cre-BAC transgene (Figure S8A). This combination, which is anticipated to result in Nanog^{ΔEsrmbKI} PGCs, was identified by genotyping somatic tissue (Figure S8B). Immunofluorescence of genital ridges from E12.5 Nanog^{ΔEsrmbKI} and control embryos. GFP expression is from the conditionally deleted (Δ) allele and is specific to germ cells of the genital ridge (scale bar, 50 μm).

(C) Cell numbers were counted from Nanog^{ΔEsrmbKI} and control genital ridges. PGCs are identified by Dazl expression. The mean (± SD) of three biological replicates for control and Nanog^{ΔEsrmbKI} are shown. n.s., not significant.

(D) Table of breeding data for adult Nanog^{ΔEsrmbKI} and control mice. Both male (row 3) and female (row 4) Nanog^{ΔEsrmbKI} mice are fertile.

See also Figures S7 and S8.

**DISCUSSION**

Although conditional knockout approaches have shown that the Oct4 and Sox2 are essential for PGC development (Campolo et al., 2013; Kehler et al., 2004), studies on other pluripotency TFs have proved challenging. Here, we use conditional knockout strategies, mouse chimeras, and the recently developed PGCLC system (Hayashi et al., 2011) to assess the function of the Nanog-Esrrb axis in PGCs. This study establishes a role for Nanog in regulating PGC numbers in vivo. However, as for ESCs (Chambers et al., 2007), Nanog is strictly dispensable for PGC function. We show that knockin of Esrrb to the Nanog locus can complement the Nanog defect and is sufficient to rescue PGC numbers in vivo. Our study also supports a recently proposed role for Nanog in the maintenance of PGCLCs from pluripotent stem cells in vitro (Murakami et al., 2016), but expands on these findings, establishing that Esrrb can substitute for Nanog function in this system. Therefore, in addition to ESC self-renewal and iPSC reprogramming, Esrrb can functionally substitute for Nanog in PGC development. This strengthens the hypothesis that aspects of the naive pluripotency network are re-established in PGCs (Leitch and Smith, 2013). It will therefore be interesting to see whether the recently reported mitotic bookmarking activity of Esrrb in ESCs is also conserved in PGCs. Previous experiments have suggested that Nanog is required for PGC development (Chambers et al., 2007; Yamaguchi et al., 2009). Although chimera experiments showed that Nanog^{−/−} ESCs could form nascent PGCs at E11.5, Nanog-null PGCs
were not observed one day later at E12.5 (Chambers et al., 2007). This loss of PGCs was shown to be due to Nanog mutation, since repair of Nanog by homologous recombination restored E12.5 PGCs (Chambers et al., 2007). These findings were largely supported by a study in which induced knockdown of Nanog led to PGC death (Yamaguchi et al., 2009). More recently, however, Nanog\textsuperscript{−/−} iPSCs were reported to be capable of germ line transmission based on expression of a GFP transgene in tissues of chimera-derived offspring (Carter et al., 2014). Our present findings provide unequivocal evidence that PGC development can be completed in the absence of Nanog by showing that two newly derived Nanog\textsuperscript{−/−} ESC lines exhibit germ line transmission, as judged by coat color and the presence of Nanog-null alleles in F1 pups. Together with the severe reduction in PGC numbers observed in our conditional deletion experiments, this clarifies that the absence of NANOG compromises the development of the PGC population, but that individual PGCs can acquire full functionality in the absence of NANOG. This germ line phenotype may render Nanog\textsuperscript{−/−} PGCs disadvantaged compared with wild-type PGCs in the context of chimeras and reduce the frequency with which germ-line competency is observed. Our previous Nanog-null chimera experiments were performed using ESCs cultured in LIF/fetal calf serum (FCS). In contrast, both current examples of germ line transmission were obtained using cells cultured in 2i/LIF, which may have enhanced the degree of chimerism, thereby increasing the likelihood of observing germ line transmission, as previously shown for 3i/LIF culture medium (Kiyonari et al., 2010).

The fact that Esrrb can substitute for Nanog provides functional evidence that the naive pluripotency network may be conserved in PGCs. It is of interest that Esrrb fully restored PGC numbers by E12.5 when expressed from Nanog, Tetraploid embryos complemented by morula aggregation with Esrrb-null ESCs showed a reduction in PGC numbers of 50%–80% between E13.5 and E15.5 (Mitsunaga et al., 2004). Esrrb transcripts were first detected by real-time (RT)-PCR at E11.5 (Mitsunaga et al., 2004), with Nanog expression detected earlier in PGCs (Yamaguchi et al., 2005). However, re-analysis of published single-cell RNA sequencing (RNA-seq) data from PGCs (Hackett et al., 2013; Magnusdottir et al., 2013) shows that Esrrb and Nanog mRNAs both increase in expression from E6.5 to E7.5, remaining relatively steady thereafter until E12.5 (Figure S9). This suggests that ESRRB may function in PGCs before E11.5. Esrrb is also expressed during PGCLC differentiation, but at a reduced level in Nanog\textsuperscript{−/−} PGCLCs relative to wild-type cells. The wild-type expression level of Esrrb mRNA is restored in Nanog\textsuperscript{−/−} PGCLCs by Nanog induction. These results indicate that NANOG controls Esrrb expression in PGCs, but that, as is the case in ESCs, positive inputs in addition to NANOG also contribute to Esrrb expression (Festuccia et al., 2012; Martello et al., 2012).

Nanog\textsuperscript{−/−} cells undergoing PGCLC differentiation showed increased apoptosis and reduced proliferation, validating previous important observations using an in vivo conditional knockdown approach (Yamaguchi et al., 2009). Consistent with this seminal study, apoptotic cells positive for active caspase-3 were invariably either OCT4 low or OCT4 negative. Restoring either Nanog or Esrrb expression in Nanog\textsuperscript{−/−} PGCLCs is sufficient to reverse both the apoptosis and proliferation defects. Together, these studies add to the evidence that Esrrb is a physiologically relevant mediator of PGCLC function (Mitsunaga et al., 2004).

A limited number of studies have focused on other naive pluripotency factors in the germ line. In addition to Oct4 and Sox2, conditional knockout of Sall4 in PGCs does appear to affect gonadal PGC numbers, although interpretation is complicated by the mosaic deletion brought about by TNAP-Cre (Yamaguchi et al., 2015). The extent to which other pluripotency factors influence germ line competence, PGC specification, and subsequent development is of significant interest. The PGCLC system may be an ideal tool to assess these factors. Recently, it was reported that induced expression of Nanog is sufficient to induce PGCLCs from epiblast-like cells (EpiLCs) (Murakami et al., 2016). Together with our data, this may indicate that Nanog has a dose-dependent influence on both the specification and maintenance of PGCLCs. This is reminiscent of the role of Nanog in ESCs, in which Nanog is not absolutely required, but functions as a pluripotency rheostat (Chambers et al., 2007; Mullin et al., 2017; 2008). In this regard, it is notable that Prdm14 is a direct Nanog responsive gene in ESCs (Festuccia et al., 2012; 2013) and responds to Nanog in EpiLCs (Murakami et al., 2016). The ability of Esrrb to restore function in vitro to Nanog\textsuperscript{−/−} PGCLCs further underscores the similarities between naive pluripotency and germ line development. It would be interesting to assess whether elevated levels of Nanog or Esrrb in vitro might enhance PGC specification and germ cell numbers. How such manipulations of the pluripotency gene regulatory network might affect PGC identity is also of interest. This will enable us to reveal how the pluripotency gene regulatory network interacts with germ-cell-specific genes during PGC development and so build on the remarkably insightful studies that first pioneered the connection between pluripotency and the germ line more than half a century ago (Stevens, 1983).

**EXPERIMENTAL PROCEDURES**

Animal studies were authorized by a UK Home Office Project License and carried out in a Home-Office-designated facility.

**PGCLC Differentiation**

PGCLC differentiation was performed essentially as described previously (Hayashi et al., 2011). Briefly, ESCs were cultured in 2i/LIF medium (as above) for several passages. Cells were then seeded onto fibronectin-coated plates at \(1 \times 10^5\) cells/12 well in N2B27/1%KSR/Activin A to obtain EpiLCs. Two days later, EpiLCs were collected and aggregated at 2,000 cells/well in PGCLC medium (50 ng/mL bone morphogenetic protein (BMP)4, 50 ng/mL BMP8a, 10 ng/mL stem cell factor (SCF), 10 ng/mL epidermal growth factor (EGF), and 1,000 U/mL LIF) using U-bottom 96-well plates (Thermo Fisher Scientific, 174925). For induction of gene expression, 1 \(\mu\)g/mL Dox (Sigma, D9891) was added at day 2 of PGCLC differentiation.

Further methods can be found in Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, nine figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.060.
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AUTHOR CONTRIBUTIONS

I.C., H.G.L., N.F., A.S., and M.A.S. conceived the project. H.G.L. performed the Nanog conditional knockout analyses. J.N. and H.G.L. derived the ESC lines. N.F. constructed the NanogESrrbKI allele and performed initial analyses. M.Z. analyzed the NanogESrrbKI phenotype and performed PGCLC experiments. W.W.C.T. and E.H.P. provided technical support. H.G.L., M.Z., and I.C. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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