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Nanog is required for primitive endoderm formation through a non-cell autonomous mechanism

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ABSTRACT

Early lineage segregation in mouse development results in two, either CDX2- or OCT4/NANOG-positive, cell populations. CDX2-positive cells form the trophectoderm (TE), OCT4/NANOG-positive cells the inner cell mass (ICM). In a second lineage decision ICM cells segregate into Epiblast (EPI) and primitive endoderm (PE). EPI and PE formation depend on the activity of the transcription factors *Nanog* and Gata4/6. A role for *Nanog*, a crucial pluripotency factor, in preventing PE differentiation has been proposed, as outgrowths of mutant ICMs result in PE, but not EPI derivatives. We established *Nanog*-mutant mouse lines and analyzed EPI and PE formation *in vivo*. Surprisingly, *Gata4* expression in mutant ICM cells is absent or strongly decreased, thus loss of *Nanog* does not result in precocious endoderm differentiation. However, *Nanog*-deficient embryos retain the capacity to form PE in chimeric embryos and, in contrast to recent reports, in blastocyst outgrowths. Based on our findings we propose a non-cell autonomous requirement of *Nanog* for proper PE formation in addition to its essential role in EPI determination.

Introduction

The first lineage segregations in the pre-implantation embryo establish the basis for mouse embryonic development. At the compacting morula stage, trophectoderm (TE) and inner cell mass (ICM) emerge; later the ICM gives rise to primitive endoderm (PE) and epiblast (EPI) (Gardner and Beddington, 1988). Recent evidence suggests that unlike TE/ICM segregation in the morula (Ralston and Rossant, 2008), lineage-specific gene expression precedes EPI- and PE-cell sorting in the ICM. Before the PE becomes evident, the ICM is already composed of two, committed cell types arranged in a 'salt-and-pepper-like' distribution (Chazaud et al., 2006). Transcription factors determine the adopted fate: PE cells express *Gata6* and *Gata4* (Koutsourakis et al., 1999; Morrisey et al., 1998), and EPI cells the homeobox transcription factor *Nanog* (Chambers et al., 2003; Mitsui et al., 2003). *Nanog* expression commences at the morula stage and becomes gradually restricted to the ICM during blastocyst formation and expansion (Hart et al., 2004; Mitsui et al., 2003; Zernicka-Goetz et al., 2009). Disruption of *Nanog* results in peri-implantation lethality and although mutant blastocysts are morphologically normal and induce decidualization they don't form a proper EPI. Embryonic stem (ES) cells cannot be isolated from *Nanog*-deficient blastocysts; the cells recovered from blastocyst outgrowths display a parietal endoderm-like morphology (Mitsui et al., 2003). *Gata4/6*-deficient embryos show deficits in establishing PE and fail to maintain PE derivatives (Koutsourakis et al., 1999; Morrisey et al., 1998; Soudais et al., 1995), while over-expression of *Gata4/6* in ES cells is sufficient to induce PE differentiation (Fujikura et al., 2002). A direct link between *Nanog* and *Gata6* was proposed and NANOG was shown to bind the *Gata6* promoter in ES cells (Mitsui et al., 2003; Singh et al., 2007).

While early knock-out studies reported endodermal differentiation of *Nanog*-mutant ICMs, a recent study addressing the role of *Nanog* in the generation of pluripotency suggested that *Nanog*-mutant ICM cells are blocked in a transitional stage and are not capable of acquiring the pluripotent ground state and lose their differentiation potential (Silva et al., 2009). These authors propose that *Nanog*-null ICM cells are thus only capable of TE differentiation or else undergo apoptosis, as opposed to the original description of PE outgrowth (Mitsui et al., 2003).

We established and examined *Nanog* gene-trap mouse lines and found that surprisingly PE formation in mutant blastocysts is profoundly repressed rather than precociously activated. However, in this study we took the analysis from *Nanog* mutants further, showing that surviving ICM cells from *Nanog*-null embryos retain the capacity for PE formation *in vivo* and at least transiently *in vitro*. Thus, our findings help resolve the recently emerged controversy of *Nanog* action in the early mouse embryo (Mitsui et al., 2003; Silva et al., 2009), and moreover, we demonstrate that PE formation depends on a functional EPI, hence directly or indirectly, on *Nanog* expression. In the model we propose, *Nanog* expression is required for the formation of the EPI lineage, which in turn through a non-cell autonomous mechanism, possibly mediated by FGF/Erk signaling (Chazaud et al., 2006; Nichols et al., 2009), is vital for proper PE formation.

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Materials and methods

Generation of gene-trap mice and genotyping

Two ES cell lines (D042B07 [GT1] and D047A12 [GT2]) with disrupted *Nanog* alleles were obtained from the GSF (Munich) (Schnutgen et al., 2005) (Supplementary Fig. S1) and used for blastocyst injection. Chimeras were mated with C57BL/6 females to generate the $Nanog^{gt1/+}$ and $Nanog^{gt2/+}$ mouse lines. Genotyping was performed by PCR on genomic DNA isolated from embryos, yolk sacs or mouse-tail biopsies using primers specific for the GT1- or GT2-allele. For blastocyst complementation the Gt(Rosa)26Sor/J (ROSA26) line (Zambrowicz et al., 1997) was bred onto the GT1-Nanog background and matings performed as detailed in supplementary Fig. S2.

Whole-mount immunodetection

Embryos were flushed (E3.5) or dissected (E4.5) from the uterus following natural matings, counting noon of the day the plug was detected as E0.5. Antibody detection assays were performed as described in (Kan et al., 2007). Self-made affinity-purified rabbit antibody for NANOG was used at a dilution of 1:50, OCT4 (monoclonal mouse, Santa Cruz Biotechnology) at 1:200, CDX2 (monoclonal mouse, BioGenex) at 1:200 and GATA4 (polyclonal rabbit, Santa Cruz Biotechnology) at 1:100. Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse (Molecular Probes; 1:500). Nuclei were stained with 10 µM DAPI (Molecular Probes) added to the last wash. For double staining of blastocyst outgrowths with GATA4 an additional α-NANOG antibody (R&D, polyclonal goat, 1:10) was used.

ES cell culture, blastocyst outgrowths and embryo culture

ES cells were cultured on feeder cells in ES cell medium (DMEM with 15% FCS, supplemented with 500 U/ml LIF [Chemicon, ESGRO #ES1107]). For blastocyst outgrowths embryos were recovered at E3.5 and cultured in ES cell medium on gelatinized tissue culture chambers (Lab-tek #177402). After 5 days, attached outgrowths were fixed in 4% PFA/PBS, permeabilized in 0.5% Triton X-100/PBS and blocked in 10% FCS in 0.1% Triton X-100/PBS (PBSTx). First and second antibody incubation was performed in blocking solution as described above. Embryos were cultured in KSOM microdrops covered with mineral oil at 37 °C and 5% CO_2 atmosphere. Inhibitor treatment on wild-type embryos was performed in KSOM microdrops containing 20 µM SU5402 in DMSO or DSMO only for controls. FGF treatment was performed on embryos from heterozygous intercrosses isolated at E2.5 and cultured for 48 in the presence of human recombinant FGF4 (10^{-8} M; Sigma) in KSOM microdrops. Blastocysts were PCR genotyped after GATA4 immunostaining.

Generation and analysis of chimeric embryos

Wild-type W4 or stable pCAGGS-eGFP transfected W4 ES cells were injected into E3.5 blastocysts obtained from matings according to supplementary Fig. S2. All embryos resulting from these matings carry a ubiquitously expressed β-galactosidase reporter, allowing tracing of embryo-derived cells. After ES cell-injection the blastocysts were retransferred into pseudo-pregnant females and embryos isolated at indicated time points. LacZ staining was performed as described previously (Stemmler et al., 2005). For IF, embryos and yolk sacs were frozen in OCT (Tissue-tek) and sectioned at 14 µm using a cryostat (Leica). Sections were blocked in 10% FCS/PBSTx and incubated with α-GFP (polyclonal sheep, 1:400; AbD) and α-β-galactosidase (polyclonal rabbit, 1:1000; MPBiomedicals) antibodies for 1 h at RT. GFP was visualized with biotin-conjugated donkey-anti-sheep Ig (Jackson Labs; 1:300) and FITC-conjugated streptavidin (Jackson Labs; 1:400); β-GAL with Cy3-conjugated donkey-anti-rabbit Ig (Jackson Labs; 1:300).

Results and discussion

Nanog gene-trap embryos

Despite the relatively early expression of *Nanog* in pre-implantation embryos, *Nanog* mutants form normal morulae, which compact and give rise to morphologically normal blastocysts (Hart et al., 2004; Mitsui et al., 2003). We established two independent gene-trap mouse lines carrying a *β-Geo* insertion in the first intron of the *Nanog* locus (Supplementary Fig. S1A, B). We first investigated homozygous offspring of both lines for NANOG expression and found that embryos homozygous for either allele ($Nanog^{gt1/gt1}$ or $Nanog^{gt2/gt2}$) failed to express NANOG at pre-implantation stages (supplementary Fig. S1C). We failed to derive live homozygous offspring from either gene-trap line, and did not succeed in isolating homozygous mutants at E7.5, whereas phenotypically undistinguishable heterozygous and wild-type embryos were present. The number of empty deciduomas found, matched the expected Mendelian ratio of 25%. Thus, we concluded that, although genetically different in terms of the gene-trap integration site within the first intron, both, $Nanog^{gt1/gt1}$ and $Nanog^{gt2/gt2}$ embryos phenocopied the previously described *Nanog*-null mutant (Mitsui et al., 2003) and have identical phenotypes. Further, blastocysts carrying both gene-trap alleles ($Nanog^{gt1/gt2}$) were indistinguishable from homozygous embryos for either allele ($Nanog^{gt1/gt1}$ or $Nanog^{gt2/gt2}$) (Fig. 1 A, G and supplementary Fig. S1C). All breeding for experiments shown was carried out using intercrosses of both strains allowing accurate identification of null-mutants by PCR. Thus, embryos carrying both disrupted alleles are referred to as 'mutants', and heterozygous and wild-type embryos are grouped as 'controls'.

TE/ICM lineage segregation is independent of Nanog

We analyzed TE and ICM formation by examining expression of the TE-marker CDX2 and the ICM-marker OCT4 in mutant and control E3.5 blastocysts. CDX2 was restricted to the nuclei of the TE cells in both mutants (Figs. 1A–C) and controls (Figs. 1D–F). Though NANOG was absent in the mutants, we did not find CDX2 in ICM cells as has been described recently (Chen et al., 2009). OCT4 was detectable in all cells of the early blastocyst stage (Figs. 1H, K), thus CDX2 expression does not inevitably drive *Oct4* repression *in vivo*. Only later at implantation does OCT4 become restricted to the ICM (Dietrich and Hiiragi, 2007). *Oct4* expression, however, was unaffected by the loss of *Nanog* (Figs. 1G–I). To ascertain whether loss of *Nanog* affects cell proliferation, we counted TE and ICM cells in mutant and control blastocysts on stacks of confocal z-series images of CDX2/DAPI stained blastocysts (Fig. 1M). CDX2-positive cells were subtracted from the total cell number to determine the number of ICM cells. Mutant embryos were compared to their control littermates to assure stage/age-matched blastocysts. In one litter ICM cell numbers were 20.3 ± 2.1 (standard deviation, sd) in mutant ($n = 3$) and 22.3 ± 3.9 (sd) in control embryos ($n = 9$). TE cell numbers were 32.3 ± 2.3 (sd) in mutants and 30.1 ± 2.4 (sd) in controls. Therefore, we did not find significant differences in the number of ICM (Student's *t*-test, $p = 0.3$) or TE ($p = 0.23$) cells in mutants and controls. In a second litter ICM cell numbers were 24 (mutant, $n = 1$) and 24.5 ± 1.9 (sd) (controls, $n = 8$) and TE cell numbers were 32 (mutant) and 33 ± 5.5 (sd) (control). We concluded that, whilst *Nanog* is expressed at the morula stage, it is neither required for segregation of TE and ICM, nor for cell proliferation until the E3.5 blastocyst stage.

Nanog mutant ICMs show defects at implantation

The same marker-gene analysis was conducted on implanting embryos (E4.5), which were directly dissected from the uterus. All embryos had hatched and attached to the uterine wall at the time of isolation. In control embryos NANOG-positive and NANOG-negative ICM cells were distinct populations (Figs. 2E, H), whereas at E3.5 they