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Primitive Endoderm Differentiates via a Three-Step Mechanism Involving Nanog and RTK Signaling

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SUMMARY

During preimplantation mouse development, the inner cell mass (ICM) differentiates into two cell lineages—the epiblast and the primitive endoderm (PrE)—whose precursors are identifiable by reciprocal expression of Nanog and Gata6, respectively. PrE formation depends on Nanog by a non-cell-autonomous mechanism. To decipher early cell- and non-cell-autonomous effects, we performed a mosaic knockdown of Nanog and found that this is sufficient to induce a PrE fate cell autonomously. Strikingly, in *Nanog* null embryos, Gata6 expression is maintained, showing that initiation of the PrE program is Nanog independent. Treatment of *Nanog* null embryos with pharmacological inhibitors revealed that RTK dependency of Gata6 expression is initially direct but later indirect via Nanog repression. Moreover, we found that subsequent expression of Sox17 and Gata4—later markers of the PrE—depends on the presence of Fgf4 produced by Nanog-expressing cells. Thus, our results reveal three distinct phases in the PrE differentiation program.

INTRODUCTION

Preimplantation development in the mouse is characterized by the differentiation of two extraembryonic lineages, the trophectoderm and primitive endoderm (PrE), and of a pluripotent cell population, the epiblast, that gives rise to the embryo proper. Positional signals via the Hippo pathway direct outer cells of the late morula to form the trophectoderm, which surrounds the inner cell mass (ICM) after blastocyst formation (Nishioka et al., 2009). The mechanisms governing PrE and epiblast lineage segregation within the ICM remain less understood but are known to involve a progressive emergence of differential gene expression in their respective precursors followed by a combination of cell sorting and cell death (Gerbe et al., 2008; Meilhac et al., 2009; Plusa et al., 2008). By the time of implantation at embryonic stage (embryonic day [E]) E4.5, the PrE is a morphologically distinct epithelium separating the epiblast from the blastocyst cavity and expresses markers such as Gata6, Gata4, Sox17, and Pdgfra (Chazaud et al., 2006; Morris et al., 2010; Niakan et al., 2010; Plusa et al., 2008). Gata6 has also been shown to be required for PrE epithelium formation (Cai et al., 2008). Expression of the homeobox transcription factor Nanog is specific to the early epiblast but subsequently downregulated after implantation (Chambers et al., 2003). Nanog appears to be crucial for cells to attain a state of pluripotency that is characteristic of embryonic stem cells (ESCs), which are considered to be most similar to the early "naive" epiblast of the late preimplantation conceptus (Nichols et al., 2009). In the embryo, Nanog is required for formation of both epiblast and PrE cell lineages at E4.5 (Messerschmidt and Kemler, 2010; Silva et al., 2009). Moreover, blastocyst complementation experiments with wild-type ESCs gave evidence of a non-cell-autonomous requirement for Nanog in visceral endoderm formation (Messerschmidt and Kemler, 2010). Recent and earlier studies suggest that Fgf4 could be the non-cell-autonomous factor required for PrE differentiation (Arman et al., 1998; Cheng et al., 1998; Feldman et al., 1995; Goldin and Papaioannou, 2003; Yamanaka et al., 2010). Indeed, transcriptional profiling of single ICM cells revealed that *Fgf4* is expressed at E3.5 only in pre-epiblast cells (Guo et al., 2010; Kurimoto et al., 2006). Moreover, Fgf4 administration in embryo cultures drives ICM cells toward a PrE fate (Yamanaka et al., 2010), whereas receptor tyrosine kinase (RTK)/Mek pathway inhibition (Nichols et al., 2009; Yamanaka et al., 2010) or inactivation through *Grb2* knockout (Chazaud et al., 2006) prevents PrE differentiation and induces pan-ICM Nanog expression.

During late morula and early blastocyst stages, expression of Nanog is widespread and overlapping with expression of Gata6 and Pdgfra (Plusa et al., 2008). By E3.75 (~64 cells), expression of Nanog and PrE markers is largely mutually exclusive, marking epiblast and PrE precursors, respectively. Expression of Gata4 or Sox17 is initiated later than Gata6, marking cells that subsequently sort to form the PrE layer lining the blastocyst cavity. The mechanisms by which ICM cells interact to establish the

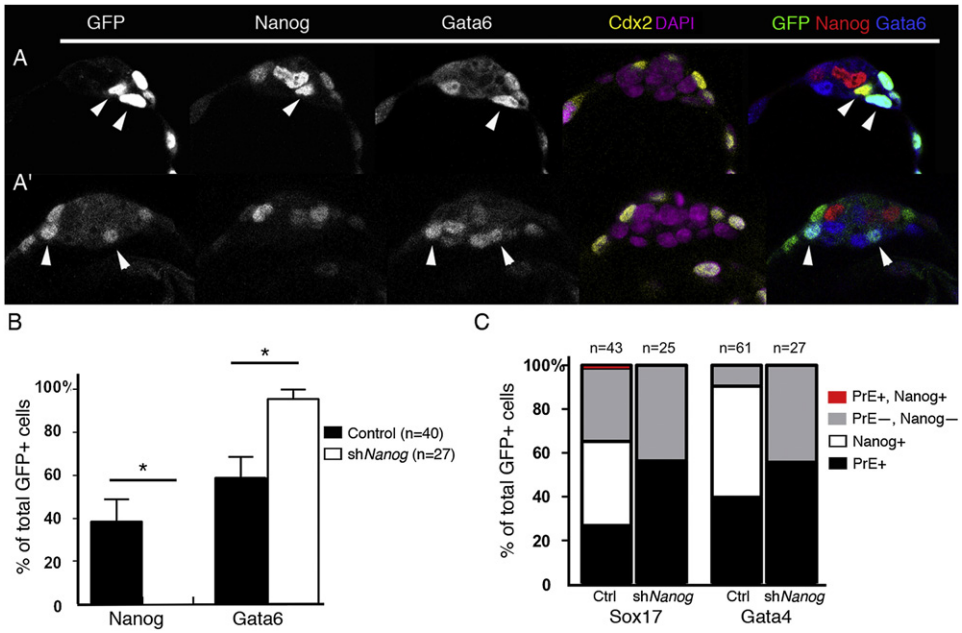

Figure 1. PrE Markers Are Induced in *Nanog* Knockdown Cells

(A and A′) Five-color labeling of 37 hr cultured embryos electroporated with control (A) or sh*Nanog* (A′) vectors. Arrowheads indicate GFP-positive cells in the ICM.

(B) Histogram showing the percentage of Nanog- and Gata6-positive cells among GFP-positive/Cdx2-negative cells in 37 hr cultured embryos (*$p < 0.05$, Wilcoxon test; error bars, SEM).

(C) Histogram showing the distribution of Nanog- and Sox17- or Gata4-positive cells among GFP-positive/Cdx2 negative cells. Embryos labeled for Sox17 were cultured for 37 hr, whereas for Gata4 labeling, a 45-hr culture was necessary to obtain sufficient numbers of labeled cells.

See also Figure S1.

two precursor populations are poorly understood but are likely to involve mutual inhibition between Nanog and Gata6 as well as phosphorylation of Erk (p-Erk) downstream of Fgf4 and Fgfr2 (Chazaud et al., 2006; Nichols et al., 2009; Singh et al., 2007; Yamanaka et al., 2010).

In this study, we examined the role of intercellular interactions in the establishment of epiblast and PrE precursors. We show that knocking down *Nanog* expression within a random subset of cells of the late morula is sufficient to enable induction of a PrE fate. By examining the development of *Nanog* mutant conceptuses, we also found that maturation of PrE precursors, characterized by Gata4 and Sox17 expression, is dependent on a non-cell-autonomous requirement for Nanog mediated by Fgf4 signaling. Finally, we found that early expression of Gata6 depends directly on p-Erk, whereas at later stages an absence of Nanog appears to be sufficient. Ultimately, absence of both Nanog and p-Erk, in the presence of Gata6, results in cell death.

RESULTS

Nanog Knockdown Is Sufficient to Induce Primitive Endoderm Formation in Targeted Cells

To analyze cell- and non-cell-autonomous effects at early stages, we inactivated *Nanog* by a mosaic RNAi knockdown in wild-type embryos. This strategy allows knockdown of the gene within the embryo before cell commitment, in contrast to embryo complementation assays using ESCs that are already committed to epiblast.

To introduce double-stranded RNA into cells, we performed embryo electroporation (Grabarek et al., 2002; Keramari et al., 2010; Soares et al., 2005) using expression plasmids. We used shRNAs to target *Nanog*, and the progeny of electroporated cells was identified by H2B-EGFP fluorescence. We used eight-cell stage embryos to achieve knockdown in progeny ICM cells because mainly surface cells are electroporated. Moreover, this stage is well before epiblast and PrE determination occurs, providing sufficient time for the treatment to potentially influence cell fate. After electroporation, embryos were cultured to the equivalent of E4.0–E4.5, when most ICM cells express either Nanog or Gata6 (Guo et al., 2010; Plusa et al., 2008). Fluorescence immunohistochemistry followed by five-channel confocal microscopy was then performed to detect GFP, Nanog, Gata6 (or other PrE marker), Cdx2, and nuclei in each embryo. ICM cells were identified by absence of Cdx2 protein because expression of Nanog or Gata6 can persist in the trophoblast after blastocyst formation (Dietrich and Hiiragi, 2007).

Although in control electroporated embryos, GFP-expressing cells were distributed in both of the ICM cell lineages (Figures 1A and 1B), electroporation of sh*Nanog* induced a knockdown of Nanog in GFP-expressing cells and activated Gata6 expression (Figures 1A′ and 1B; see Figure S1 available online). There was no statistically significant difference in the sorting of the Gata6-expressing cells that were transfected or not (data not shown). This result suggests a diversion toward PrE fate; however, Gata6 upregulation might only reflect a release of Nanog repression, and not a full PrE differentiation program.

Thus, expression of Sox17 and Gata4, which are known PrE markers, was also examined (Figure 1C). Gata6 is expressed earlier than Sox17 and Gata4, with Sox17 expressed slightly earlier than Gata4 (Artus et al., 2011; Morris et al., 2010; Niakan et al., 2010; Plusa et al., 2008). The number of GFP-positive cells expressing Sox17 and Gata4 increased when Nanog was knocked down. Most of the electroporated cells were positioned at the surface of the ICM (17 of 20); however, some of them did not express these proteins, suggesting that they might not represent fully mature PrE. Indeed, a difference can be seen in the number of Sox17- and Gata4-expressing cells compared with Gata6-expressing cells, regardless of *Nanog* knockdown, that probably reflects their later onset of expression. Such cells without Nanog and Gata4 expression can also be found quite late in freshly dissected embryos (Plusa et al., 2008; data not shown). To our current knowledge, it is not clear whether these cells will eventually express Sox17 and Gata4, undergo apoptosis (Plusa et al., 2008), or both. Unfortunately, cleaved caspase-3 or TUNEL staining does not allow identification of cells in combination with transcription factor expression because these methods identify cells in late apoptosis after the nucleus has usually already degenerated. In conclusion, these electroporation experiments demonstrate that a mosaic knockdown of *Nanog* cell autonomously induces expression of Gata6, Sox17, and Gata4, implying a full diversion toward PrE identity.

Nanog Mutant Embryos Express Gata6 in the Whole ICM

Our results above show that when Nanog expression is impaired in a subset of cells, they differentiate into PrE. By contrast, no or very few Gata4-expressing cells are present when whole embryos are mutant for *Nanog* (Messerschmidt and Kemler, 2010; Silva et al., 2009), suggesting that the PrE differentiation pathway is induced but not maintained. Thus, an analysis of earlier heterozygous and homozygous *Nanog* mutant embryos was carried out. We did not observe any difference in the number of Nanog-expressing cells between wild-type and heterozygous embryos, suggesting that one copy of the gene is sufficient to fulfill Nanog function. Because Gata6 and Sox17 are earlier markers than Gata4 (Niakan et al., 2010; Plusa et al., 2008), their expression was analyzed in *Nanog* null embryos from early E3.5. At E3.5 and E4.0, Gata6 was expressed in all ICM cells (Figures 2A′ and 2B′; n = 10/10 and 3/3, respectively). Thus, as in the electroporation experiments, *Nanog* inactivation induces Gata6 expression. As previously published (Messerschmidt and Kemler, 2010; Silva et al., 2009), only a few cells expressed Gata4 at E4.5–E4.75 (n = 4/4). Similarly to Gata4, Sox17 was expressed in very few cells at E3.75 and E4.5–E4.75 (Figures 2D′ and 2E′; n = 4/4 and 3/3, respectively). Pdgfra expression was also strongly impaired in *Nanog* mutants, with only a few weakly positive cells at E4.0 and E4.75 (Figure S2A; n = 5/5), whereas Oct4 expression was not affected (Figures 2C′ and 2E′; n = 8/8). Surprisingly, Gata6 was still expressed in all ICM cells at E4.5–E4.75 (Figure 2C′; n = 4/5) or in the majority of them (Figure S2B; n = 1/5), demonstrating that Gata6 expression does not require Nanog activity, in contrast to Gata4, Sox17, and Pdgfra expression. Thus, unlike in the electroporation experiments, Gata6 is not sufficient to induce Sox17 and Gata4 expression. This suggests that although repression of Nanog is required cell autonomously to activate *Gata6*, a non-cell-autonomous factor is required to induce Sox17 and Gata4 expression. In mosaic sh*Nanog* embryos, this is most likely mediated by the neighboring *Nanog*-expressing cells.

Because the number of pyknotic nuclei is high in E4.5–4.75 mutant embryos (Figure 2C′), we performed TUNEL staining to analyze cell death. Prolific cell death was observed in all tissues of mutant embryos at this stage of development (Figure 2E′; n = 5/5; Messerschmidt and Kemler, 2010). To check whether this wave of apoptosis induced by the absence of Nanog starts earlier, we analyzed E3.75 embryos. At this stage, previous reports showed that in wild-type embryos a small subset of cells undergoes apoptosis during the sorting of epiblast and PrE cells (Copp, 1978; Handyside and Hunter, 1986; Meilhac et al., 2009; Morris et al., 2010; Plusa et al., 2008). TUNEL staining showed that whereas some cells were apoptotic in control littermate embryos (Figure 2D), apoptosis was absent in E3.75 *Nanog* mutant ICMs (Figure 2D′; n = 3/3), suggesting that they are somewhat protected from the normally occurring apoptosis. It was proposed that in wild-type embryos cell death might occur in cells expressing both Nanog and Gata6 (Plusa et al., 2008). Thus, the sole Gata6 expression may prevent ICM cells from apoptosis at this stage.

Although previously reported (Chen et al., 2009; Silva et al., 2009), the lack of Nanog did not seem consistently to induce trophoblast identity. Mosaic *Nanog* knockdown did not induce Cdx2 expression in ICM cells, probably due to a late knockdown but also to the CD1 genetic background. Indeed, we observed differences analyzing either the mixed 129/B6/CD1 background embryos issued from *Nanog*$^{+/-}$ intercrosses compared to the CD1 embryos. Cdx2 expression in the ICM, although at lower levels than in trophoblast cells, was not uncommon in wild-type and heterozygous embryos produced from *Nanog*$^{+/-}$ intercrosses. In *Nanog*$^{-/-}$ embryos, Cdx2-expressing cells could be observed in many embryos while not in others (n = 6/17), suggesting that an absence of Nanog does not systematically induce Cdx2 in inner cells. We also found that the ratio of epiblast to PrE precursor cells was higher in the 129-mixed background, consistent with a previous report (Batlle-Morera et al., 2008), and that the mutually exclusive expression of Nanog and Gata6 developed a few hours later. Moreover, the sorting between epiblast and PrE cells was slower and sometimes not complete because some Gata6-, Sox17-, and Gata4-expressing cells could still be observed deep in the ICM of 129-mixed wild-type and heterozygous embryos at E4.5 (Figure 2C; n = 6). These effects tended to disappear while backcrossing on the CD1 background. These observations highlight the variability in differentiation of lineage precursors with respect to their proportions and their positions.

Fgf4 Is Expressed in Epiblast Precursors and Can Rescue Sox17 and Gata4 Expression in *Nanog* Mutants

As a secreted factor, Fgf4 is likely to activate PrE differentiation in a non-cell-autonomous manner. Single-cell RT-qPCR analysis has shown that *Fgf4* transcripts are highly enriched in pre-epiblast compared to pre-PrE cells (Guo et al., 2010). We confirmed this finding by in situ hybridization, showing that *Fgf4* expression is restricted to Nanog-positive cells at E3.5 (thus in pre-epiblast cells) and to the epiblast at E4.5 (Figures 3A and 3B). *Fgf4* expression was not detected in *Nanog* mutant

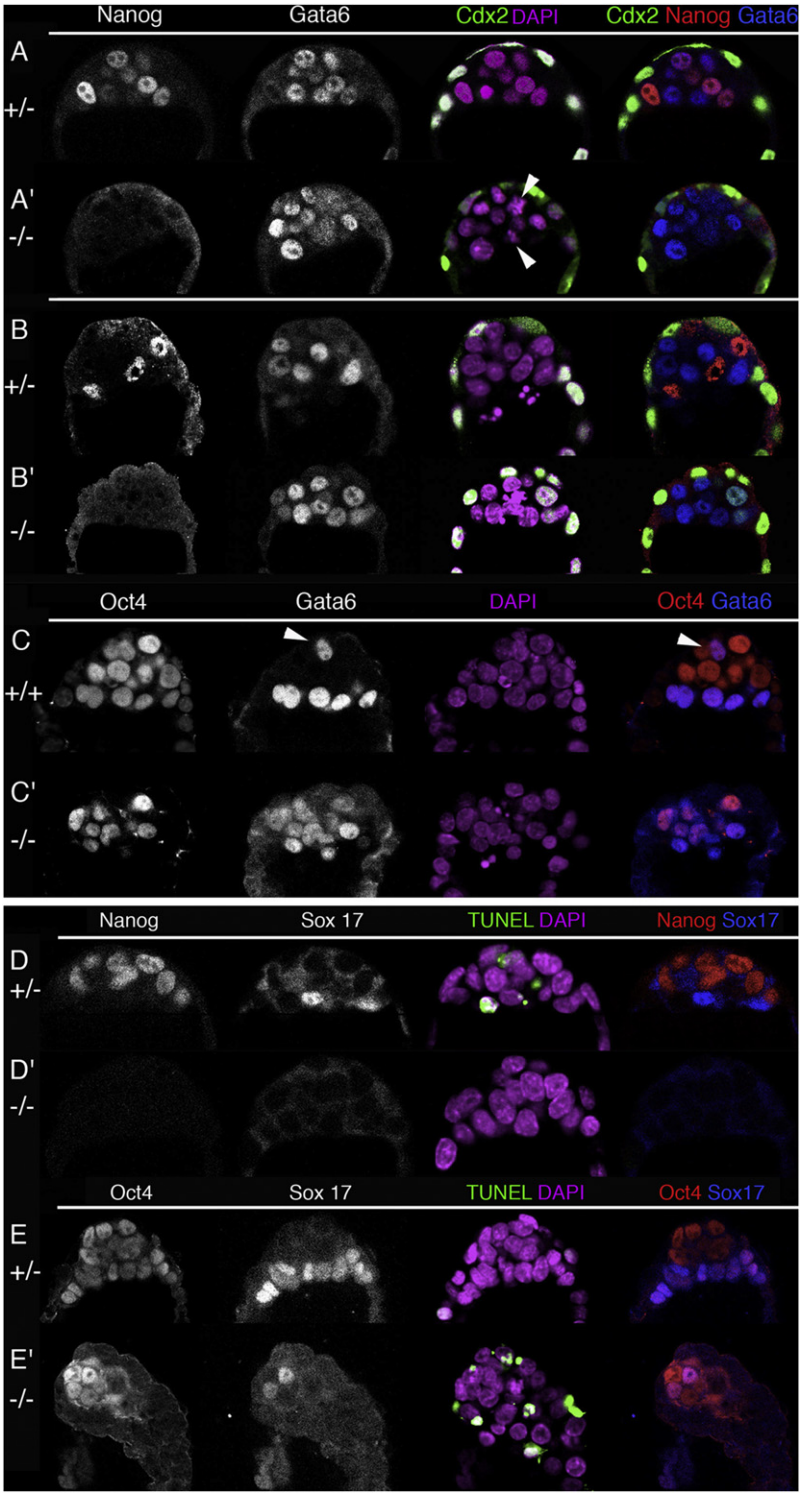

Figure 2. Analysis of PrE Markers in *Nanog*$^{-/-}$ Embryos

(A–B′) Nanog, Gata6, and Cdx2 immunolocalization at E3.5 (A and A′) and E4.0 (B and B′) in control and *Nanog*$^{-/-}$ embryos. Arrowheads indicate mitotic cells in which Gata6 is not nuclear localized.

(C–C′) Expression of Oct4 and Gata6 at E4.5 in control and *Nanog*$^{-/-}$ embryos. Arrowheads indicate an unsorted Gata6-expressing cell.

(D–E′) Nanog or Oct4, Sox17, and TUNEL staining in control and mutant embryos at E3.75 (D and D′) and E4.75 (E and E′).

See also Figure S2.

embryos at both stages (Figures 3A′ and 3B′; n = 3/3 and 3/3) and was not observed in *Nanog* knockdown cells (n = 15/16; Figure S3). Thus, *Fgf4* expression depends on the presence of Nanog.

Therefore, Fgf4 might be the non-cell-autonomous factor required to induce Sox17 and Gata4 expression in *Nanog* mutant embryos. To test this hypothesis, we cultured embryos from *Nanog*$^{+/-}$ intercrosses in the presence or absence of exogenous recombinant Fgf4. Because Gata6 is already expressed in *Nanog* mutant embryos, we speculated that treating embryos from the early blastocyst stage (E3.25) to E4.0 should be sufficient to induce Sox17 and Gata4. Although almost no cells were expressing Sox17 in *Nanog* mutants of the control culture (n = 5/5), most ICM cells expressed Sox17 in Fgf4-treated mutant embryos (n = 6/6, 93% of 59 ICM cells) (Figures 3C and 3C′). With this time window treatment, Gata4 was not detected (n = 3/3). However, Gata4 expression was detected in Fgf4-treated *Nanog* mutant ICM when embryos were cultured till E4.5 (Figure 3C″; n = 4/4, 80% of 55 ICM cells). Thus, Fgf4 is sufficient to rescue the non-cell-autonomous requirement for *Nanog* to induce Sox17 and Gata4 expression in PrE precursors.

RTK-Direct and -Indirect Activation of Gata6 Expression in the ICM

We and others have shown that the RTK pathway, through p-Erk activation, is required for Gata6 and PrE gene expression (Chazaud et al., 2006; Nichols et al., 2009; Yamanaka et al., 2010), although we were unable to detect p-Erk expression immunohistochemically at the blastocyst stage probably due to low and transient levels (Figure S4). Because Nanog can bind the Gata6 promoter and repress its expression (Singh et al., 2007), we wondered whether RTK activation of Gata6 occurs directly or only via inhibition of Nanog-mediated repression (scheme on Figure 4). To remove this repression, we used *Nanog*$^{-/-}$ embryos and cultured them with Fgf receptor and Mek inhibitors (Kunath et al., 2007; Ying et al., 2008). An absence of Gata6 expression in these embryos would show that *Gata6* is directly induced by the RTK pathway. By contrast, if Gata6 is still expressed in *Nanog*$^{-/-}$ embryos treated with the inhibitors, this would suggest that it is solely due to the relief of Nanog-mediated

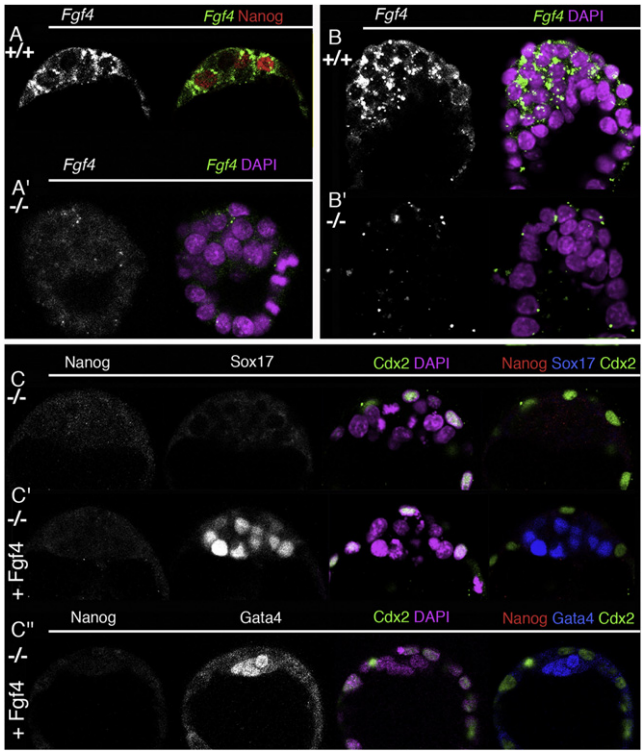

Figure 3. Fgf4 Treatment Rescues Sox17 and Gata4 Expression in *Nanog*$^{-/-}$ Embryos

(A–B′) *Fgf4* RNA labeling in wild-type and *Nanog*$^{-/-}$ embryos at E3.75 (A and A′) and E4.5 (B and B′), and coupled to Nanog immunofluorescence (A). See also Figure S3.

(C–C″) *Nanog*$^{-/-}$ embryos cultured in the absence (C) or presence (C′ and C″) of exogenous Fgf4 from E3.25 to E4.0 (C and C′) or E4.5 (C″) and immunolabeled.

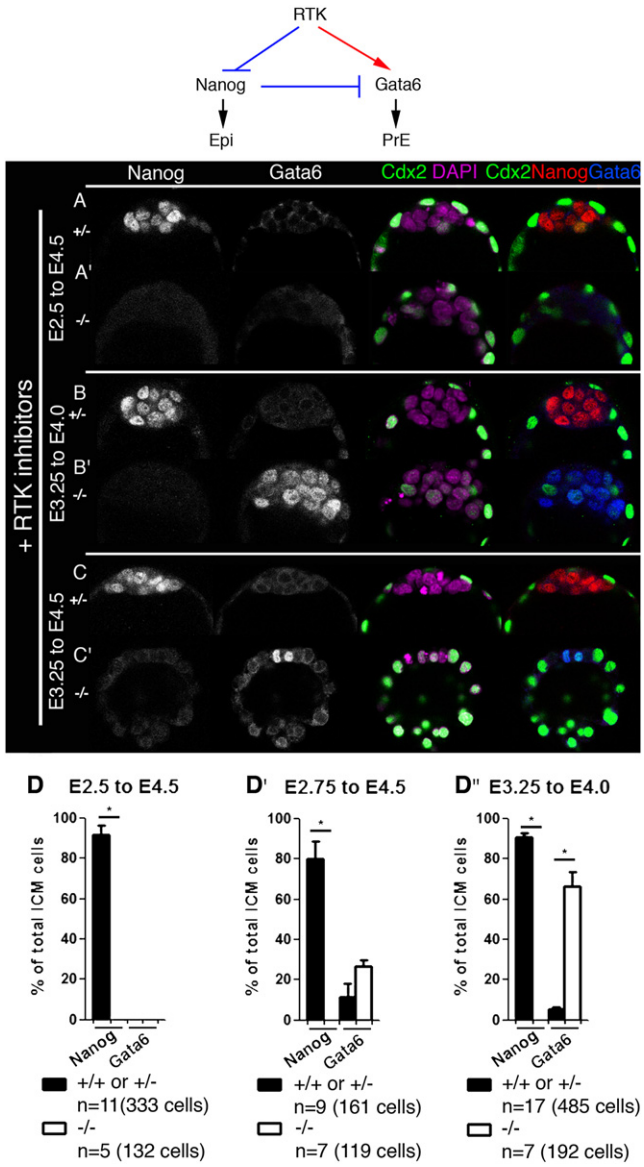

Figure 4. Gata6 Expression Is RTK Independent in *Nanog*$^{-/-}$ Embryos after Blastocyst Formation

Top: scheme representing the two distinct paths of Gata6 activation by RTKs: direct (red) and indirect (blue).

(A–C′) Control and mutant embryos were cultured in the presence of PD0325901 and PD173074 from E2.5 to E4.5 (A and A′), from E3.25 to E4.0 (B and B′), and from E3.25 to E4.5 (C and C′), and labeled with Nanog, Gata6, and Cdx2 antibodies. See also Figure S4.

(D–D″) Cell composition of embryos after different time windows of inhibitor treatment. Control and mutant embryos were cultured from E2.5 to E4.5 (D), from E2.75 to E4.5 (D′), and from E3.25 to E4.0 (D″), and stained for Nanog and Gata6 expression. (*$p < 0.05$, Wilcoxon test; error bars, SEM).

repression on the *Gata6* promoter. We carried out treatments through different time windows corresponding to the different phases of Gata6 expression: from E2.5, at the onset of Gata6 expression; from E2.75, when Gata6 is coexpressed with Nanog; and from E3.25, when reciprocal expression of Gata6 and Nanog begins to emerge. When embryos were treated from E2.5 to E4.5, no Gata6-expressing cells were observed in wild-type or mutant embryos (Figures 4A, 4A′, and 4D), showing that the RTK pathway is required directly for the onset of Gata6 expression. We then treated embryos from E2.75 because it was previously shown that Nanog and Gata6 are coexpressed in most, if not all, cells prior to cavitation (Guo et al., 2010; Plusa et al., 2008). Thus, at this stage, Nanog repression is presumably not effective. Surprisingly, with this time window treatment, about 27% of ICM cells expressed Gata6 in the mutant embryos (Figure 4D′). After treatment from E3.25 to E4.0, Gata6 expression was repressed as previously described in wild-type embryos (Yamanaka et al., 2010). By contrast, a high proportion of ICM cells (67%) expressed Gata6 in the mutant embryos (Figures 4B, 4B′, and 4D″). Thus, at this stage, Gata6 expression does not depend on the RTK pathway when Nanog is absent. This result means that in wild-type inhibitor-treated embryos, Gata6 repression solely depends on Nanog expression, and not on the absence of RTK signaling. Interestingly, the different time window treatments reveal that there are progressively more cells that switch from RTK-direct to RTK-indirect Gata6 expression and that at E3.25, some cells are still directly activating Gata6 expression by p-Erk.

Some E3.25 wild-type and *Nanog* mutant embryos were treated with the RTK pathway inhibitors for a longer period, till

E4.5. In mutant embryos cultured with inhibitors, ICM cells were very few in number (Figure 4C′; n = 4/5) and expressed Gata6, or many contained pyknotic nuclei (n = 1/5). This phenotype was different from that of untreated mutant embryos or treated wild-type embryos, which still had an ICM at this stage (Figures 2C′ and 4C, respectively). Loss of ICM cells in untreated *Nanog* mutant embryos occurs only later because they are present at E4.5 (Figure 2C′; Figure S3). This shows that in untreated *Nanog* mutant embryos, despite undetectable *Fgf4* transcript levels, an RTK activity is present and required for cell survival. Surprisingly, this precocious cell death was not observed when the mutant embryos were treated from E2.5 or E2.75 (Figure 4B′; n = 10). The notable difference between these groups from our results above is that Gata6 expression is lower or absent in earlier-treated embryos. Thus, Gata6 expression, in the absence of Nanog and RTK activity, is associated with earlier cell death.

DISCUSSION

In this study, we characterized the role of Nanog during epiblast versus PrE determination in E3.5 blastocysts. By using complementary approaches of either mosaic knockdown or full mutant embryos, we analyzed cell-autonomous and non-cell-autonomous roles for Nanog. Previous studies have shown that *Nanog*$^{-/-}$ embryos produce very few or no Gata4-expressing cells (Messerschmidt and Kemler, 2010; Silva et al., 2009), demonstrating that PrE cells can be induced but in very low numbers. Here we show that inactivation of Nanog leads to an upregulation of Gata6 expression in targeted cells of mosaic or fully inactivated embryos. This implies that Nanog inhibits Gata6 expression, probably via direct repression on specific enhancers (Mitsui et al., 2003; Singh et al., 2007). Thus, removing Nanog primes cells toward a PrE fate. The pan-ICM Gata6 expression in Nanog mutants together with the pan-ICM Nanog expression in *Grb2* mutants (Chazaud et al., 2006) supports the model of reciprocal inhibition of Nanog and RTK pathways to induce either epiblast or PrE differentiation.

Unlike in ESCs, where Gata6 expression leads to an upregulation of several PrE genes (Fujikura et al., 2002; Wang et al., 2011), pan-ICM Gata6 expression is unable to induce efficiently Sox17, Gata4, or Pdgfra in *Nanog* mutant embryos. Furthermore, it was shown that Gata6 overexpression in the embryo does not influence cell position (Meilhac et al., 2009; Morris et al., 2010). Thus, Gata6 alone is not sufficient to induce a full PrE identity, even in a *Nanog* mutant background, despite the presence of GATA response elements in *cis*-regulatory sequences of *Gata4*, *Sox17*, and *Pdgfra* (Niakan et al., 2010; Wang and Song, 1996). Thus, another factor is required in parallel with Gata6 to induce these downstream PrE genes. Consistent with this, mosaic *Nanog* knockdown experiments, as well as ESC chimaera production (Messerschmidt and Kemler, 2010), show that the presence of Nanog-expressing cells is sufficient to induce a full PrE identity in a non-cell-autonomous manner. We demonstrate here that *Fgf4*, which is a potent PrE inducer (Yamanaka et al., 2010), is expressed in epiblast precursor cells and is regulated by Nanog. Moreover, by adding recombinant Fgf4 to *Nanog* mutant embryos in culture, we show that Fgf4 is the factor required to induce Sox17 and Gata4 expression. Similar to the wild-type embryos, the onset of Gata4 expression was later than that of Sox17 in both *Nanog* knockdown and Fgf4-rescued mutant embryos. This internal clock could be regulated by Sox17 and Gata6 levels or by some other cofactors. Thus, PrE formation depends on both Gata6 (Cai et al., 2008) and Fgf4/RTK signaling pathways.

Studies in ESCs have suggested that Gata6 expression could be induced by the RTK pathway directly (Wang et al., 2011) or indirectly via repression of Nanog (Hamazaki et al., 2006). By inhibiting the RTK pathway during several time windows in the absence of Nanog, we show that Gata6 has RTK-direct and -indirect activation phases. Indeed, around the compaction stage, in both *Nanog* mutant and wild-type contexts, Gata6 expression requires the RTK pathway, implying that RTK activation directly induces Gata6 expression. *Fgf4* and *Fgfr2* are expressed at high levels from the two-cell and one-cell stage, respectively (Guo et al., 2010; Wang et al., 2004), and thus can potentially induce *Gata6* directly, whose expression increases around the eight-cell stage (Guo et al., 2010; Plusa et al., 2008). *Fgf4* mRNA levels are high before the onset of Nanog expression, suggesting that *Fgf4* induction at the two-cell stage does not depend on Nanog. Indeed, by RT-PCR we did not observe any difference in *Fgf4* expression between wild-type/heterozygous embryos and *Nanog* mutants at that stage (data not shown). Thus, Fgf4 probably induces Gata6 expression at precavitation stages. *Fgf4* expression requires both Sox2 and Oct4 (Avilion et al., 2003; Keramari et al., 2010; Nichols et al., 1998; Yuan et al., 1995), with only Sox2 being specifically downregulated in PrE precursors (Guo et al., 2010). Therefore, early Fgf4 expression could potentially be induced by maternally supplied Sox2 and Oct4.

Later-stage treatment with Fgfr and Mek inhibitors fails to downregulate Gata6 in a *Nanog* mutant background, converse to in wild-type embryos (Nichols and Smith, 2009; Yamanaka et al., 2010). This shows that the later Gata6 RTK dependency in wild-type embryos requires Nanog, and thus RTK signaling must directly repress Nanog. Thus, whereas the RTK pathway is always necessary for Gata6 expression, it is clearly required directly for initially inducing Gata6 expression and indirectly for its maintenance after cavitation. We cannot rule out the possibility that the RTK pathway continues to contribute directly to Gata6 expression, but the indirect mechanism seems to be sufficient to maintain Gata6 expression. It is known that *Gata6* contains GATA-binding sites in *cis* (Molkentin et al., 2000); thus, after an initial induction by the RTK pathway, *Gata6* could self-maintain its expression at high levels in PrE precursors. Indeed, it was shown that *Gata6* expression remains at the same levels from the eight-cell stage until formation of PrE precursors, while being lost in epiblast precursors (Guo et al., 2010).

The remaining RTK activity present in untreated *Nanog* mutants, revealed by the presence of a few Sox17-, Gata4-, or Pdgfra-expressing cells, could depend on the early *Fgf4* expression from the two-cell stage. This early expression is independent of *Nanog* expression, which is detected only later. Thus, low residual *Fgf4* expression could explain how a few cells still express these PrE markers in untreated *Nanog* mutant embryos (Figure 3B′; Messerschmidt and Kemler, 2010; Silva et al., 2009). However, we cannot exclude activation by another RTK receptor. Also, a striking loss of ICM cells occurred in *Nanog* mutants expressing Gata6 but devoid of RTK signaling. Thus,

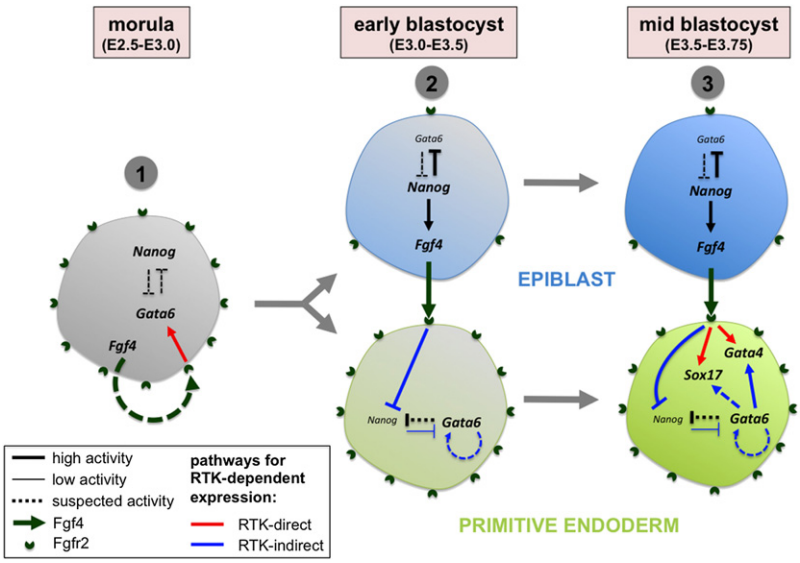

Figure 5. A Three-Step Mechanism for PrE Specification

Gata6 expression is initially induced directly by the RTK pathway (probably by Fgf4, which is present at this stage). From the morula-blastocyst transition, Gata6 expression is maintained only in Nanog-deprived cells through an indirect activation of the RTK pathway. During PrE maturation, Gata6 expression is still maintained indirectly by the RTK pathway. Conversely, Sox17 and Gata4 expression requires direct activation by Fgf4, secreted from epiblast cells. During these steps of PrE differentiation, cells are not synchronized, revealing heterochrony.

the remaining RTK activity in untreated *Nanog* mutants might slightly delay cell death, perhaps until Fgf4 depletion. These results suggest that the presence of Gata6, in the absence of Nanog and p-Erk, leads to cell death. Although the levels of RTK signaling need to be investigated in wild-type and mutant embryos (Figure S4), it is possible that such a mechanism is responsible for the wave of cell death that occurs throughout cell-sorting stages.

Intriguingly, *Fgf4*, *Fgfr2*, *Nanog*, and *Gata6* mRNAs are present at high levels in the same cells from the eight-cell stage (Guo et al., 2010), whereas the onset of mutually exclusive Gata6 and Nanog expression occurs only around E3.25. This reciprocal expression occurs first in a few cells and increases throughout the fourth day postcoitum in the whole ICM (Plusa et al., 2008). Possibly, the maturation from RTK-direct to RTK-indirect Gata6 induction results in more stable expression and allows mechanisms that establish reciprocal expression to operate. Alternatively, it could be a consequence of these mechanisms. In our experiments, some inhibitor-treated mutant cells were already resistant to RTK pathway inhibition at E2.75 (Figure 4D′), suggesting that this heterochronic maturation initiates quite early. However, the absence of Nanog repression might have accelerated the process compared to in wild-type embryos, perhaps distorting the normal timing of events. The onset of mutually exclusive Gata6 and Nanog expression could be a direct outcome of the positive and negative regulative loops influencing expression of Fgf4, Fgfr2, Nanog, and Gata6. Thus, the reciprocal gene expression would arise from fluctuating "noisy" expression (Canham et al., 2010; Chambers et al., 2007; Hayashi et al., 2008; Kalmar et al., 2009) by inducing a bistable cell fate specification, as shown in other organisms (Johnston and Desplan, 2010; Stockholm et al., 2010). A decrease in *Fgf4* expression can be observed between the two-cell stage and the morula stage (Guo et al., 2010) and might initiate differences in RTK activation and, thus, differences in Gata6 versus Nanog expression. Alternatively, the initiation of reciprocal expression could depend on an "external" switch activated by other factors (transcription, growth factors) or from cell-cell interactions as described for the Hippo pathway in ICM/trophoblast determination (Nishioka et al., 2009). Recently, two analyses examined the relative roles of two successive waves of inner cell generation (8- to 16-cell and 16- to 32-cell stages) in influencing cell fate (Lanner and Rossant, 2010; Morris et al., 2010; Yamanaka et al., 2010) and came to opposing conclusions. Posttranscriptional modifications could also play a role in regulating the Nanog/Gata6 balance. It is noteworthy that Gata6/Nanog reciprocal expression seems to begin at the stage when ICM/trophoblast determination has just been accomplished.

Our results reveal a three-step mechanism for PrE differentiation (Figure 5): (1) Gata6 is initially directly activated by Fgf4/RTK signaling from the eight-cell stage (E2.5); (2) between the morula and early blastocyst stage, a subset of cells maintains higher levels of Gata6 and reduces the levels of Nanog, which predisposes them toward the PrE program; Gata6 expression does not require direct activation by RTK and is maintained possibly via an autoregulatory mechanism; and (3) subsequent upregulation of Sox17 and Gata4 then depends on expression of Fgf4 from Nanog-positive epiblast cells to differentiate fully into PrE. The other subset of cells, engaged toward an epiblast identity, downregulates Gata6 expression while maintaining high levels of Nanog. However, cells are only engaged toward a PrE or epiblast identity but not determined yet because administration of RTK inhibitors or Fgf4 can change cell identity until at least E3.75 (Yamanaka et al., 2010).

In conclusion, our multistep model for PrE differentiation highlights the importance of continuously changing and reinforcing interactions between emerging cell types during differentiation. This has particular relevance for controlling the differentiation or maintenance of ESCs in vitro because heterogeneous cell populations would be expected to behave differently than homogeneous populations. A full understanding of the mechanisms regulating epiblast and PrE segregation is also likely to help in developing a new model for bistable cell fate specification.

EXPERIMENTAL PROCEDURES

Experiments were performed in accordance with French and EU guidelines for the care and use of laboratory animals.

Embryo Electroporation and Culture

CD1 outbred mice, kept in a 12 hr light cycle, were used for electroporation experiments. Noon of the vaginal plug was considered as E0.5. Embryos

were obtained from natural matings and flushed from oviducts with M2 (Sigma-Aldrich) at E2.5 before compaction. The zona pellucida was removed with acid Tyrode solution (Sigma-Aldrich), and embryos were electroporated in G2 medium (Vitrolife) buffered with HEPES (20 mM) under the following conditions: four pulses of 30 V and 1.5 ms duration each separated by 100 ms (BTX ECM 830). Embryos were subsequently cultured under paraffin oil in 10 μl drops for 30–45 hr at 37°C, 5% CO_2. A pCX vector (Hadjantonakis et al., 2002) containing H2B-EGFP was electroporated at 50 μg/ml to label electroporated cells with the pSuper vector (OligoEngine) empty (control) or carrying the shRNA sequence 5′-GACAGTGAGGTGCATATACTTCAAGA GAGTATATGCACCTCACTGTC-3′ targeting *Nanog* (Ivanova et al., 2006) at 100 μg/ml.

Cell sorting was analyzed counting Gata6-expressing cells remaining inside and Nanog-expressing cells being at the surface of the ICM. For control embryos: 11.8% ± 2.7% (n = 120) and 6.3% ± 2.1% (n = 130), respectively.

Nanog Mutant Mice

Homozygous embryos for *Nanog*^{tm1Yam} mutation (Mitsui et al., 2003) were produced by natural matings and genotyped after the staining procedures using primer IntAS3 (5′-CAGAATGCAGACAGGTCTACAGCCCG-3′) coupled with either 5′-AATGGGCTGACCGCTTCCTCGTGCTT-3′ for the mutant allele or 5′-GGCCCAGCTGTGTGCACTCAA-3′ for the wild-type allele.

Embryos were staged according to the time of their collection: E2.5 (before compaction), E2.75 (after compaction), E3.0 (before cavitation), E3.25 (early expanding blastocyst), E3.5 (ICM:cavity volume = 1:1), E3.75 (ICM volume less than cavity volume; lineage precursors not fully sorted), E4.0 (sorted ICM; not implanted), E4.5 (implanted; flat PrE epithelium; no parietal endoderm), and E4.75 (beginnings of parietal endoderm migration).

In Vitro Culture and Fgf4 or Inhibitor Treatment

Embryos were flushed in M2 at E2.5, E2.75, or E3.25 and cultured until stages equivalent to E4.0 or E4.5, with zona pellucida intact in G2 medium. For treatments, the Fgf receptor inhibitor PD173074 (Sigma-Aldrich) at 100 nM and Mek inhibitor PD0325901 (Axon MedChem) at 500 nM, or recombinant mouse Fgf4 (5846-F4; R&D Systems) at 1 μg/ml and heparin at 1 μg/ml, were added to equilibrated G2 medium (Nichols et al., 2009; Yamanaka et al., 2010).

In Situ Labeling

Fluorescent in situ hybridization and immunostaining were performed as described previously (Chazaud and Rossant, 2006; Chazaud et al., 2006). Primary antibodies used in this study were: Nanog (21603 [Abcam] and RCAB0002P-F [Cosmo Bio]), Gata6 (AF1700 [R&D Systems]), Sox17 (AF1924 [R&D Systems]), Gata4 (1237 [Santa Cruz Biotechnology]), GFP (13970 [Abcam]), Oct4 (19857 [Abcam]), Pdgfra (AF1062 [R&D Systems]), and Cdx2 (AM392 [BioGenex]). Secondary antibodies coupled with Alexa 488, Cy3, Cy5, and Biotin (Jackson ImmunoResearch) in conjunction with streptavidin-chromeo 494 (Chromeon) as a fourth fluorochrome were used. Nuclei were stained with DAPI (Sigma-Aldrich), and apoptotic cells were labeled using a TUNEL reaction kit (TMR red, 12156792910 [Roche]).

We attempted immunostaining of p-Erk with an antibody from Cell Signaling Technology (#9101) because it was previously described for this stage (Lin et al., 2010; Lu et al., 2008); however, the staining appears to be nonspecific (Figure S4). No signal could be detected with an antibody from Sigma-Aldrich (M-8159; data not shown). Thus, levels of p-Erk are certainly very low and transient.

The embryos were scanned with a Leica SP5 laser confocal microscope and analyzed with ImageJ (NIH). Cell counting was semiautomated with Imaris (Bitplane) software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.devcel.2011.10.019.

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