RESEARCH ARTICLE

Oct4 is required for lineage priming in the developing inner cell mass of the mouse blastocyst

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ABSTRACT

The transcription factor Oct4 is required in vitro for establishment and maintenance of embryonic stem cells and for reprogramming somatic cells to pluripotency. In vivo, it prevents the ectopic differentiation of early embryos into trophoblast. Here, we further explore the role of Oct4 in blastocyst formation and specification of epiblast versus primitive endoderm lineages using conditional genetic deletion. Experiments involving mouse embryos deficient for both maternal and zygotic Oct4 suggest that it is dispensable for zygote formation, early cleavage and activation of Nanog expression. Nanog protein is significantly elevated in the presumptive inner cell mass of Oct4 null embryos, suggesting an unexpected role for Oct4 in attenuating the ability of inner cell mass cells to adopt lineage-specific identity and acquire the molecular profile characteristic of either epiblast or primitive endoderm. Sox17, a marker of primitive endoderm, is not detected following prolonged culture of such embryos, but can be rescued by provision of exogenous FGF4. Interestingly, functional primitive endoderm can be rescued in Oct4-deficient embryos in embryonic stem cell complementation assays, but only if the host embryos are at the pre-blastocyst stage. We conclude that cell fate decisions within the inner cell mass are dependent upon Oct4 and that Oct4 is not cell-autonomous to the initial allocation of these cells to the ICM lineage occurs normally (Nichols et al., 1998; Ralston et al., 2010). The presence of Oct4 protein has been reported in developing oocytes (Schöler et al., 1989) and unfertilised eggs (Palmieri et al., 1994). To eliminate the possibility that lingering maternal Oct4 might facilitate normal gene expression during cleavage, both maternal and zygotic deletion has been performed. Interestingly, early ICM markers such as Nanog and Gata6 still localise to the inside cells in maternal-zygotic Oct4 mutants (Frum et al., 2013; Wu et al., 2013).

INTRODUCTION

A prerequisite for the viviparous development of the mouse embryo is the capacity to generate enveloping tissues to facilitate implantation in the mother, while preserving the potential to produce a fetus. The first extra-embryonic lineage, the trophectoderm, forms the outer layer of the blastocyst. The internal population of cells, termed the inner cell mass (ICM), is protected from differentiation by expression of the POU domain transcription factor Oct4 (also known as Oct3, Oct3/4 and Pou5f1). Following zygotic deletion of Oct4, the blastocyst eventually differentiates into trophectoderm (Nichols et al., 1998). However, expression of trophectoderm markers such as Troma1 (keratin 8) and the homeobox transcription factor Cdx2 is not apparent in the inside cells until after blastocyst formation, suggesting that the initial allocation of these cells to the ICM lineage occurs normally (Nichols et al., 1998; Ralston et al., 2010). The presence of Oct4 protein has been reported in developing oocytes (Schöler et al., 1989) and unfertilised eggs (Palmieri et al., 1994). To eliminate the possibility that lingering maternal Oct4 might facilitate normal gene expression during cleavage, both maternal and zygotic deletion has been performed. Interestingly, early ICM markers such as Nanog and Gata6 still localise to the inside cells in maternal-zygotic Oct4 mutants (Frum et al., 2013; Wu et al., 2013).

After segregation of the trophectoderm, the ICM becomes partitioned into epiblast, which is the founder of the foetus, and primitive endoderm (PrE), or hypoblast, which is the source of the extra-embryonic endoderm lineage. By means of immunohistochemistry, Oct4 protein has been detected in the PrE following its segregation from the epiblast prior to implantation (Palmieri et al., 1994). Intriguingly, the fluorescence appeared to be more intense in the PrE compared with the epiblast. This led to speculation that elevation of Oct4 might be a prerequisite for PrE differentiation. This hypothesis was further endorsed by the observation that transgenic enhancement of Oct4 expression in embryonic stem cells (ESCs) resulted in differentiation accompanied by the expression of markers of extra-embryonic endoderm (Niwa et al., 2000). A requirement for Oct4 in PrE specification in vivo was inferred using maternal and/or zygotic deletion (Frum et al., 2013). However, the conversion of the majority of presumptive ICM into trophectoderm before implantation in embryos lacking Oct4 (Frum et al., 2013; Nichols et al., 1998; Ralston et al., 2010) somewhat compromises the investigation of a role for Oct4 specifically in subsequent PrE differentiation and function.

Embryos lacking fibroblast growth factor (FGF) 4, a target of Oct4 (Nichols et al., 1998; Yuan et al., 1995), fail to generate PrE unless supplemented with excess FGF4 or FGF2 (Feldman et al., 1995; Kang et al., 2013). A role for FGF4 in directing differentiation of PrE has also been elegantly demonstrated by addition of high concentrations of FGF4 to embryos before blastocyst expansion.
(Yamanaka et al., 2010). Provision of FGF4 has also been shown to induce the survival of ICM cells expressing markers of PrE in embryos deficient for Nanog (Frankenberg et al., 2011). Unlike Oct4, Nanog is restricted to a subset of cells in the ICM of expanding blastocysts and subsequently localises to the epiblast before implantation (Chazaud et al., 2006). In Nanog-deficient blastocysts, functional PrE derivatives can be rescued by chimerae complementation with wild-type ESCs (Messerschmidt and Kemler, 2010). Thus, it might be speculated that the role of Nanog in the context of PrE specification is primarily for maintenance of the epiblast as a source of FGF4. Loss of FGF4 in Oct4 mutants might also be expected to result in failure in PrE segregation.

In order to clarify the role of Oct4 in early lineage specification and subsequent development we used a combination of strategies for conditional deletion, ex vivo culture and embryo complementation. Our results reveal hitherto unsuspected activities of Oct4 in the developing mouse embryo.

RESULTS
Oct4 is dispensable for oocyte maturation and the initiation of cleavage

The Cre recombinase system allows efficient recombination at LoxP sites to create null alleles (Blij et al., 2012; de Vries et al., 2004; Sauer and Henderson, 1989). Driving Cre expression from the ZP3 promoter is known to induce recombination during oocyte maturation at the primary follicle stage (Lan et al., 2004), providing a suitable mechanism for assessing the roles of maternally expressed genes during fertilisation and early cleavage (de Vries et al., 2000; Lewandoski et al., 1997). Such a system has been employed to confirm that Cdx2 is dispensable for the initial segregation of trophoderm (Blij et al., 2012). Male ZP3Cre+/- mice (kindly provided by Barbara Knowles, Institute of Medical Biology, Singapore) were intercrossed with female mice heterozygous for Oct4 (Nichols et al., 1998) to generate male progeny carrying the ZP3Cre transgene and a null Oct4 allele, which were then crossed to females in which exons 2 to 5 of both Oct4 alleles (the coding region) were flanked by LoxP sites (floxed). This breeding scheme produced female mice bearing the ZP3Cre transgene and one floxed and one null Oct4 allele (Fig. 1A). These mice were termed ODE for ‘Oct4 deletion in the egg’. In ODE females, Cre-induced recombination of LoxP sites occurs during oocyte maturation. To assess the role of maternally produced Oct4 in fertilisation and the first steps of development, ODE females were mated with wild-type (F1) males. Oocytes depleted of Oct4 could be fertilised by wild-type sperm and gave rise to litters of a size within the normal range containing viable offspring (Fig. 1B). These results suggest that maternal Oct4 is dispensable for oocyte maturation and the reprogramming events that prepare the invading sperm for fusion with the female pronucleus and initiation of cleavage, as described recently (Frum et al., 2013; Wu et al., 2013).
Embryos devoid of Oct4 delineate presumptive ICM but fail to produce PrE

To investigate the potential in vivo role of Oct4 in promoting the activation of Nanog we generated transgenic embryos in which both maternal and zygotic Oct4 are excised. ODE females were mated with male Oct4 heterozygotes (Fig. 1C) (Nichols et al., 1998). Because each ODE female possesses one floxed and one null Oct4 allele, expression of ZP3 CRE during oocyte maturation renders all ovulated oocytes Oct4 negative. It is anticipated that Oct4 null and heterozygous embryos will therefore be generated in equal proportions, as dictated by the sperm genotype. The majority of embryos isolated from ODE females mated by Oct4 heterozygous males at ~3.5 morphologically resembled normal blastocysts. Contrary to previous reports in which RNA interference was employed to reduce Oct4 levels in early embryos (Foygel et al., 2008; Tan et al., 2013), and in concordance with a recent genetic study (Frum et al., 2013), we failed to observe obvious defects in development prior to the blastocyst stage. Therefore, we carefully scrutinised lineage segregation in mutant blastocysts by immunohistochemistry. As can be seen in Fig. 1D, embryos lacking Oct4 exhibited robust expression of Nanog, which was localised to the cells of the putative ICM. Surprisingly, single-cell quantitative immunofluorescence (QIF) revealed that production of Nanog in Oct4 null embryos was higher than in their heterozygous counterparts (Fig. 1G; supplementary material Fig. S1; P=8.058x10^{-6}). We speculate that Oct4 might function as a brake to suppress uncontrolled elevation of Nanog, which could otherwise impede timely exit from pluripotency in response to developmental cues. In consideration of this hypothesis, we examined PrE specification in null ODE embryos.

Expression of Gata6 is normally observed in the majority of ICM cells in early blastocysts, then becomes confined to a subset of cells marking PrE progenitors at the mid-blastocyst stage (Chazaud et al., 2006; Plusa et al., 2008). In embryos lacking Nanog, the number of Gata6-positive cells appears to be elevated (Frankenberg et al., 2011). This provides in vivo evidence in support of the proposed role for Nanog as a PrE antagonist, which was inferred from earlier studies using Nanog null ESCs (Mitsui et al., 2003) and the demonstrable restriction of Nanog expression to the early epiblast (Chambers et al., 2003). By contrast, null ODE early blastocysts exhibited a decrease in Gata6 expression compared with heterozygous controls (Fig. 1E, H; supplementary material Fig. S1; P=7.729x10^{-6}). This reduction in Gata6 might be a consequence of Nanog derepression (Fig. 1D, G), consistent with a role for Oct4 in constraining Nanog levels to permit differentiation in the ICM. Alternatively, or additionally, it might indicate a role for Oct4 in initiating PrE differentiation. Sox17, a marker of more advanced PrE differentiation (Artus et al., 2011), was not apparent above background levels in null ODE embryos, although in heterozygotes clear nuclear fluorescence could be seen in a few ICM cells (Fig. 1F, I).

Sustained Oct4 expression is required for PrE specification

Since it has recently been revealed that at least a proportion of ICM cells remains plastic until the late blastocyst stage immediately before implantation (Grabarek et al., 2012), we reasoned that allowing expression of Oct4 until the late morula/early blastocyst stage would protect the ICM from differentiating into trophectoderm, but enable the subsequent segregation, differentiation and function of PrE to be assessed. Deficiency of PrE markers in embryos following Oct4 deletion at around the early blastocyst stage would imply that Oct4 plays an active role in the specification of this tissue, whereas an increase in PrE at the expense of epiblast would suggest that, as with trophectoderm segregation, Oct4 is required to protect the pluripotent cells from differentiation to this second extra-embryonic tissue.

In order to distinguish between these possibilities, we established a system to execute conditional deletion at the late morula/early blastocyst stage. By mating mice homozygous for an inducible Cre driven by the ROSA26 promoter (R26CreERT2) (Vooijs et al., 2001) to mice homozygous for floxed Oct4, and selecting double-homozygous mice from subsequent heterozygous crosses, we established a line of compound homozygous transgenics termed IOD for ‘inducible Oct4 deletion’. Oct4 could be excised at the morula or early blastocyst stage by administering 4-hydroxytamoxifen (4-OHT) to IOD embryos at the 8-cell stage (Fig. 2A). We confirmed deletion of Oct4 by immunohistochemistry after 24 hours of treatment, when the embryos had acquired ~32 cells (Fig. 2B). Embryos cultured first for 24 hours in the presence of 4-OHT, then a further 24 hours without 4-OHT to attain the equivalent of E4.5, exhibited localisation of Cdx2 to the outer cells and exclusion from the inner cells (Fig. 2C). Embryos undergoing Oct4 zygotic deletion would be expected to exhibit ectopic expression of trophectoderm markers in the inner cells by this stage (Frum et al., 2013; Nichols et al., 1998; Ralston et al., 2010). We were thus confident that our inducible system was appropriate for investigating the role of Oct4 in PrE formation without the complication of premature differentiation of putative ICM cells to trophectoderm.

IOD embryos were isolated at the 8-cell stage and cultured, with or without 4-OHT, for 24 hours, then without 4-OHT for a further 24 hours before fixation and immunohistochemistry. IOD embryos that had not been exposed to 4-OHT exhibited immunoreactivity to antibodies raised against Nanog and Sox17 in a non-overlapping pattern within the ICM, demonstrating that segregation of epiblast and PrE had commenced (Fig. 2D, upper two panels). By contrast, the majority of treated embryos expressed Nanog in most ICM cells, with only occasional Sox17-positive cells (Fig. 2D, lower two panels). The frequency of Sox17-positive cells in treated embryos was significantly lower than in controls (Oct4-deleted mean=1.77±1.88, n=13; control mean=9.6±8.5, n=8; P=0.0001, Student’s t-test; Fig. 3B). The total cell number was not significantly different (Oct4-deleted mean=54±10.1; control mean=56.6±13.3; P=0.6137, Student’s t-test; Fig. 3B). However, the mean number of ICM cells was slightly lower in the treated embryos (Oct4-deleted mean=6.15±5.67; control ICM mean=9.94±3.59; P=0.0219, Student’s t-test; Fig. 3B), which might reflect a slight toxicity of the 4-OHT treatment, but supports the hypothesis that Oct4 is required for PrE specification. We investigated the expression of another PrE marker, Gata4, which appears later than Sox17 (Artus et al., 2011). Whereas untreated IOD embryos exhibited Gata4 staining in 50% of ICM cells (7/14), only 5.6% of ICM cells in treated embryos (2/36) were Gata4 positive (data not shown). These results lend weight to the hypothesis that expression of Oct4 is a prerequisite for segregation of PrE. The few Sox17 and Gata4 double-positive cells observed in treated embryos might be attributed to a more advanced stage of development that had already begun to specify PrE at the time of Oct4 deletion. Alternatively, they might suggest an Oct4-independent mechanism by which the PrE programme may occasionally be initiated. As expected, and in agreement with a previous study (Frum et al., 2013), we failed to detect significant activation of Sox17 in Oct4 null ODE embryos following FGF pathway activation (mean Sox17^+ cells in null embryos=1.22±1.56, n=9; mean Sox17^+ cells in heterozygous embryos=6.875±4.03,
Oct4 is required for lineage segregation in the ICM

The apparent lack of PrE in 4-OHT-treated IOD embryos might be indicative of dysregulation of lineage segregation in ICM cells. To assess the role of Oct4 in initiating lineage-specific molecular programmes, single-cell transcriptional profiling was performed on individual ICM cells disaggregated from IOD embryos flushed from oviducts at the 8-cell stage and cultured for 24 hours, with or without 4-OHT. During normal development at E3.5 epiblast precursors emerge, exhibiting high levels of Nanog and FGF4 and negligible expression of the receptors for platelet derived growth factor (Pdgfrα) and FGF (Fgfr2), while PrE precursors acquire the reciprocal pattern (Guo et al., 2010; Kurimoto et al., 2006). As expected, this reciprocal expression profile was observed in 9/10 ICM cells from the untreated embryos (Fig. 2E). We anticipated that, as a target of Oct4, FGF4 would not be expressed in cells from treated embryos. Accordingly, all (10/10) of the ICM cells from 4-OHT-treated embryos lacked significant expression of FGF4, whereas most (9/10) expressed Fgfr2. Only a small minority of the cells from treated embryos exhibited reciprocal expression of Nanog and Pdgfra, whereas several showed co-expression (Fig. 2E). We thus propose that Oct4 is required to facilitate preparatory differential gene expression in ICM cells prior to overt lineage segregation, potentially by an FGF dependent mechanism.

FGF pathway activation can rescue specification but not differentiation of PrE in Oct4-deleted IOD embryos

Modulation of FGF activity can influence the production of PrE (Yamanaka et al., 2010). Since strong expression of Fgfr2 was detected in the majority of ICM cells from 4-OHT-treated IOD embryos (Fig. 2E), we investigated whether PrE formation could be rescued by provision of the missing ligand. Supplementation of the culture medium with FGF4 (or FGF2) resulted in a robust and significant increase in the number of Sox17-positive cells in 4-OHT-treated IOD embryos (mean Sox17+ cells in FGF2-supplemented deleted embryos=11.17±3.92, n=6; mean Sox17+ cells in non-supplemented deleted embryos=1.77±1.88, n=13; P<0.0001, Student’s t-test; Fig. 3A,B). The highly significant increase in the numbers of Sox17-positive cells seen in IOD embryos treated with FGF2 following Oct4 deletion as compared with non-supplemented deleted embryos contrasts strikingly with the response of maternally and/or zygotically deleted embryos to the provision of FGF2 or FGF4 presented in the previous section and in an alternative study.

Fig. 2. Deletion of Oct4 at the morula/blastocyst stage to investigate specification of PrE. (A) Scheme for inducible deletion of Oct4 in IOD embryos isolated at the 8-cell stage and subsequent culture. (B) Confocal images of IOD embryos. The top row shows an untreated embryo with 36 cells following culture from the 8-cell stage (E2.5) for 24 hours; the bottom row is a 32-cell embryo following treatment with 4-OHT from the 8-cell stage for 24 hours, showing immunofluorescence for Oct4 (red) and DAPI staining (blue). (C) Single confocal section of IOD embryo treated from E2.5 with 4-OHT for 24 hours, then cultured for a further 24 hours without 4-OHT showing Oct4 (white), Nanog (green), Cdx2 (red) and DAPI (blue). (D) Confocal images of two untreated IOD embryos (top two panels) and two 4-OHT-treated embryos (bottom two panels) cultured from the 8-cell stage (E2.5) for 48 hours, showing Oct4 (white), Nanog (green), Sox17 (red) and DAPI (blue). Arrowhead indicates Sox17-positive cells. Mean embryo cell numbers are presented in Fig. 3B. (E) Single-cell gene expression analysis on single ICM cells isolated from IOD embryos with or without 4-OHT treatment from the 8-cell stage (E2.5) for 24 hours. Gene expression values were normalised to Gapdh. Error bars indicate s.d.
(Frum et al., 2013), in which very few Sox17-positive cells were detected upon treatment. Our Oct4 inducible deletion system (IOD) therefore provides an excellent tool for assessing the potential role of Oct4 in PrE specification and function as development progresses.

To ascertain whether the Sox17-positive cells could progress to acquire morphological and behavioural characteristics of PrE derivatives, IOD embryos were cultured with or without 4-OHT and FGF2 and explanted on to gelatin in GMEM supplemented with 20% foetal calf serum and LIF. Following 5 days of culture, outgrowths were assessed for the appearance of cells bearing the morphological characteristics of trophoblast giant cells, ICM clumps and PrE derivatives (Nichols et al., 1998). In non-treated control IOD outgrowths, 6/8 embryos produced sheets of trophoblast giant cells, prominent ICMs and small migratory cells that were assumed to be parietal endoderm (Fig. 3C), whereas 4/4 of the 4-OHT-treated embryos produced sheets of trophoblast giant cells with no discernible ICM or PrE derivatives (Fig. 3D). Although supplementation of deleted IOD embryos with high concentrations of FGF2 produced small lumps of tissue overlying the trophoblast giant cells (8/10; Fig. 3E), these developed neither parietal endoderm migratory behaviour nor morphology, and were assumed to indicate proliferation of the diploid polar trophectoderm in response to FGF2, as reported previously (Nichols et al., 1998). This result implies that, although Oct4-deleted IOD embryos can activate the PrE programme when administered to Oct4-deleted embryos at the blastocyst stage.

To reconcile these hypotheses, we repeated the above experiments, but instead of transferring the embryos to foster mothers they were cultured for another 24 hours and processed for immunohistochemistry. The mean number of Sox17-positive cells per embryo did not differ significantly from that observed in un.injected embryos (Fig. 4B; control mean=9±1.85, n=14; un.injected mean=1.80±1.88, n=13; P=0.8138, Student’s t-test), but was significantly lower than in control undeleted, un.injected embryos (Fig. 4B; control mean=9±1.85, P<0.0001). The lack of significant activation of Sox17 in embryos injected with ESCs strongly suggests that recreation of the physiological environment at the blastocyst stage by supplementation with ESCs is insufficient to activate the PrE programme in the absence of endogenous Oct4. That the majority of the Sox17-positive cells (15/21; data not shown) did not express Gata4 suggests that further differentiation of the PrE lineage requires Oct4 cell-autonomously. This contrasts with the role of Nanog in PrE specification, which has been attributed to survival of the tissue (epiblast) required for secretion of PrE-
inducing ligands such as FGF4 (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010).

We next explored the role of Oct4 in PrE specification by combining induction of Sox17 expression in Oct4-deleted embryos by addition of FGF2 with subsequent provision of ESCs and embryo transfer. Of 12 IOD Oct4-deleted embryos treated with FGF2 prior to ESC injection that implanted in the uterus, none contained any embryonic tissue, whereas undeleted IOD embryos treated with FGF2 prior to injection produced three normal chimaeric embryos from four implantations (Table 1), providing reassurance that the developmental failure of the deleted embryos was likely to be attributable to a requirement for Oct4 in this context, rather than a possible toxicity effect of treatment with excess FGF2.

To summarise the results so far, deletion of Oct4 by addition of 4-OHT at the 8-cell stage produces blastocysts with significantly fewer Sox17-positive cells, but the normal number can be restored by addition of excess FGF2 from the early blastocyst stage (24 hours later). However, providing a substitute epiblast at this stage in the form of ESCs does not provide the environment to facilitate normal developmental progression of the PrE in a chimaeric conceptus.

**Provision of ESCs to early embryos prior to Oct4 deletion activates PrE-specific genes and enables functional differentiation of PrE derivatives**

A final set of experiments was performed to interrogate the developmental potential of Oct4-deleted IOD embryos using a system in which a substitute epiblast could be established in IOD embryos before deletion of Oct4. Thirty-three IOD embryos were each injected with eight H2B Tomato-labelled ESCs at the 8-cell stage. Twenty-two were then incubated with and 11 without 4-OHT for 24 hours. Both groups were cultured in non-supplemented medium for a further 24 hours and processed for immunohistochemistry using antibodies raised against Oct4, Sox17 and Gata4. Surprisingly, in Oct4-deleted IOD embryos we observed expression of both Sox17 and Gata4 in numbers of cells not significantly different from those of control injected embryos (Oct4-deleted mean Sox17 and Gata4 double-positive cells=7.32±2.82; control mean Sox17 and Gata4 double-positive cells=9.09±4.83; P=0.19097, Student’s t-test; Fig. 5A,B). Confirmation of deletion of Oct4 can be seen in the treated host embryos by absence of Oct4 immunoreactivity in cells negative for the reporter (Fig. 5A, top panel).

To ascertain whether the robust activation of PrE-specific genes, including Gata4, would be a prediction of functional differentiation, an additional set of injected embryos was transferred to pseudopregnant recipients and dissected at E7. Of 16 Oct4-deleted uninjected embryos that implanted in the uterus, only two small, abnormal embryonic structures were observed, whereas 23 deleted injected embryos produced 12 chimaeras, of which ten were overtly normal (Fig. 5C) and a further two produced abnormal non-chimaeric embryonic structures (Table 2). The small proportion of abnormal embryos observed from both groups might have arisen as a result of incomplete Oct4 deletion or slight variability of the developmental stage at the time of treatment. However, that the vast majority of uninjected 4-OHT-treated embryos implanted but failed to produce any embryonic tissue, whereas a substantial proportion of the injected 4-OHT-treated embryos produced normal chimaeras, suggests that Oct4 is not essential for the differentiation of functional PrE as long as a suitable supportive environment is provided.

**DISCUSSION**

A role for Oct4 in protecting the embryo from differentiating into trophoderm has been shown previously (Nichols et al., 1998). However, even in the absence of both maternal and zygotic Oct4, ICM identity is at least transiently established, as indicated by activation of Nanog (Fig. 1D,F) (Frum et al., 2013). This intriguing observation suggests that acquisition of ICM identity is not directed by Oct4, as previously proposed (Niwa et al., 2005), but must

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**Table 1. Outcome of dissections at E6.5 of IOD embryos treated as indicated and transferred to pseudopregnant recipient females**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transferred</th>
<th>Implanted</th>
<th>Embryos</th>
<th>Chimaeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 4-OHT +ESCs</td>
<td>22</td>
<td>22</td>
<td>20</td>
<td>18</td>
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<tr>
<td>+4-OHT</td>
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<td>0</td>
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<tr>
<td>+4-OHT +ESCs</td>
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<td>15</td>
<td>0</td>
<td>0</td>
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<tr>
<td>No 4-OHT +FGF +ESCs</td>
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<td>4</td>
<td>3</td>
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</tr>
<tr>
<td>+4-OHT +FGF +ESCs</td>
<td>25</td>
<td>12</td>
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</table>

See Fig. 4.
require some hitherto unknown inducing factor or event. The unexpected elevation in levels of Nanog immunofluorescence detected in \textit{Oct4} null ODE embryos suggests a role for \textit{Oct4} in regulating the accumulation of Nanog, which might have implications for initiating differential expression patterns leading to epiblast and PrE segregation. Sox17 was not detected above background levels in null ODE blastocysts. This might result from failure to activate FGF4, a known target of \textit{Oct4} in the embryo (Nichols et al., 1998), in the absence of which PrE would not be expected to develop (Nichols et al., 2009; Yamanaka et al., 2010). However, we and others observe that provision of FGF2 or FGF4 does not result in significant rescue of Sox17 expression when \textit{Oct4} is deleted at this early stage (Frum et al., 2013).

In order to assess the role of \textit{Oct4} in specification and differentiation of PrE in more detail we generated compound transgenic embryos (IOD) in which deletion of \textit{Oct4} can be induced by application of 4-OHT. Although insignificant expression of Sox17 was observed in IOD embryos exposed to 4-OHT for 24 hours from the 8-cell stage (Fig. 2D), the number of Sox17-positive cells could be restored to normal by provision of FGF2 or FGF4 (Fig. 3A,B). This confirms that in the murine embryo, Sox17 is not dependent upon direct activation by \textit{Oct4}, and that FGF signalling is functional in the absence of \textit{Oct4}. Fgfr2 expression was detected in 9/10 ICM cells randomly selected from IOD embryos 24 hours after 4-OHT administration, but at variable levels (Fig. 2E). In contrast to the untreated IOD ICM cells, expression of Nanog and Pdgfra in \textit{Oct4}-deleted ICMs was not mutually exclusive, suggesting that, in the absence of \textit{Oct4}, lineage segregation is not initiated. Paradoxically, ‘salt and peppering’ of Gata6 and Nanog was observed in ICMs following maternal and/or zygotic deletion of \textit{Oct4} (Frum et al., 2013). Our IOD single-cell qPCR results suggest that sustained expression of \textit{Oct4} beyond the late morula/early blastocyst stage is required to coordinate appropriate divergence of epiblast and PrE lineages. The molecular confusion apparent in ICM cells of \textit{Oct4}-deleted IOD embryos might be a contributory factor in their inability to produce PrE derivatives following FGF pathway activation, either in blastocyst explant cultures (Fig. 3E) or ESC injection chimaeras (Fig. 4B, Table 1).

This latter observation contrasts with results of similar experiments performed in \textit{Nanog} null embryos (Messerschmidt and Kemler, 2010) and highlights the possibility that the functions of these two ‘pluripotency’ factors in the developing epiblast are different, despite the fact that \textit{Oct4} and Nanog frequently operate in partnership to activate target genes (Boyer et al., 2005). Whereas

**Table 2. Outcome of dissections at E7 of IOD or wild-type embryos treated as indicated and transferred to pseudopregnant recipient females**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transferred</th>
<th>Implanted</th>
<th>Normal embryos</th>
<th>Abnormal embryos</th>
<th>Chimaeras</th>
</tr>
</thead>
<tbody>
<tr>
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<td>11</td>
<td>10</td>
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<tr>
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<tr>
<td>+4-OHT (IOD) +ESCs</td>
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<td>23</td>
<td>10</td>
<td>4*</td>
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<tr>
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<td>8</td>
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<td>5</td>
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</tr>
</tbody>
</table>

The bottom line shows the fate of wild-type (WT) embryos treated with 750 μM 4-OHT for 24 hours from the 8-cell stage to test toxicity.

*Two of these abnormal embryos were chimaeric.

See Fig. 5.
Nanog is essential for sequestering a subpopulation of cells for acquisition of epiblast identity (Mitsui et al., 2003; Silva et al., 2009), its role in PrE development is inferred to be the non-cell-autonomous provision of paracrine factors, such as FGFR4 (Frankenberg et al., 2011). Oct4, however, appears to play a more complicated role in the generation and function of PrE that is in some respects cell-autonomous. Consistent with this, it has recently been shown that Oct4 partners Sox17 in the induction of differentiation to endodermal lineages, and is required for differentiation of PrE-like cells from F9 embryonic carcinoma cells in vitro (Aksoy et al., 2013). Thus, the role of Oct4 to safeguard the pluripotent compartment against diversion into extra-embryonic lineages appears to be confined to the first differentiation event during development, i.e. the segregation of ICM from trophectoderm, as shown previously (Nichols et al., 1998) (Fig. 6).

The importance of establishing an instructive embryonic niche in preparation for optimal developmental progression was highlighted by the surprising finding that ESCs can rescue PrE segregation and differentiation in Oct4-deleted IOD embryos if they are donated in the 8-cell stage (Fig. 5). That provision of excess recombinant FGF2 at this stage, followed by ESC injection 24 hours later, is not compatible with PrE rescue provokes the intriguing speculation that PrE differentiation depends upon a hitherto unsuspected chemical phenomenon further.

MATERIALS AND METHODS

Cloning and vector production

A 5.0 kb BamHI-BamHI fragment of the Oct4 genomic locus containing part of exon 1 and the whole of exons 2-5 was subcloned into the BamHI site of pBluescript KS, resulting in pOct4-BamHI. The synthetic LoxP sequences flanked by HindIII and NheI sites was subcloned between HindIII (in exon 1) and XbaI (in intron 1) of pOct4-BamHI, resulting in pOct4-LoxP-XbaI. A 2.4 kb HindIII (in exon 1)-XbaI (in intron 1) fragment of pOct4-BamHI and a 4.0 kb XbaI-HindIII (with partial fill in by Ag nucleotides) fragment downstream of exon 5 of pOct4-IREsbgGEOKO vector was subcloned between HindIII and XbaI (with partial fill in by TC nucleotides) of pBluescript KS, resulting in pOct4 5′−3′. A 5.0 kb HindIII fragment containing the LoxP sequence in intron 1 and exons 2-5 was subcloned into the XbaI site of pOct4 5′−3′; then the SacII (in the multicloning site flanked by the 5′-end of the genome)-NsiI (in exon 5) fragment and the 2.1 kb NsiI-EcoRI fragment downstream of exon 5 were inserted into SacII-EcoRI of pUC19, resulting in pOct4-LoxP-SacII-EcoRI. Finally, the XbaI fragment of Lox4-ko-locx containing HSV-Δk and PGK-neo-pA (kindly provided by Andrew Smith, University of Edinburgh, UK) was inserted into XbaI downstream of exon 5, resulting in pOct4-LoxP KO.

Mice and embryos

Experiments were performed in accordance with EU guidelines for the care and use of laboratory animals, and under the authority of appropriate UK governmental legislation. Use of animals in this project was approved by the ethical review committee for the University of Cambridge and relevant Home Office licences are in place. The majority of mice used in this study were transgenic strains with mixed genetic backgrounds. They were: Oct4+/− (Nichols et al., 1998), ZP3CreTg+ (Lewandoski et al., 1997), R26CreERT2 (Vooijs et al., 2001) and Oct4LoxPflox. Correctly targeted ESC clones were confirmed by Southern blot analysis and transgenic mice were generated via germline transmission through chimaeras. Compound transgenic mice were generated from crosses of these lines.

Genotyping was performed by PCR analysis using DNA extracted from ear biopsies. Primer sequences (5′-3′) are as follows: Oct4LoxP, CTCAAACCCCAGGTGATCTTCAAAAC and GGATCCCATGCCCTCTTCCTGGT; Oct4 null, GCCCTCTTCTAGTTGGGTCACACCC, GGCGTGACCGCTCTCGTGTCTTACGC and GAGGATGTGACCTACTCTTCTGC; Oct4LoxP recombined, ACTGAGAAGAAGGCAGCCTTAGC and GGATCCCATGCCCTCTTCTGGT; Cre transgene, GGCGTGCTGGCCGCTAAACTAGC and GTGAAACAGCCTGTGC.
Embryo explant culture

Embryos of compound homozygous transgenics, homozygous for both R26CreER(T2 and Oct4 dn3aP/LnaP), termed IOD for 'inducible Oct4 deletion' were flushed from oviducts at E2.5, cultured for 24 hours, with or without 750 nM 4-OHT and with or without 1 μg/ml FGF2. Zona pellucidae were removed by brief incubation in acid tyrodes solution (Sigma) and the blastocysts explanted on to gelatin in GMEM (Sigma) supplemented with 20% foetal calf serum (Gibco), LIF (made in house) and FGF2 for 5 days.

Chimera analysis

IOD embryos were harvested and treated as above. Some were then un.injected to recipient females at 2.5 days of pseudopregnancy following mating with vasectomised males. Others were injected with ESCs, derived in house from Tg(cag-dsred*mst) embryos using 2i+LIF, as described previously (Ying et al., 2008), or H2B-Tomato, kindly donated by Joshua Brickman (University of Copenhagen, Denmark), either at the 8-cell or blastocyst stage, with or without treatments. For some experiments, chimaeras were matured in vitro for a few days then fixed, processed for immunohistochemistry and examined by confocal microscopy. Decidual swellings from transferred embryos were dissected from recipient uteri at E6.5-7.0 and carefully analysed for the presence of embryos. Chimaerism was assessed by detection of red fluorescence.

Immunohistochemistry

Embryos were prepared for immunohistochemistry as previously described (Nichols et al., 2009). Primary antibodies used were: anti-Nanog (eBiosciences 14-5761, 1:100), anti-Oct4 (Santa Cruz 5279, 1:100), anti-Sox17 (R&D Systems AF1924, 1:200), anti-Gata6 (Santa Cruz AF1700, 1:200), anti-Gata4 (Santa Cruz SC1237 or SC9053, 1:200) and anti-Cdx2 (Biogenex CDX2-88, 1:200).

Imaging and image analysis

Images were acquired using either a Leica TCS SP5 or Zeiss 700 confocal microscope using a Plan-Apochromat 63×/1.40 objective, with optical section thickness of 2 μm. All images subsequently used for fluorescence quantification were obtained using the sequential scanning mode, under the same conditions of laser intensity, gain and pinhole, and were processed in exactly the same way. An automated image-processing workflow based on scale-space Hessian analysis and geodesic distance transformation was designed for parallelized segmentation of multiple cell nuclei. The segmentation result was used for quantification of the immunofluorescence data. The frontend software Modular Interactive Nuclei Segmentation (MINIS; X.L., M. Kang, P. Xenopoulos, S.M.-D. and A.-K.H., unpublished) is a MATLAB-based graphical user interface that performs basic file loading, image transformation and also has advanced features such as nuclei segmentation and classification (e.g. multiple embryo recognition and trophoderm versus ICM classification). The core algorithms were implemented in C++ for better computational efficiency and parallelized in shared memory workstations. As part of pre-processing, background levels were subtracted, defined by the mean fluorescence intensity of a line plotted along the background of the maximum projected z-stack image and measured using ImageJ. The fluorescence intensity from each cell was normalised against the median of the cell population from all embryos imaged within the same confocal session. To assess significance of protein level differences between populations we performed Anova among all pairs of populations (P-values are shown in supplementary material Fig. S1). Paleontological statistics (PAST) data analysis was used for this analysis (Hammer et al., 2001).

Single-cell analysis

ICMs were isolated from IOD blastocysts following 24 hours of culture in KSOM, with or without 4-OHT, by immunosurgery (Sotter and Knowles, 1975). Single cells were isolated by trypsinisation. Generation of cDNAs and subsequent amplification were performed as described (Tang et al., 2010). The following TaqMan probes were used: Pou5f1, Mm003053917_g1; Nanog, Mm02019550_s1; Pdgfrα, Mm00440701_m1; Fgf4, Mm00438916_g1; Fgf2, Mm01269930_m1.

Acknowledgements

We thank Sam Jameson and staff for animal husbandry; Peter Humphreys for assistance with imaging; András Nagy, Anton Berns and Barbara Knowles for provision of transgenic mouse strains; Josh Brickman for Tomato ESCs; and Alfonso Martinez-Arias, Paul Bertone, Thorsten Boroviak, Kevin Chalut, Pau Rue, Christian Schroeter and Austin Smith for helpful discussion.

Competing interests

The authors declare no competing financial interests.

Author contributions

G.C.L.B., S.M.-D., A.K., H.L. and N.G. carried out experiments and data analysis, X.L. devised the quantification of immunofluorescence, W.M. and C.E.-D. performed microinjection and surgery, C.M. performed data analysis, H.N. designed and generated fixed fixed Oct4 ESCs, A.-K.H. provided discussion and diagrams, and J.N. conceived the project, performed experiments and data analysis and wrote the manuscript.

Funding

This work was supported by the Wellcome Trust and the Medical Research Council [079249/Z/06/Z, 091484/Z/10/Z and 097922/Z/11/Z to G.C.L.B, A.K, H.L, W.M. C.E.-D, J.N.]; the US National Institutes of Health [RO1-HD052115 and RO1-DK084391 to A.-K.H, S.M.-D, X.L]; and the University of Cambridge. Deposited in PMC for immediate release.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096875/-/DC1

References


