

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

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 level of Nanog, which might be significant for priming differentiation during epiblast maturation. Induced deletion of Oct4 during the morula to blastocyst transition disrupts 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-autonomously required for the differentiation of primitive endoderm derivatives, as long as an appropriate developmental environment is established.

KEY WORDS: Blastocyst, Chimaera, Nanog, Oct4 (Pou5f1), Primitive endoderm, Sox17

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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 foetus. The first extra-embryonic lineage, the trophoblast, 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 trophoblast (Nichols et al., 1998). However, expression of trophoblast 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 trophoblast, 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 trophoblast 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 chimaera 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 trophoblast (Blij et al., 2012). Male *ZP3Cre^{Tg/+}* 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).

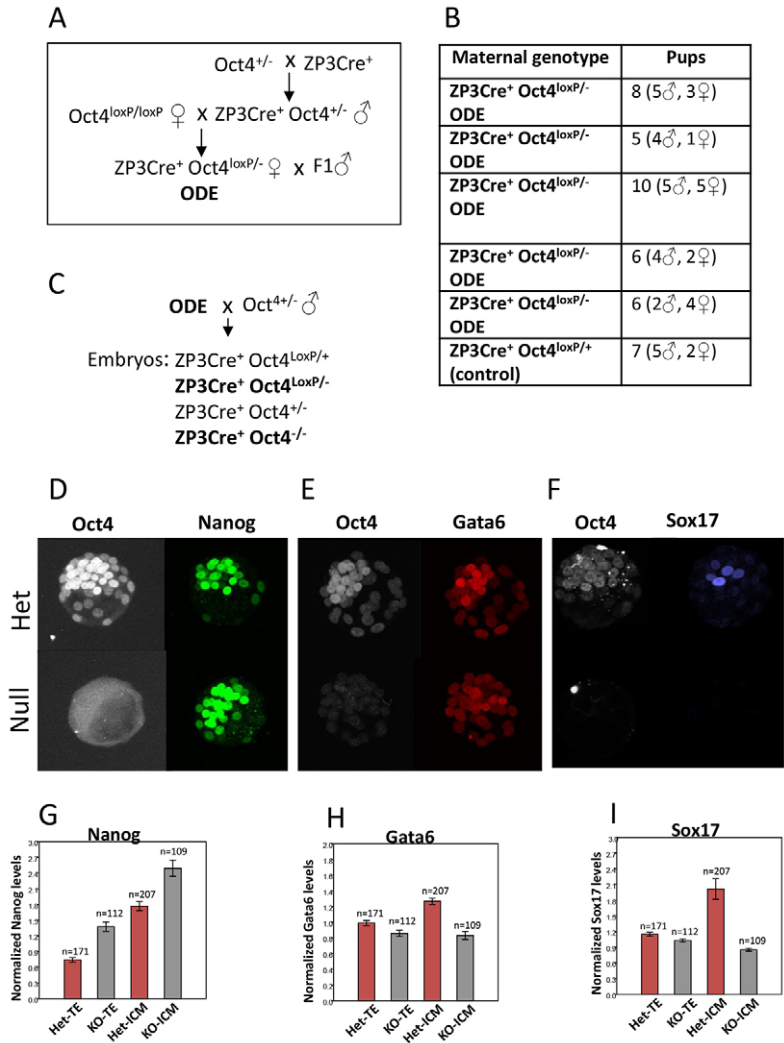


Fig. 1. Consequences of deleting Oct4 in the developing oocyte and zygote. (A) Breeding scheme for the generation of female mice in which *Oct4* is deleted during oocyte maturation by recombination of LoxP sites in one *Oct4* allele mediated by Cre recombinase driven by the *ZP3* promoter. These females are termed ODE for ‘*Oct4* deletion in the egg’. (B) The number of offspring produced from ODE females following mating with F1 males, as compared with a control female from the same breeding programme but carrying a wild-type *Oct4* allele. (C) Scheme to illustrate genotypes of embryos generated by mating ODE females with males heterozygous for *Oct4*. (D-F) Example confocal images, maximum projection of *Oct4*^{+/-} heterozygote (Het, top) and *Oct4* maternal-zygotic mutant embryos (Null, bottom) at E3.5 stained for Oct4 (white) and (D) Nanog (green), (E) Gata6 (red) or (F) Sox17 (blue). All embryos contained 34–58 cells. (G–I) Mean Nanog (G), Gata6 (H) and Sox17 (I) levels in trophoblast (TE) or ICM cells obtained by QIF analysis from *Oct4*^{+/-} (Het, red bars) and *Oct4* maternal-zygotic mutant embryos (KO, grey bars). Single-cell fluorescence levels were quantified for seven *Oct4*^{+/-} and five *Oct4* maternal-zygotic mutant embryos. Error bars indicate s.e. The raw data are presented in supplementary material Fig. S1.

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 *ZP3Cre* 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 ~E3.5 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; $P=8.058 \times 10^{-6}$). 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; $P=7.729 \times 10^{-6}$). 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 *ROS426* 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±1.85, $n=8$; $P<0.0001$, Student's *t*-test; Fig. 3B). The total cell number was not significantly different (*Oct4*-deleted mean=54±10.1; control mean=56.62±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 ICM 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,

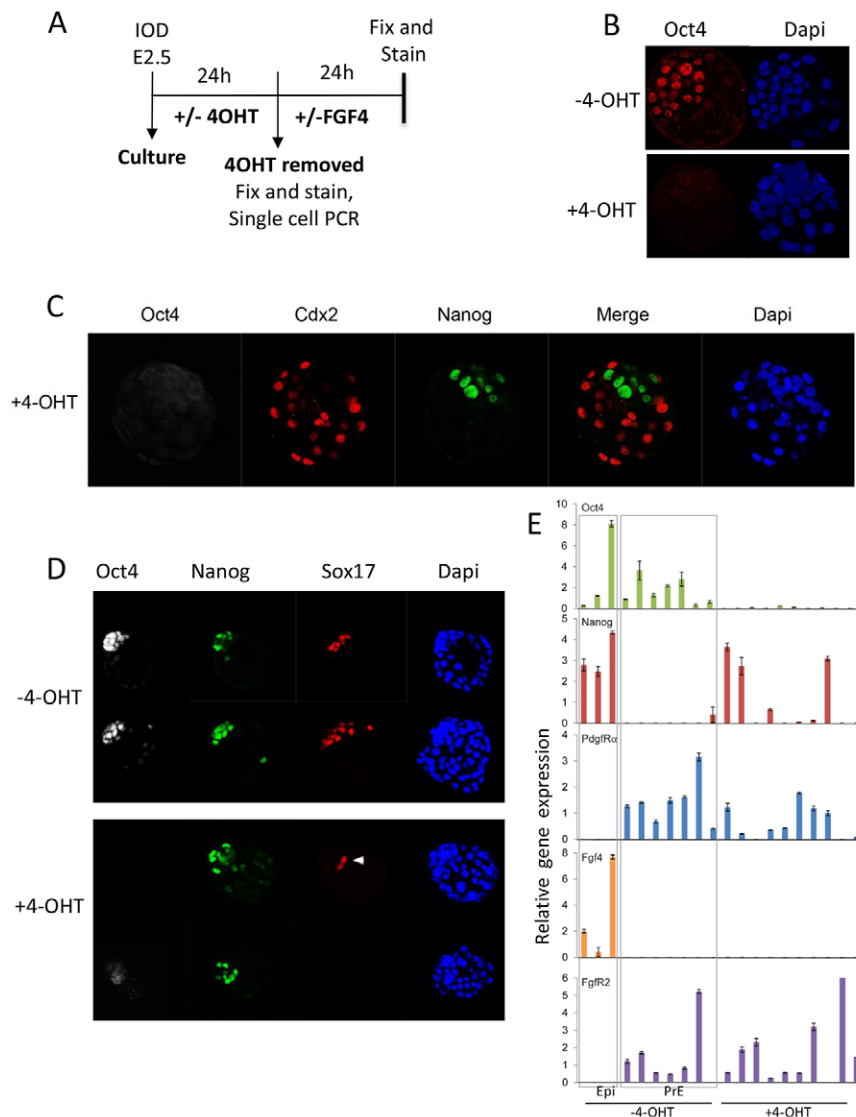


Fig. 2. Deletion of *Oct4* at the morula/blastocyst stage to investigate specification of PrE. (A) Scheme for inducible deletion of *Oct4* in IOD embryos isolated at the 8-cell stage and subsequent culture. (B) Confocal images of IOD embryos. The top row shows an untreated embryo with 36 cells following 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.

$n=16$; $P=5.5 \times 10^{-4}$, Student's *t*-test). Together, these results suggest that sustained Oct4 is required for PrE specification.

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 (*Pdgfra*) 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

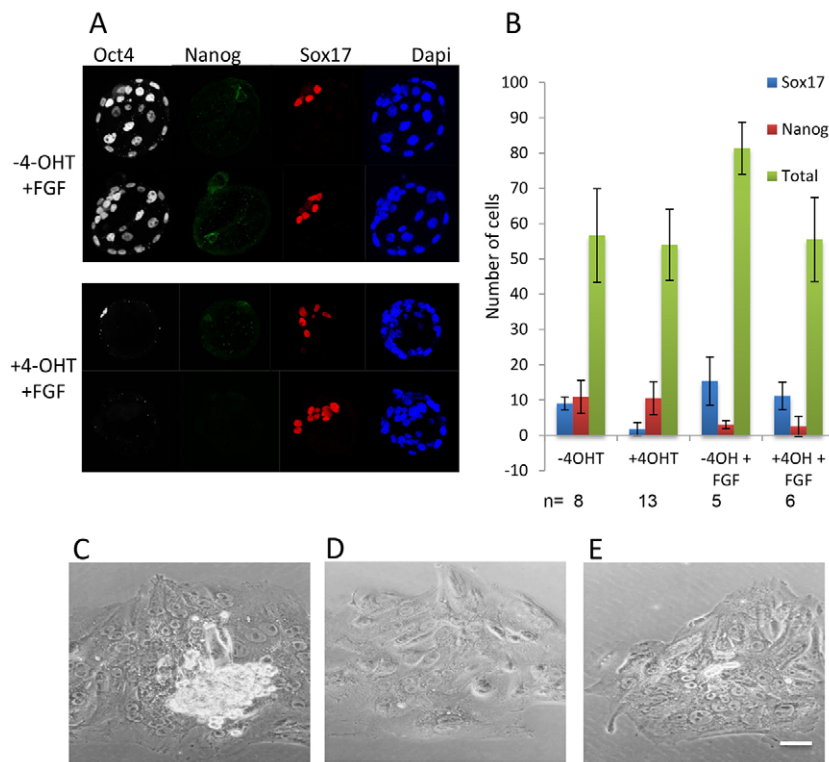


Fig. 3. Effect of addition of excess FGF2 or FGF4 to *Oct4*-deleted IOD embryos. (A) Confocal images of two untreated IOD embryos (top two panels) and two 4-OHT-treated embryos (bottom two panels) cultured from the 8-cell stage (E2.5) for 48 hours, as indicated in Fig. 2A, supplemented with 1 mg/ml FGF4 (or FGF2) from 24–48 hours showing Oct4 (white), Nanog (green), Sox17 (red) and DAPI (blue). (B) Average numbers of cells expressing Sox17, Nanog and total cell number. The number of embryos used for each data point is indicated beneath the x-axis. Error bars represent s.d. (C–E) Phase contrast images of whole blastocyst outgrowths from IOD embryos following 5 days in culture after (C) culture for 24 hours from the 8-cell stage with treatment, (D) 24 hours with 4-OHT, (E) 24 hours with 4-OHT and FGF2. Scale bar: 50 μ m.

(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 trophoderm in response to FGF2, as reported previously (Nichols et al., 1998). This result implies that, although *Oct4*-deleted IOD embryos can activate the PrE transcriptome in response to stimulation of FGF signalling, Oct4 expression is required for overt differentiation of PrE derivatives.

To probe this possible conclusion further in a more physiologically relevant environment, we performed chimaera assays. It has been reported that *Nanog* null embryos at the peri-implantation stage (E4.5) exhibit reduced numbers of Sox17-positive cells compared with wild types (Frankenberg et al., 2011). However, PrE function can be rescued by provision of ESCs in blastocyst injection chimaeras (Messerschmidt and Kemler, 2010).

We attempted a similar assay. IOD embryos were isolated at the 8-cell stage, incubated for 24 hours with or without 4-OHT, and 15–20 dsRed-labelled ESCs were injected into some of the resulting blastocysts. Embryos were then transferred to pseudopregnant recipients and subsequently dissected at E6.5. Untreated IOD embryos injected with ESCs produced 11 decidual swellings, of which ten contained morphologically normal chimaeras (Fig. 4A, Table 1). No embryonic or extra-embryonic tissue, however, could be detected in 15/15 decidua from the treated injected embryos, suggesting that the injected ESCs were unable to induce PrE in host embryos (Table 1). We conclude that *Oct4*-deleted blastocysts are unable to produce functional PrE derivatives in response to paracrine signals from ESCs, suggesting either that Oct4 is required cell-autonomously for PrE differentiation or that the ESCs produce insufficient FGF4 to initiate the PrE developmental 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 uninjected embryos (Fig. 4B; injected mean = 1.56 ± 2.63 , $n=14$; uninjected mean = 1.80 ± 1.88 , $n=13$; $P=0.8138$, Student's *t*-test), but was significantly lower than in control undeleted, uninjected 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-

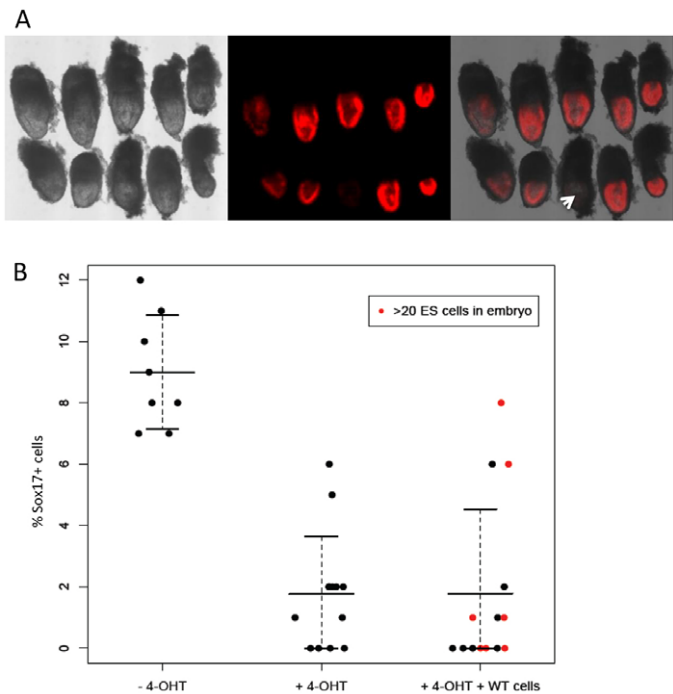


Fig. 4. IOD blastocyst complementation with ESCs. (A) Digital microscopy images of embryos dissected at E6.5 following injection of ESCs into untreated IOD embryos cultured from E2.5 for 24 hours. Images (left to right): brightfield, fluorescence, merge. The level of chimaerism is low but detectable in the embryo indicated by an arrow. Scale bar: 200 μ m. (B) Percentage Sox17⁺ cells in control IOD embryos cultured from the 8-cell stage for 48 hours (–4-OHT), IOD embryos cultured with 4-OHT from the 8-cell stage for 24 hours then without 4-OHT for a further 24 hours (+4-OHT) and IOD embryos cultured with 4-OHT from the 8-cell stage for 24 hours then injected with 15–20 Tg(cag-dsred^{*}mst) ESCs each, and cultured for a further 24 hours prior to immunohistochemistry. Each dot represents an embryo. Red dots represent embryos in which more than 20 ESCs could be identified.

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 \pm 2.82; control mean Sox17 and Gata4 double-positive cells=9.09 \pm 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 trophectoderm 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

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

Treatment	Transferred	Implanted	Embryos	Chimaeras
No 4-OHT +ESCs	22	22	20	18
+4-OHT	23	21	0	0
+4-OHT +ESCs	20	15	0	0
No 4-OHT +FGF +ESCs	8	4	3	3
+4-OHT +FGF +ESCs	25	12	0	0

See Fig. 4.

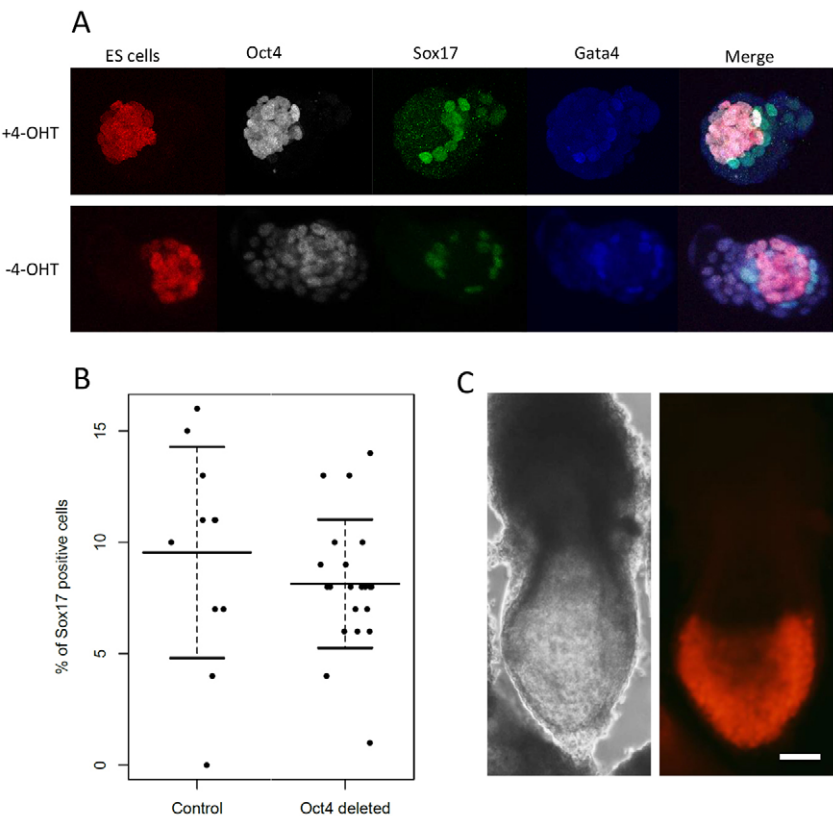


Fig. 5. Provision of ESCs to IOD embryos prior to *Oct4* deletion. (A) Maximum projection confocal images of embryos injected with eight H2B Tomato ESCs each at the 8-cell stage, followed by culture with 4-OHT for 24 hours, then without for a further 24 (top panel) or 48 (bottom panel) hours prior to immunohistochemistry for Oct4 (white), Sox17 (green) or Gata4 (blue). The red channel shows H2B Tomato from the injected ESCs. (B) Percentage of Sox17⁺ cells. Each dot represents an embryo. (C) Representative digital micrographs of a chimaeric E7 egg cylinder generated from an *Oct4*-deleted IOD embryo injected with ESCs. Left, brightfield; right, fluorescence. Scale bar: 100 μ m.

require some hitherto unknown inducing factor or event. The unexpected elevation in levels of Nanog immunofluorescence detected in *Oct4* null ODE embryos suggests a role for 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 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 *Oct4* is deleted at this early stage (Frum et al., 2013).

In order to assess the role of Oct4 in specification and differentiation of PrE in more detail we generated compound transgenic embryos (IOD) in which deletion of *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 Oct4, and that FGF

signalling is functional in the absence of 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 *Oct4*-deleted ICMs was not mutually exclusive, suggesting that, in the absence of 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 *Oct4* (Frum et al., 2013). Our IOD single-cell qPCR results suggest that sustained expression of *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 *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 *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 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

Treatment	Transferred	Implanted	Normal embryos	Abnormal embryos	Chimaeras
No 4-OHT +ESCs	18	11	10	0	8
+4-OHT (IOD)	18	16	0	2	0
+4-OHT (IOD) +ESCs	30	23	10	4*	12
+4-OHT (WT)	8	8	5	0	0

The bottom line shows the fate of wild-type (WT) embryos treated with 750 μ M 4-OHT for 24 hours from the 8-cell stage to test toxicity.
*Two of these abnormal embryos were chimaeric.
See Fig. 5.

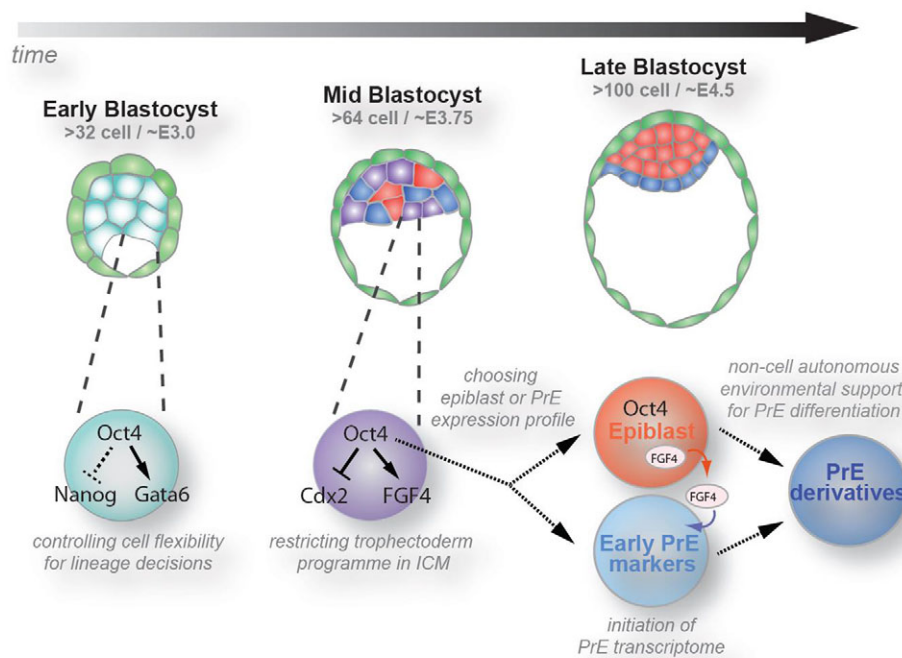


Fig. 6. Model of Oct4 functions during preimplantation development. As shown in Fig. 1, levels of Nanog protein are higher when Oct4 is subject to maternal-zygotic deletion, implying that Oct4 plays a role in restricting Nanog to maintain flexibility for formation of PrE. It is known from previous work (Nichols et al., 1998; Niwa et al., 2005) that Oct4 is required to suppress Cdx2 and trophectoderm differentiation in the mid-late ICM. Single-cell expression analysis (Fig. 2C) suggests that sustained expression of Oct4 beyond the 8-cell stage is required to enable the segregation of molecular programmes for either epiblast or PrE. However, expression of Sox17 can be induced by addition of excess FGF2 or FGF4, and overt differentiation of PrE derivatives is possible in cells from which Oct4 was conditionally deleted from the morula stage following ESC complementation from the 8-cell stage.

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 FGF4 (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 at 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 factor or a specific physical interaction facilitated by the presence of epiblast-like cells within the embryo. Our Oct4-deletable IOD embryos provide an ideal system with which to investigate this phenomenon further.

MATERIALS AND METHODS

Cloning and vector production

A 5.0 kb *Bam*HI-*Bam*HI fragment of the Oct4 genomic locus containing part of exon 1 and the whole of exons 2-5 was subcloned into the *Bam*HI site of pBluescript KS, resulting in pOct4-BamHI. The synthetic LoxP sequences flanked by *Hind*III and *Nhe*I sites was subcloned between *Hind*III (in exon 1) and *Xba*I (in intron 1) of pOct4-BamHI, resulting in pOct4-LoxP-XbaI. A 2.4 kb *Hind*III (in exon 1)-*Xba*I (in intron 1) fragment of pOct4-BamHI and a 4.0 kb *Xba*I-*Hind*III (with partial fill in by AG nucleotides) fragment downstream of exon 5 of pOct4-IRESbgeoKO vector was subcloned between *Hind*III and *Xba*I (with partial fill in by TC nucleotides) of

pBluescript KS, resulting in pOct4 5'+3'. A 5.0 kb *Hind*III fragment containing the LoxP sequence in intron 1 and exons 2-5 was subcloned into the *Xba*I site of pOct4 5'+3', then the *Sal*I (in the multicloning site flanked by the 5'-end of the genome)-*Nsi*I (in exon 5) fragment and the 2.1 kb *Nsi*I-*Eco*RI fragment downstream of exon 5 were inserted into *Sal*I-*Eco*RI of pUC19, resulting in pOct4-LoxP-SalI-EcoRI. Finally, the *Xba*I fragment of Lox-tk-neo-lox containing HSV-tk and PGK-neo-pA (kindly provided by Andrew Smith, University of Edinburgh, UK) was inserted into *Xba*I downstream of exon 5, resulting in pOct4-3LoxP 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), ZP3Cre^{Tg/+} (Lewandoski et al., 1997), R26CreERT2 (Vooijs et al., 2001) and Oct4^{LoxP/LoxP}. 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, CTCA-AACCCAGGTGATCTTCAAAC and GGATCCCATGCCCTCTTC-TGGT; Oct4 null, GCCTTCCTCTATAGTTGGGCTCCAACC, GGGC-TGACCGTTCCTCGTCTTTACG and GAGCTTATGATCTGATGT-CCATCTCTGTGC; Oct4LoxP recombined, ACTGAGAAGAAGGCAG-CCTTAGC and GGATCCCATGCCCTCTTCTGGT; Cre transgene, GCGGTCTGGCAGTAAAACTATC and GTGAAACAGCATTGCTG-TCACTT.

Wild-type F1 mice generated from C57BL/6 × CBA crosses were used occasionally. Mice were maintained by in-house breeding on a lighting regime of 14 hours light and 10 hours darkness with food and water supplied *ad libitum*. Embryos were generated by natural mating. Detection of a copulation plug confirmed successful mating; the resulting embryos were then considered to be embryonic day (E) 0.5. Embryos were isolated in M2 medium (Quinn et al., 1982), made in house and cultured in KSOM (Millipore) or Blastassist (Origio) from E2.5 to E3.5, and in N2B27 (Ying et al., 2003) thereafter. Supplements included FGF4 (1 µg/ml; R&D Systems), FGF2 (1 µg/ml; made in house), 4-OHT (250-750 nM, depending on the batch in use; Sigma).

Embryo explant culture

Embryos of compound homozygous transgenics, homozygous for both R26CreERT2 and *Oct4^{LoxP/LoxP}*, 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.

Chimaera analysis

IOD embryos were harvested and treated as above. Some were then transferred uninjected 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 (MINS; 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 trophectoderm 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 (Solter 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, Mm03053917_g1; Nanog, Mm02019550_s1; Pdgfra, Mm00440701_m1; Fgf4, Mm00438916_g1; Fgfr2, Mm01269930_m1.

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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 floxed *Oct4* ESCs, A.-K.H. provided discussion and diagrams, and J.N. conceived the project, performed experiments and data analysis and wrote the manuscript.

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Supplementary material

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

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