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Differential plasticity of epiblast and primitive endoderm precursors within the ICM of the early mouse embryo

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SUMMARY

Cell differentiation during pre-implantation mammalian development involves the formation of two extra-embryonic lineages: trophoblast and primitive endoderm (PrE). A subset of cells within the inner cell mass (ICM) of the blastocyst does not respond to differentiation signals and forms the pluripotent epiblast, which gives rise to all of the tissues in the adult body. How this group of cells is set aside remains unknown. Recent studies documented distinct sequential phases of marker expression during the segregation of epiblast and PrE within the ICM. However, the connection between marker expression and lineage commitment remains unclear. Using a fluorescent reporter for PrE, we investigated the plasticity of epiblast and PrE precursors. Our observations reveal that loss of plasticity does not coincide directly with lineage restriction of epiblast and PrE markers, but rather with exclusion of the pluripotency marker Oct4 from the PrE. We note that individual ICM cells can contribute to all three lineages of the blastocyst until peri-implantation. However, epiblast precursors exhibit less plasticity than precursors of PrE, probably owing to differences in responsiveness to extracellular signalling. We therefore propose that the early embryo environment restricts the fate choice of epiblast but not PrE precursors, thus ensuring the formation and preservation of the pluripotent foetal lineage.

KEY WORDS: Lineage specification, Mouse blastocyst, ICM, Primitive endoderm, Epiblast, Pdgfra, Pluripotency, Cell plasticity

INTRODUCTION

Pre-implantation mammalian development is primarily dedicated to the allocation of two extra-embryonic lineages: trophoblast and PrE. Signalling pathways operating in the early mammalian embryo direct differentiation and segregation of extra-embryonic lineages (Chazaud et al., 2006; Nishioka et al., 2008). However, a subset of cells within the embryo must remain in an undifferentiated state to ensure formation of the pluripotent epiblast, which gives rise to tissues of the future body and is also the founder lineage for embryonic stem (ES) cells (Chazaud et al., 2006; Nishioka et al., 2008). Emergence of a pluripotent cell population is a crucial event in mammalian development, underscoring our understanding of the origin of pluripotency. Nonetheless, how this allocation is achieved remains unsolved.

The first extra-embryonic lineage, the trophoblast, is specified by differential positional signals between inner and outer cells of the morula, acting via the Hippo pathway, that induce epithelialisation in the outer layer of cells (reviewed by Sasaki, 2010). Cavitation of the morula results in the formation of the blastocyst, where the trophoblast surrounds the remaining inner cells (inner cell mass, ICM) and the blastocyst cavity. Maintenance of the trophoblast depends on lineage-specific transcription factors, most notably Cdx2 and Gata3 (Chawengsaksophak et al., 2004; Home et al., 2009; Ralston et al., 2010; Ralston and Rossant, 2008; Strumpf et al., 2005). From the morula to mid blastocyst stage (up to ~64 cells) different lineage-specific transcription factors are co-expressed. In embryos with more than 64 cells, two cell populations can be distinguished within the ICM. One starts to exclusively express markers of PrE, such as Gata4, Gata6 and Pdgfra, whereas the other expresses markers of epiblast, such as Nanog and Sox2 (Chazaud et al., 2006; Guo et al., 2010; Kurimoto et al., 2006; Plusa et al., 2008). Initially, these two populations of cells are positioned in an apparently salt-and-pepper manner within the ICM. Only later (embryos with more than ~100 cells) do they become segregated to their respective layers by mechanisms involving random cell movements, positional signals and selective apoptosis (Plusa et al., 2008; Meilhac et al., 2009; Morris et al., 2010; Yamanaka et al., 2010).

Experiments using embryos carrying mutations in components of the FGF/Erk signalling pathway, such as *Fgf4*, *Fgfr2* and *Grb2* (Arman et al., 1998; Chazaud et al., 2006; Cheng et al., 1998; Feldman et al., 1995), and pharmacological alterations of FGF/Erk signalling (Guo et al., 2010; Nichols et al., 2009; Yamanaka et al., 2010) have shown that activation of this pathway is necessary for correct specification of PrE. By analogy, blocking the FGF/Erk pathway also has a strong tendency to reduce the differentiation of ES cells. Although these recent studies detail spatially and temporally the events leading to PrE and epiblast formation, they do not reveal how changes in gene expression and cell position correspond to lineage commitment and cell plasticity. Here, we define plasticity as distinct from potency: while potency describes the repertoire of potential fates of a cell that can be revealed in appropriate environment (Slack, 1991), plasticity describes the relative ease with which a cell can switch between these fates.

It is not clear whether the early, overlapping expression of PrE- and epiblast-specific markers represents a period when cells retain high plasticity, and whether the mutually exclusive expression in

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later stages represents lineage commitment. In addition, it remains unknown how apparently identical cells of the ICM acquire the differential response to FGF/Erk that establishes the PrE and epiblast lineages. Furthermore, it is unknown when each lineage becomes finally committed and what molecular events can be linked to complete loss of cell plasticity within the ICM.

Observations of cell behaviour within intact embryos allow investigations of cell fate but do not reveal whether this behaviour is due to the influence of the embryonic micro-environment (e.g. proximity to blastocyst cavity or trophoblast) or to the existence of intrinsic/functional differences in cell potency between different populations of cells within the ICM. A classical test of these properties is to change the position and the environment of a cell. If this alteration does not result in a change of fate, the cell can be said to be committed. This assay can be used to assess the developmental potency of different populations of ICM cells.

Therefore, we selectively isolated epiblast and PrE precursors from blastocysts and transferred them to recipient morulae. Epiblast and PrE precursors were defined on the basis of the absence or presence of histone H2B-GFP reporter, expressed from the *Pdgfra* locus (*Pdgfra*$^{H2B\text{-}GFP}$) (Hamilton et al., 2003), at defined stages of blastocyst development (Plusa et al., 2008). Using this experimental design, we investigated whether epiblast and PrE precursors differ in their developmental plasticity, and at what stage these differences were first apparent.

Our results reveal that ICM cells differ in their developmental plasticity by early stages of blastocyst formation, prior to establishment of a mutually exclusive pattern of epiblast- and PrE-specific marker expression. Unexpectedly, we noted that the developmental plasticity of pluripotent epiblast precursors appears more restricted compared with that of PrE precursors, which have a more restricted fate during later development. Furthermore, we found that the developmental plasticity of transplanted epiblast and PrE precursors could be modified by manipulation of FGF signalling, suggesting that the early embryo microenvironment limits the fate choices of epiblast precursors, with PrE precursors maintaining greater plasticity.

Based on our findings, we propose that in the early blastocyst a group of ICM cells becomes sheltered from the differentiation signals present in the embryo micro-environment by virtue of their position and low responsiveness to differentiation signals, which ensures allocation of a pluripotent population. The remaining ICM cells (Pdgfra-positive) are able to contribute to all three lineages of the blastocyst.

MATERIALS AND METHODS

Embryo collection

Embryos were collected from natural matings. *Pdgfra*$^{H2B\text{-}GFP}$ (Hamilton et al., 2003), *CAG:mRFP1*$^{Tg/+}$ (Long et al., 2005) and CD1 strains were used for experiments.

Morulae were collected at 2.5 dpc, blastocysts at 3.25, 3.45, 3.5 and 4.3 dpc from *Pdgfra*$^{H2B\text{-}GFP/+}$ and *CAG:mRFP1*$^{Tg/+}$ females mated with *CAG:mRFP1*$^{Tg/+}$ and *Pdgfra*$^{H2B\text{-}GFP/+}$ males, respectively. Embryo handling and culture was performed in M2 and KSOM-AA medium, respectively (both with 4 mg/ml BSA; Sigma). Mouse studies were carried out in a designated facility under licenses issued by the United Kingdom Home Office.

Chimaera assay

Preparation of donor cells

Blastocysts were pre-selected. Only those positive for both mRFP and H2B-GFP fluorescence were used for further experiments. Littermates were used as controls. Donor cells were obtained from the following groups of blastocysts, based on the time of collection, number of nuclei (counted in littermates) and pattern of *Pdgfra*$^{H2B\text{-}GFP}$ expression: (1) early blastocysts – collected at 3.25 dpc, mean cell number less than 64 and *Pdgfra*$^{H2B\text{-}GFP}$ expressed heterogeneously throughout the ICM; (2) mid blastocysts – collected at 3.5 dpc, mean cell number greater than 64 and *Pdgfra*$^{H2B\text{-}GFP}$ expressed heterogeneously throughout the ICM; (3) late blastocysts – collected at 3.45 dpc, cultured overnight in KSOM and subsequently selected as blastocysts, mean cell number more than 100 and *Pdgfra*$^{H2B\text{-}GFP}$-expressing cells sorted to line blastocyst cavity; (4) peri-implantation ('E4.5') embryos – collected at 4.3 dpc, with PrE clearly sorted to line cavity and signs of early PrE migration often present (as visualised by *Pdgfra*$^{H2B\text{-}GFP}$ expression).

To prepare donor cells, the zona pellucida was removed from early to late blastocysts by treatment with acidic Tyrode's solution (Sigma). ICMs were isolated by immunosurgery (Hogan and Tilly, 1978; Solter and Knowles, 1975). ICMs were thoroughly cleaned of remaining trophoblast by vigorous pipetting, and disaggregated as described previously (Gardner and Rossant, 1979) by incubation in 1× trypsin-EDTA (Invitrogen). Single cells were sorted using a confocal microscope according to levels of H2B-GFP (*Pdgfra*$^{H2B\text{-}GFP}$) expression. GFP-negative cells were designated 'GFP-N'. GFP-positive cells were separated into two groups based on the intensity of GFP fluorescence, as quantified using Fluoview 1000 confocal software. Intensity measurements were normalised against background (taken as 0%) and the highest intensity measurement in the group (taken as 100%). Cells with GFP intensity of greater than 70% were designated 'GFP-H' (GFP-high). Cells with GFP intensity of less than 70% were designated 'GFP-L' (GFP-low) donors. As an additional control, we also performed the chimaera assays on non-selected (random) early and late blastocyst donors (supplementary material Fig. S1B).

Recipient embryos and creation of chimaeras

Recipient morulae were placed in M2 medium without Ca^{2+} and Mg^{2+} for 10-15 minutes, to induce de-compaction. For aggregation, zona pellucidae were removed from recipients prior to decompaction. Single donor cell and single morula pairs were then placed in small indentations in plastic dishes in drops of KSOM, brought together using a glass pipette, and cultured for 48 hours.

For micromanipulation chimaeras, both donor cells and de-compacted recipient embryos were placed in a micromanipulation chamber and a single donor cell was introduced between morula blastomeres using a micromanipulation pipette.

Chimaeras produced by micromanipulation were observed every 12 hours during the 48-hour culture.

Inhibitors

Chimaeras were treated with 1 μM MEK inhibitor PD0325901 and 3 μM Gsk3 inhibitor Chir99021 (Division of Signal Transduction Therapy, Dundee, UK) (2i) (Nichols et al., 2009) or 100 ng/ml recombinant human FGF4 (R&D Systems) in KSOM medium.

Immunostaining

Embryos were fixed in 3.7% paraformaldehyde overnight at 4°C and processed as described previously (Plusa et al., 2008). Primary antibodies used were: mouse anti-Cdx2 (BioGenex) 1:1, goat anti-Gata4 (C-20, Santa Cruz) 1:100, rabbit anti-Nanog (Cosmo Bio) 1:300, mouse anti-Oct4 (C-10, Santa Cruz), goat anti-Oct4 (N-19, Santa Cruz) and rabbit anti-Sox2 (Abcam) 1:100. All secondary antibodies (Alexa Fluor) were purchased from Invitrogen.

Microscopy, data acquisition and analysis

Micromanipulations were performed using a Leica DMI 6000B inverted microscope. For live visualisation, embryos were placed in drops of medium under paraffin oil on a glass bottom dish (MatTek).

For imaging, embryos were placed in Vectashield mounting medium (Vector Labs) on a glass bottom dish and visualised using an Olympus inverted confocal microscope (Fluoview FV1000) with Olympus Fluoview v2.1 software or a Leica inverted SP5 confocal microscope with Leica LAS software. Intensity of GFP fluorescence in live *Pdgfra*$^{H2B\text{-}GFP}$ embryos was quantified using Olympus Fluoview v2.1 software. Analysis of images was performed using IMARIS and Fluoview v2.1 software.

Statistical analysis was performed using the QuickCalc GraphPad website (http://www.graphpad.com/quickcalcs/index.cfm). Two-tailed chi-square tests with Yates correction were performed. *P* value (*P*) is shown wherever the difference between compared groups was significant.

RESULTS

ICM cells retain pluripotency until the late blastocyst stage

Recent data indicate that cells within the ICM do not restrict their fate until after they reach the E4.0 stage of development (Nichols et al., 2009; Yamanaka et al., 2010). However, these experiments do not show how the restriction of potency in ICM cells correlates with the pattern of expression of lineage-specific markers and the spatial arrangement of cells within the ICM. To assess the developmental potential of presumptive epiblast and PrE precursors at different stages of blastocyst development, we devised the following experimental scheme (Fig. 1). To obtain donor cells, we crossed *Pdgfra*$^{H2BGFP/+}$ mice with mice that ubiquitously express red fluorescent protein (RFP) and collected early, mid, late and E4.5 blastocysts. Cell numbers of surplus (GFP-negative and/or RFP-negative) littermates were counted to estimate cell numbers of donor blastocyst groups. Mean cell numbers (±s.e.m.) were 44.2±2.9 (early), 74.1±6.1 (mid), 121.5±4.3 (late) and 178.0±7.7 (E4.5), with some overlap between groups (supplementary material Fig. S2A). As previously described (Plusa et al., 2008), the selected stages correlate with changing expression of PrE and epiblast markers Gata4 and Nanog, respectively (supplementary material Fig. S3). The trophoblast of donor blastocysts was removed by immunosurgery, followed by dissociation of ICMs. The level of GFP fluorescence for each ICM cell was then scored as negative (‘GFP-N’, presumptive epiblast precursors), low (‘GFP-L’) or high (‘GFP-H’, presumptive PrE precursors). All cells obtained from E4.5 blastocysts were scored as either GFP-N or GFP-H, as the epiblast and PrE layers are fully differentiated by this stage and no GFP-L cells could be identified. The proportions of GFP-N, GFP-L and GFP-H cells isolated from ICMs within each donor stage (group) are presented in supplementary material Fig. S2C. The total number of chimaeras obtained for each stage is summarised in supplementary material Table S1. Individual donor GFP-N, GFP-L and GFP-H cells were aggregated with recipient *Pdgfra*$^{H2BGFP/+}$ morulae (Fig. 1A), then cultured to ~E4.5, when an overt PrE layer lines the cavity roof. The mean cell number of chimaeras at the end of culture was 134.5±6.3 (s.e.m.) (supplementary material Fig. S2B). The contribution of donor cell progeny (RFP-positive) to trophoblast, PrE and epiblast was assessed, using the following criteria: (1) GFP-negative cells within the trophoblast epithelium were scored as trophoblast; (2) GFP-negative cells lying deep within the ICM were scored as epiblast; (3) GFP-positive ICM cells positioned in proximity to the blastocyst cavity were scored as PrE (Fig. 1B).

Analysis of the results revealed a high-level of multi-lineage contribution from individual donor cells (Fig. 2A). Donor cells from early blastocysts gave rise to more than one lineage in 41% (24/59) of cases, including tri-lineage contribution. Similarly, mid-blastocyst donor cells gave rise to multiple lineages in 45% (25/56) of cases. Notably, 17% (6/36) of the late blastocyst donor cells still contributed to more than one lineage, despite being derived from a stage when Gata6/4 and Nanog expression is mutually exclusive and the PrE and epiblast have separated to their final positions in the embryo (Plusa et al., 2008) (supplementary material Fig. S1A). Complete loss of plasticity in 100% of donor cells was observed only in the E4.5 donor group.

Lineage contribution with respect to the level of *Pdgfra*$^{H2B\text{-}GFP}$ expression of donor ICM cells was also analysed (GFP-N, GFP-L or GFP-H). Donor cells from all stages preceding E4.5 could contribute to a lineage other than that predicted by the initial level of GFP expression, irrespective of whether they contributed to multiple lineages or only one lineage (Fig. 2A). Only donor cells from E4.5 contributed exclusively to their predicted lineage.

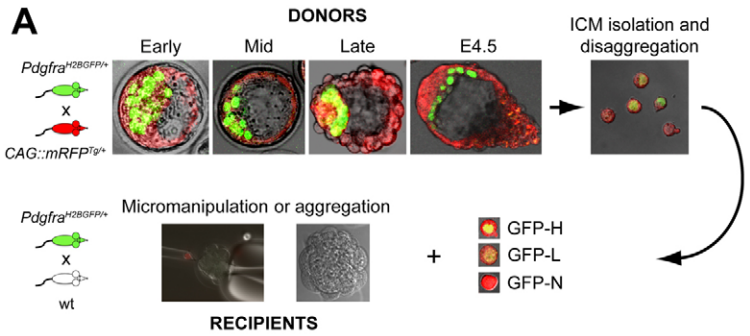
Thus, the ability of cells to give rise to multiple lineages decreases from the mid to late blastocyst stage. Notably, complete restriction of developmental potency does not correlate with establishment of mutually exclusive expression of Nanog and Gata6 (mid blastocyst). Some degree of plasticity exists even after PrE and epiblast cells are segregated to their final positions.

Epiblast and PrE precursors differ in their plasticity at the early blastocyst stage

Our experiments confirmed that ICM cells lose plasticity gradually and that complete loss of plasticity occurs between late blastocysts stage and E4.5. Unexpectedly, we noted a marked difference in cell plasticity depending on *Pdgfra*^{H2BGFP} expression. GFP-N progeny contributed to PrE in relatively few chimaeras [9% (2/22) of early, 24% (5/21) of mid and 20% (3/15) of late chimaeras] and never when derived from E4.5 blastocysts (0/7) (Fig. 2A). Instead, GFP-N progeny contributed to epiblast more often than to PrE for each donor stage [50% (11/22, P=0.0082) of early, 43% (9/21) of mid, 80% (12/15, P=0.0035) of late, and 100% (7/7, P<0.0001) of E4.5 chimaeras]. This suggests that GFP-N cells are biased against a PrE fate from the early blastocyst stage. In a number of chimaeras, the progeny of GFP-N cells from early, mid and late donors also contributed to trophoblast (Fig. 2A). This is consistent with previous reports that isolated ICMs from early blastocysts are able to regenerate trophoblast – a property that is lost in late blastocysts (Handyside, 1978; Hogan and Tilly, 1978; Rossant and Lis, 1979; Spindle, 1978; Stephenson et al., 2010).

Many of these tendencies were more pronounced when we compared the total number of donor progeny contributing to each lineage. No more than 15% of GFP-N donor progeny contributed to PrE [3% (3/105) of early, 15% (12/80) of mid and 13% (9/69) of late donor cells] and never when donor cells were derived from E4.5 embryos. Instead, GFP-N donor progeny contributed predominantly to either epiblast [57% (60/105) of early, 42.5% (34/80) of mid and 75% (52/69) of late donor cells] or to trophoblast [40% (42/105) of early, to 42.5% (34/80) of mid and 12% (8/69) of late donor cells] (Fig. 2B). The apparently higher contribution of GFP-N cells to epiblast when scoring total number of donor progeny, rather than lineage composition of donor clones, suggests a higher proliferation rate of epiblast-fated cells. This was confirmed when single-lineage clone sizes were compared (supplementary material Fig. S4B). Furthermore, GFP-N donors from early blastocysts produced fewer PrE cells in total than did those from mid and late blastocysts (Fig. 2B). Although the sample size was too small to be significant, this was surprising considering that early stage ICM cells would be expected to be more plastic than later stage ICM cells.

In contrast to GFP-N donor progeny, GFP-H cells exhibited greater flexibility in fate choice. Relative contribution to epiblast versus trophoblast was generally similar within donor stage groups [26% (20/76) versus 29% (22/76) for early donors; 15% (10/65) versus 26% (17/65) for mid donors; 6% (5/77) versus 4% (3/77) for late donors]. GFP-H donor progeny contributed substantially to PrE (Fig. 2B), with a gradual increase in PrE contribution correlating with donor cell stage [45% (34/76) of early; 58%



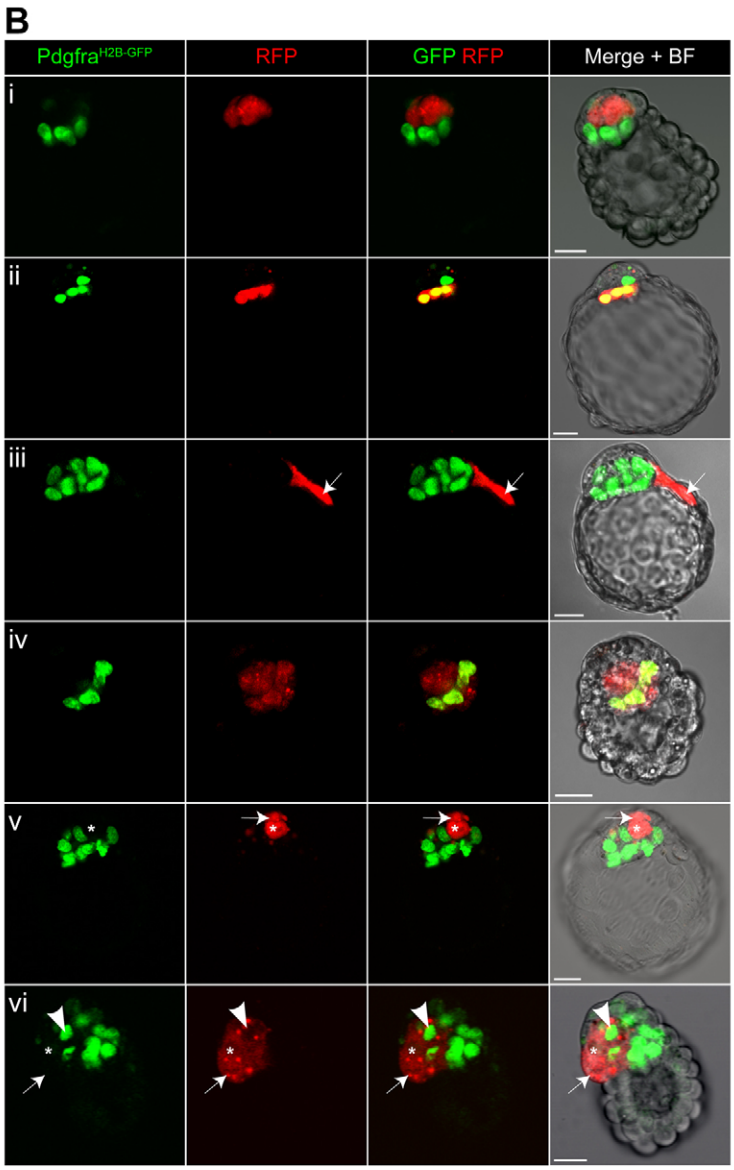


Fig. 1. Progeny of ICM cells contribute to epiblast, PrE and trophoblast lineages when aggregated with morulae. (**A**) Experimental scheme. ICM cells from donor embryos were obtained by immunosurgery and disaggregation, selected on intensity of GFP signal and reaggregated with recipient morulae. (**B**) Examples of clonal contribution of donor cells in chimaeras after 48 hours in culture (end point): (i) epiblast clone; (ii) PrE clone; (iii) trophoblast clone; (iv) epiblast + PrE clone; (v) epiblast + trophoblast clone; (vi) tri-lineage clone. Arrows indicate a trophoblast cell; arrowheads indicate a PrE cell; asterisks indicate an epiblast cell. Scale bars: 20 μm.

(38/65) of mid; 90% (69/77) of late; 100% of E4.5 donor cells]. Interestingly, although early GFP-L and GFP-H donors both contributed to PrE and epiblast lineages in similar proportions [43% (38/88) and 40% (35/88), respectively, for GFP-L; 45% (34/76) and 50% (38/76), respectively, for GFP-H], GFP-L donors exhibited a gradual increase in epiblast contribution correlating with donor cell stage [50% (60/121) epiblast versus 26% (32/121) PrE for mid donors; 70% (30/43) epiblast versus 28% (12/43) PrE for late donors]. This is consistent with the notion that a proportion of *Pdgfra*^{H2BGFP}-expressing cells that do not occupy the blastocyst cavity lining can downregulate GFP expression and then presumably contribute to epiblast (Plusa et al., 2008).

In general, we noticed a disproportionate contribution to PrE between GFP-N donors and GFP-L or GFP-H donors from early blastocysts. GFP-N donors contributed significantly less often to PrE than did GFP-L and GFP-H donors (3% versus 43% and 45%,

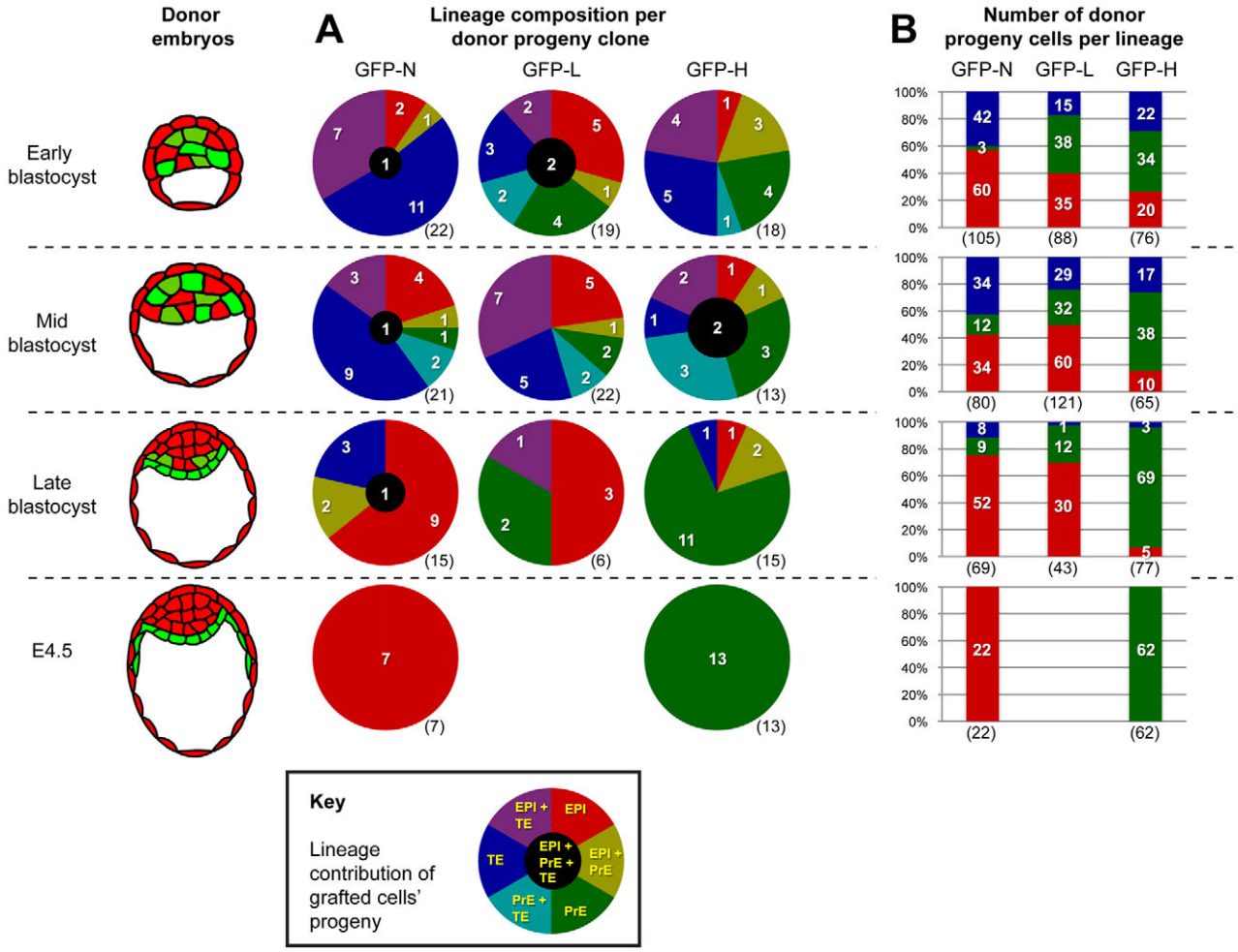


Fig. 2. Profile of lineage contribution of transplanted cells in experimental groups. (Left) schematic representation of blastocyst stages from which donor cells were isolated. (**A**) Proportions of donor cells contributing to specific combinations of lineages. (**B**) Proportions of donor cell progeny contributing to each lineage. Absolute numbers are indicated in white; sample sizes are in parentheses.

respectively; $P<0.0001$). We also noticed that the proportion of cells allocated to different lineages varied substantially for GFP-N donors from the data obtained for non-preselected (random) cells (supplementary material Fig. S1B). GFP-N donors exhibited a significantly higher contribution to epiblast than did random cells (57% and 75% versus 32% and 35% for early and late donors, respectively; $P<0.0001$), and a significantly lower contribution to PrE than did random cells (3% and 13% versus 50% and 45% for early and late donors, respectively; $P<0.0001$).

The fate of donor cells was followed by time-lapse microscopy to confirm the modes of lineage contribution (supplementary material Fig. S5). GFP-H and GFP-L donor progeny that contributed to PrE always maintained GFP fluorescence throughout the culture period. GFP-N donor progeny that contributed to epiblast sometimes upregulated GFP expression transiently, but in the majority of cases failed to maintain GFP expression during the culture period.

Collectively, these data suggest that early GFP-H cells exhibit greater developmental plasticity than GFP-N cells, whereas GFP-L cells are intermediary between GFP-N and GFP-H cells. Thus, ICM cells start to differ in their plasticity before establishment of a mutually exclusive pattern of epiblast- and PrE-specific marker expression. Unexpectedly, the developmental plasticity of pluripotent epiblast precursors (GFP-N) appears more restricted compared with that of PrE precursors (GFP-H).

Immunolocalisation of lineage-specific markers is generally consistent with the position of donor progeny within blastocyst

To verify that donor cell progeny expressed markers appropriate to the tissue to which they contributed, thereby confirming their lineage identity, we performed immunostaining for Nanog (epiblast), Gata4 (PrE) and Cdx2 (trophoblast). In all chimaeras analysed, all donor cell progeny that contributed to PrE expressed Gata4 (GFP-N, 1/1; GFP-L, 3/3; GFP-H, 12/12) (Fig. 3B). Similarly, in the majority of chimaeras in which donor cell progeny contributed to trophoblast, these cells expressed Cdx2 (GFP-N, 13/17; GFP-L, 10/12; GFP-H, 6/6) (Fig. 3A). By contrast, Nanog was not detected in donor progeny contributing to epiblast in the majority of chimaeras from GFP-N donors (10/13), although it was present in the epiblast progeny of GFP-H (6/9) and GFP-L (12/13) donors (Fig. 3C). However, an epiblast marker Sox2 was present in all progeny of GFP-N donor cells contributing to epiblast (4/4) (Fig. 3D).

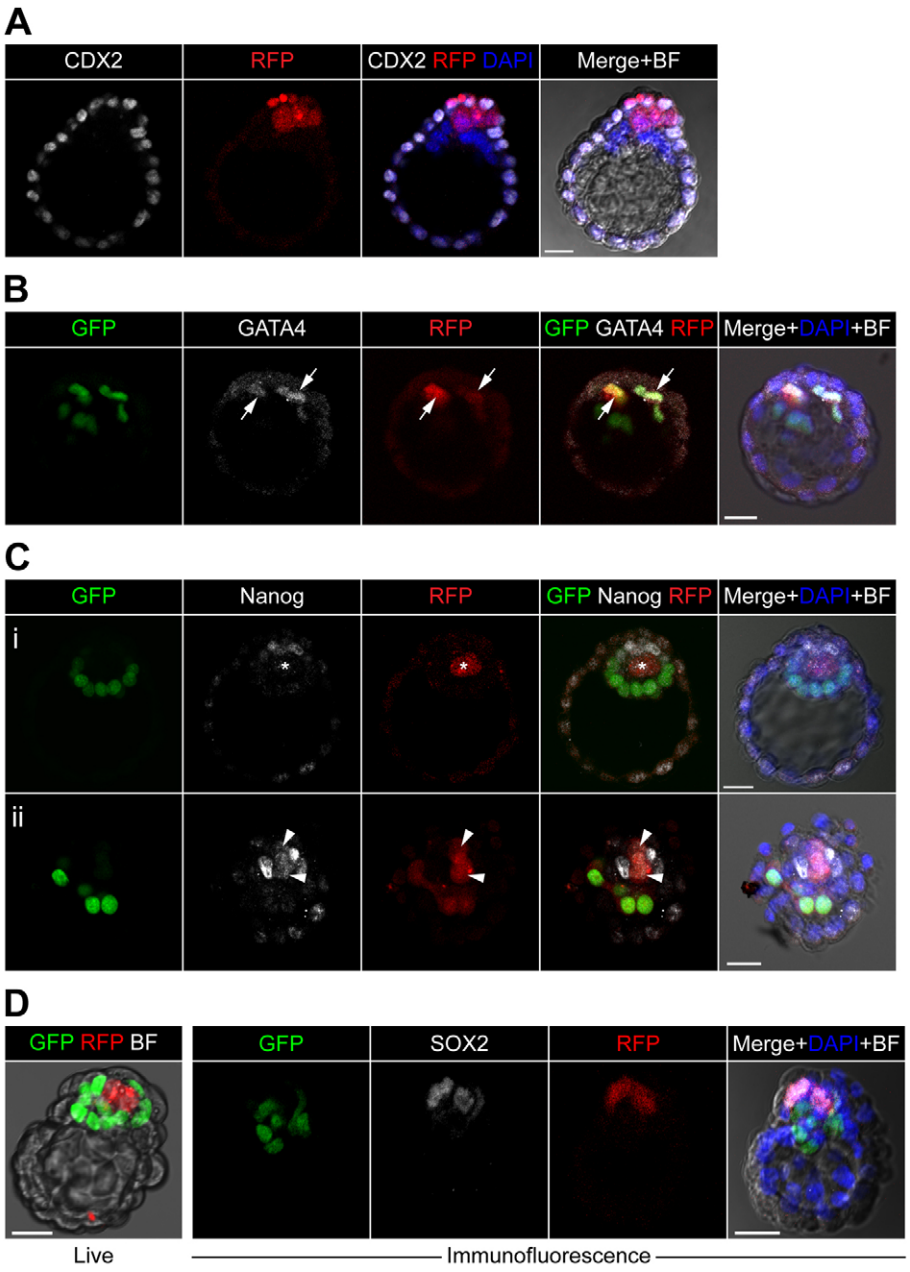


Fig. 3. Expression of lineage markers in chimaeras. Donor progeny clones visualised as RFP-expressing (red) cells. Green fluorescence represents expression of *Pdgfra*$^{H2B\text{-}GFP}$. (**A**) Cdx2 expression in a clone contributing to both epiblast and trophoblast. All trophoblast cells are positive for Cdx2, although the level of expression differs. Epiblast cells are all negative for Cdx2. (**B**) Gata4 expression in a clone contributing only to PrE. Gata4 is present in all PrE cells and overlaps with expression of GFP (arrows). (**C**) Two examples of Nanog expression in chimaeras. (i) GFP-N donor progeny contributed only to epiblast, in which Nanog is absent in donor progeny (asterisk) but present in recipient epiblast (GFP negative and RFP negative) cells. (ii) GFP-H donor progeny contributed to both epiblast and PrE. Nanog is present in donor cells in the epiblast part of the clone (GFP negative and RFP positive, arrowheads), but not in donor cells in the PrE part of the clone (GFP positive and RFP positive). (**D**) Example of Sox2 expression in chimaera where GFP-N donor progeny contributed to epiblast. First on the left is a live image of the same embryo after 48 hours of culture. Scale bars: 20 μm.

Nanog is known to be a transitory marker of early epiblast (Chambers et al., 2003), upregulated again later at gastrulation (Hart et al., 2004; Morkel et al., 2003). Because donor cells were derived from stages later than the recipient morulae, their progeny presumably represented a more advanced stage at the end of culture than their neighbouring recipient cells.

To test this hypothesis, we asked whether donor cell progeny of transplanted GFP-N cells occupying the epiblast would express Nanog when embryos were cultured with inhibitors of FGF/Erk signalling. Erk signal inhibition in pre-implantation embryos results in Nanog expression throughout the ICM and concomitant absence of a Gata6- and Gata4-positive PrE, suggesting that all cells adopt an epiblast state (Nichols et al., 2009; Yamanaka et al., 2010). Additionally, Nanog expression is not downregulated after epiblast formation in embryos treated with Mek inhibitor (Nichols et al., 2009). We cultured chimaeras with Mek/Erk inhibitor PD0325901 and Gsk3 inhibitor Chir99021 (2i). Under these conditions, we found that RFP-labelled GFP-N donor progeny always expressed Nanog, as did all recipient ICM cells (Fig. 4). This confirmed that GFP-N progeny are capable of maintaining Nanog expression, and that Nanog-negative GFP-N-derived epiblast cells observed in our experiments were probably at a more advanced stage, with downregulated Nanog.

Modulation of FGF/Erk signalling influences the developmental plasticity of transplanted epiblast and PrE precursors

Our results suggest that ICM cells differ in their developmental plasticity, with epiblast precursors appearing more restricted than PrE precursors. This fact may be due to a differential response of the ICM cells to their micro-environment. *Pdgfra* expression in PrE precursors specifically correlates with expression of *Fgfr2*, implicating differential responsiveness to Fgf4 signalling between PrE and epiblast precursors (Guo et al., 2010; Kurimoto et al., 2006).

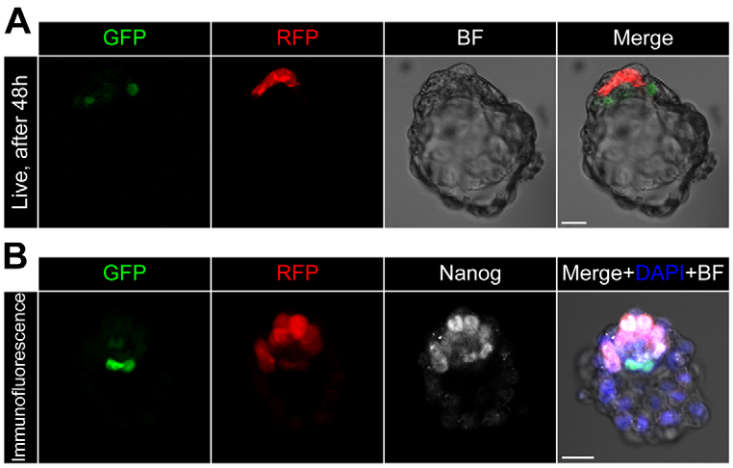


Fig. 4. Donor cells in chimaeras treated with inhibitors contribute solely to epiblast and express Nanog. (**A**) Live chimaeric embryo after 48 hours of culture. (**B**) Expression of Nanog visualised by immunofluorescence. Donor progeny are RFP expressing. GFP represents expression of *Pdgfra*$^{H2B\text{-}GFP}$. Scale bars: 20 μm.

We therefore asked whether attenuating or inhibiting the Fgf4 signalling pathway in morulae would influence the developmental plasticity of GFP-N and GFP-H donor cells from early blastocysts. Experiments were performed as above, except that chimaeras were cultured in the presence of FGF4 or 2i (Silva et al., 2008; Ying et al., 2008; Nichols et al., 2009) until formation of the blastocyst cavity, after which they were returned to standard medium. Culture of embryos in the presence of FGF4 or 2i before the mid blastocyst stage has been shown not to affect normal segregation of epiblast and PrE lineages (Nichols et al., 2009; Yamanaka et al., 2010). Chimaeras developed normally, as confirmed by the presence of fully formed and sorted PrE in *Pdgfra*$^{H2BEGFP/+}$-recipient embryos (Fig. 5A,B).

Total numbers of chimaeras and donor cell progeny in this experiment are presented in supplementary material Table S2. The progeny of early blastocyst GFP-N cells cultured in the presence of FGF4 contributed to PrE in chimaeras significantly more often [8/20 (40%)] (Fig. 5C) than in chimaeras not exposed to FGF4 [2/22 (9%) P=0.03] (Fig. 2A). Strikingly, contribution of GFP-N donor progeny to PrE (calculated in the total cell number) after FGF4 exposure was substantially higher [44% (40/91)] than without FGF4 [3% (3/105), P<0.0001], although the contribution to epiblast was still relatively high (46%) (Fig. 2B, Fig. 5D). Notably, production of chimaeras from GFP-H donor cells after FGF4 treatment was unsuccessful. Of 10 chimaeras that still retained donors progeny after 12 hours of culture, nine contained only apoptotic donor progeny at the end of the total culture period (48 hours).

Inhibition of FGF signalling with 2i in chimaeras from early GFP-N donors resulted in a high frequency of contribution to epiblast [15/17 (89%), P=0.03] (Fig. 5C), which was further confirmed by analysing total donor cell contribution to each lineage [89% contribution to epiblast (61/69), P<0.0001] (Fig. 5D). A similar increase in frequency of epiblast contribution was observed for 2i-treated chimaeras of GFP-H cells (6/8, 75%). This trend was also confirmed by analysis of total donor cell contribution to each lineage, with 68% (25/37, P<0.0001) of GFP-H donor progeny contributing to epiblast. For both GFP-N and GFP-H donors, 2i treatment also resulted in a reduction in contribution to trophoblast. Thus, 2i treatment resulted in a similar profile of lineage contribution for both GFP-N and GFP-H cells.

Notably, modulation of FGF/Erk signalling did not result in transplanted cells forming exclusive single-lineage clones, as occurs when the cells are subjected to prolonged FGF or 2i exposure until E4.0.

In summary, transient treatment of chimaeras with either FGF4 or 2i during the morula stage biases the fate of early blastocyst-derived donor cells. The effect of treatment with 2i is similar on both GFP-N and GFP-H donors, suggesting that it erases any prior differences between these cells. The increased PrE contribution after FGF4 treatment of GFP-N chimaeras suggests that early GFP-N cells are capable of responding to Fgf4, but the level of active Fgf4 in the morula environment is insufficient to induce development to PrE.

Establishment of the PrE layer and Oct4 levels may be linked to restriction of potency

Our results suggest that restriction of potency in ICM cells does not correlate with mutually exclusive expression of Nanog and Gata4/6. We therefore examined whether it correlated with the expression of Oct4, a transcription factor involved in specifying pluripotency within the ICM (Nichols et al., 1998) (Fig. 6). In littermates of early blastocysts (n=7), Oct4 was present in all ICM cells as well as the majority of trophoblast cells, and Gata4 was not detected. In littermates of mid blastocyst donors (n=7), Oct4 showed a similar pattern of expression in the ICM (Fig. 6). In littermates of late blastocyst donors (n=11), we observed two classes of Oct4 localisation, suggesting a transitional phase. In approximately half (n=6), the levels of Oct4 remained fairly uniform throughout both the epiblast and PrE layers. In only a minority of Gata4-positive PrE cells, Oct4 levels were low or absent. By contrast, in the remaining blastocysts (n=5), the majority of Gata4-positive cells forming the PrE exhibited lower levels of Oct4 expression than the deeper-lying Gata4-negative cells, and a few of these cells were Oct4 negative. Complete downregulation of Oct4 expression in Gata4-positive PrE cells was observed in the majority of E4.5 donor littermates (n=8). Weak Oct4 signal was detected in some of the early migrating parietal endoderm cells, whereas PrE cells still in contact with the epiblast were always Oct4 negative (Fig. 6). Our observations suggest that downregulation of Oct4 in presumptive PrE cells occurs after they become positioned on the cavity. This phase also correlates with the stage at which the plasticity of donor ICM cells decreases. Subsequent to this stage (E4.5), when ICM cell plasticity was lost, Oct4 was generally absent throughout non-migrating PrE.

DISCUSSION

In this study, we investigated how changes in gene expression and cell position are related to changes in developmental plasticity and the onset of a divergence in potency within the ICM.

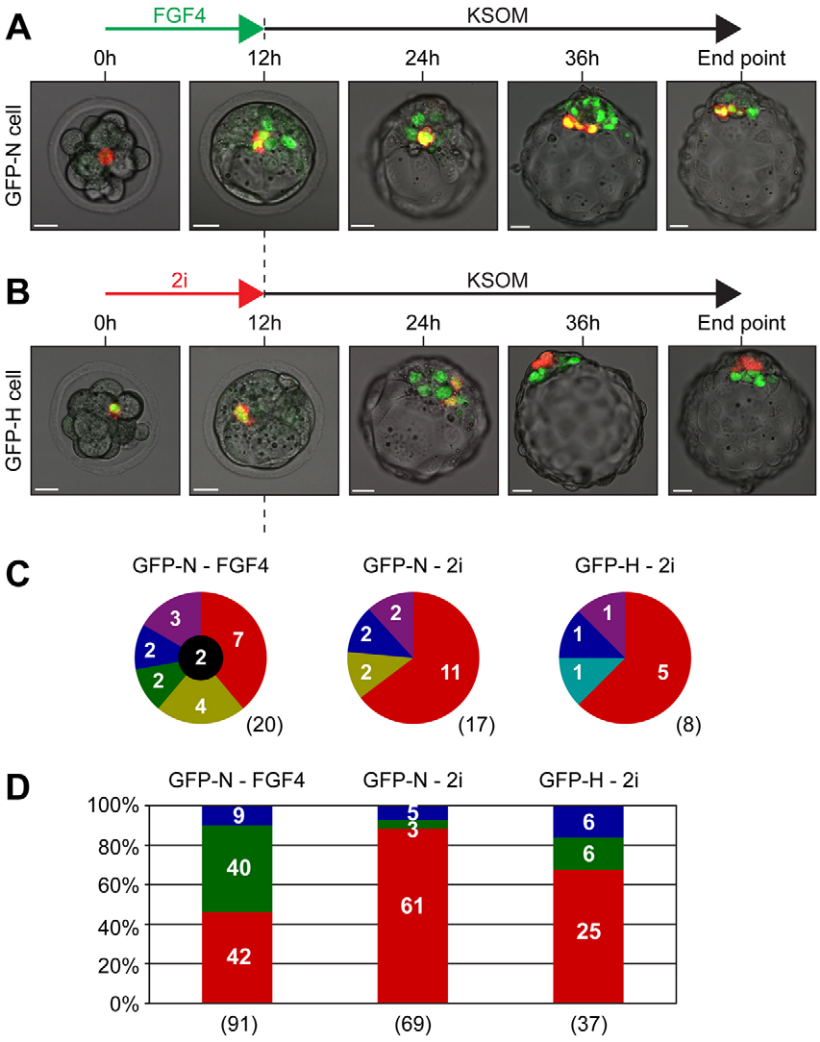


Fig. 5. Early transient FGF4 or 2i treatment influences lineage contribution of donor progeny. (**A**) Time-lapse imaging of chimaera from early GFP-N donor cell treated with FGF4 for 12 hours until early blastocyst formation. (**B**) Time-lapse imaging of chimaera from early GFP-H donor cell treated with 2i for 12 hours until early blastocyst formation. (**C**) Lineage contribution per donor progeny clone for each treatment. (**D**) Number of donor cell progeny per lineage for each treatment. Absolute numbers are indicated in white; sample sizes are in parentheses. Scale bars: 20 μm.

Our data suggest that the potency of epiblast and PrE precursors at various embryonic stages is broader than their undisturbed fate. Moreover, restriction in potency does not correlate directly with mutually exclusive expression of Nanog and Gata6. Instead, final commitment is not fully established until E4.5, in agreement with previous studies (Gardner and Rossant, 1979). Even at the late blastocyst stage, when epiblast and PrE precursors are already sorted (>100 cells), some cells maintain the capacity to contribute to multiple lineages or to a lineage other than their predicted one. Only in E4.5 embryos (more than ~140 cells) in which migration of parietal endoderm has commenced, lineage commitment of ICM cells appears to be complete. Similarly, experiments by Nichols et al. and Yamanaka et al. (Nichols et al., 2009; Yamanaka et al., 2010) showed that cells within the ICM remain plastic until E4.25. However, the correlation between changes of expression of lineage-specific markers, timing of formation of PrE and restriction of potency was not directly addressed. Our data show that restriction of potency does not correlate with changes in expression of Nanog or Gata4/6. Interestingly, we noticed that it coincides with a change in levels of Oct4. Currently, the link between Oct4 activity and cell potency within the ICM is not understood and further studies are required to investigate this connection.

The fact that ICM cells retain plasticity until the PrE is formed might suggest that their potency should remain similar throughout the process of lineage formation. However, our results show that the plasticity of ICM cells varies qualitatively and correlates with their level of *Pdgfra*$^{H2B\text{-}GFP}$ expression. Early epiblast precursors (GFP-N donor cells) are capable of contributing substantially to epiblast and trophoblast, but not to PrE, whereas PrE precursors (GFP-H donor cells) more readily contribute to all three lineages. This was unexpected because PrE is thought to represent a more developmentally restricted and differentiated lineage compared with the epiblast, which is pluripotent.

Previous work (Plusa et al., 2008; Guo et al., 2010) demonstrated that *Pdgfra*$^{H2B\text{-}GFP}$ expression is a strong predictor of PrE fate. However, it may not be an absolute predictor, especially at early stages with widespread and possible fluctuating levels of GFP. However, lack of predictive power of GFP expression in early cells would only randomise the data and thus no differences in the properties of GFP-H, -L and -N cells would then be expected. We propose that our results can be explained by differential abilities of precursor types to respond to signals in their environment. Experiments by Nichols et al. and Yamanaka et al. (Nichols et al., 2009; Yamanaka et al., 2010), in which they modulated Fgf4 signalling suggested that activation of this pathway, which is important for generation of the PrE progenitors, takes place after the early blastocyst stage. A recent study (Guo et al., 2010) reported that *Fgf4* is continuously expressed, but at its minimum level at the 16-cell stage. Moreover, cell-specific expression of *Pdgfra* positively correlated with expression of *Fgfr2* and

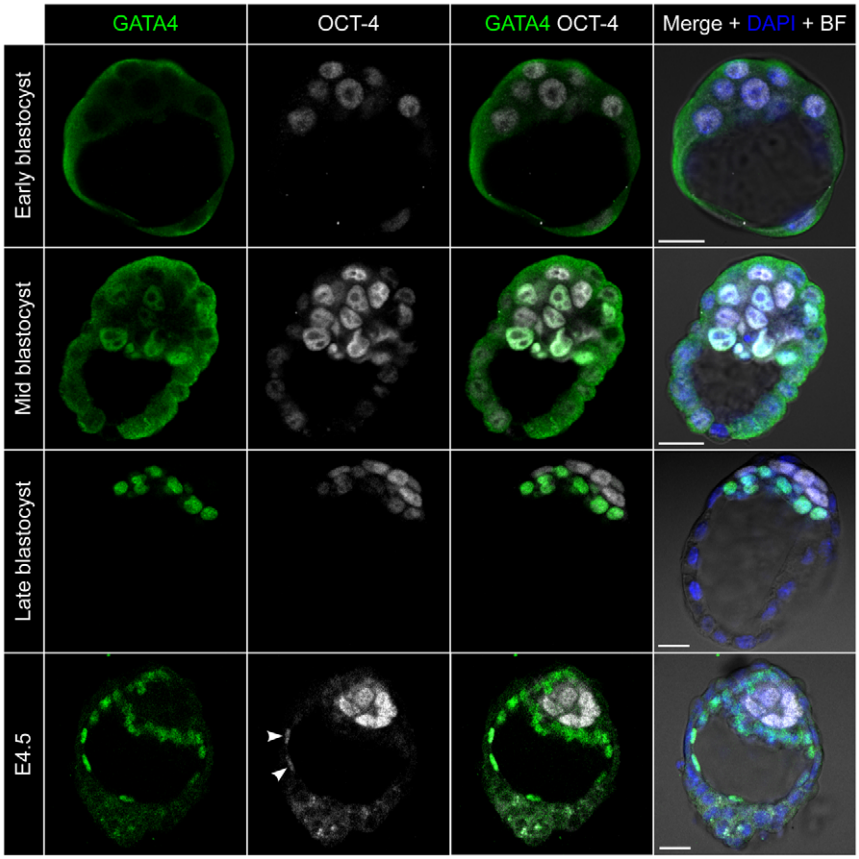


Fig. 6. Expression of Oct4 and Gata4 in donor littermates. In early and mid blastocysts, Oct4 is expressed throughout the ICM and more weakly in trophoblast cells, whereas Gata4 is absent. From the mid blastocyst stage, Gata4 is detected in nuclei of the nascent PrE layer and more weakly in some deeper cells. By the late blastocyst, Gata4 is restricted to the PrE layer, in which Oct4 staining is weaker than in the epiblast and is fully restricted to the epiblast by E4.5 (arrowheads). Scale bars: 20 μm.

negatively correlated with expression of *Fgf4* as early as the 32-cell stage. Therefore, the observed link in our study between levels of $Pdgfra^{H2B\text{-}GFP}$ expression and developmental plasticity may reflect differences between GFP-N and GFP-H in their ability to respond to FGF signalling. Increased expression of *Fgfr2* in ICM cells (Guo et al., 2010; Kurimoto et al., 2006) depends on continued Erk activity (Yamanaka et al., 2010), most probably establishing a positive-feedback loop. With insufficient Erk-activating signals in the morula and early blastocyst, Nanog levels may continue to rise such that some GFP-H cells can repress *Gata6* (Singh et al., 2007) and commit to an epiblast fate. Conversely, epiblast precursors (GFP-N) exhibit low responsiveness to PrE-inducing activity at the morula and early blastocyst stage (probably owing to insufficient expression of *Fgfr2*). Therefore, GFP-N donors give rise to few PrE progeny, especially when donor cells are collected from early blastocysts (less than 3% contribution). Mid and late blastocyst donors maintain this tendency, although the proportion of the progeny of GFP-N cells contributing to PrE slightly increases (15% for the mid and 13% for the late GFP-N donors). This may be due to the fact that other molecules required for Fgf4 activity, such as modifiers of heparan sulphate (Johnson et al., 2007; Kraushaar et al., 2010) may be limiting at earlier stages.

To confirm that the differences in plasticity of GFP-N and GFP-H cells were related to their differing responsiveness to signalling within the embryo, we transiently modified the early embryo environment by adding FGF4 to the culture or inhibiting Fgf-RTK signalling with an inhibitor of Erk1/2. Our results demonstrate that donor cells are already sensitive to this pathway at an early blastocyst stage. Treatment with 2i appeared to erase any prior differences between GFP-N and GFP-H cells, suggesting that the basis of these differences lies in Erk signalling. FGF4 treatment was partly able to bias fate of GFP-N cells towards PrE, suggesting some low-level responsiveness of GFP-N cells to FGF4. Unexpectedly, FGF4 treatment of GFP-H chimaeras led to apoptosis of donor progeny. We propose that this is due to precocious development of PrE identity, which requires survival factors that depend on the presence of epiblast or more mature epiblast precursors. A previous study demonstrating that proper development of PrE depends on the presence of Nanog-expressing cells within the ICM (Messerschmidt and Kemler, 2010) supports our hypothesis.

Our observations highlight the importance of the environment when assessing the potency of cells. For example, late epiblast-derived stem cells (EpiSCs) rarely contribute to chimaeras when injected to blastocysts, despite being pluripotent (Brons et al., 2007; Tesar et al., 2007). This may be partly due to insufficient Fgf signals within the pre-implantation embryo environment normally required to maintain pluripotency in EpiSCs.

We propose a revised model of embryonic lineage formation (Fig. 7) in which initially (before the morula stage) all cells have a similar developmental potential. Establishment of inside and outside cell populations then creates differences in cell environment. Outside cells receive polarity cues, whereas the inside cells are sheltered from this signalling. Next, a subpopulation of inside cells (which express *Fgfr2* and *Pdgfra* at higher levels) reaches the threshold necessary for Erk activation (via Fgf4) that allows PrE precursors to emerge. Inside cells expressing *Fgfr2* below a threshold level cannot acquire a PrE fate, thus forming epiblast precursors by default. This state is similar to the ground state proposed for ES cells, in which absence

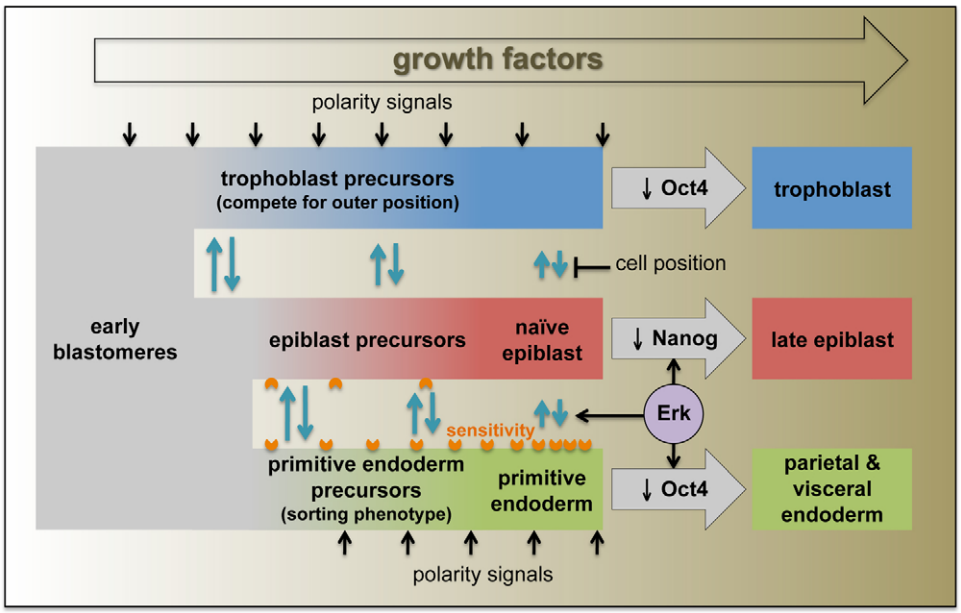


Fig. 7. Model for changing cell states during pre-implantation development. Epiblast precursors, characterised by Nanog expression, represent a naïve totipotent state that can convert to primed PrE precursors in response to Erk activation. The properties of PrE precursors predisposes them to cell sorting, after which positional signals reinforce commitment to a PrE fate. Likewise, eventual loss of Nanog in epiblast precursors, which are protected from differentiation signals by their inside position, leads to epiblast commitment. We speculate that, in the absence of Erk activation, naïve epiblast precursors can also pass through a trophoblast precursor state that becomes reinforced by positional signals.

of growth factor signalling is sufficient to maintain pluripotency (Ying et al., 2008; Silva et al., 2008). Positive-feedback mechanisms in PrE precursors help reinforce an expression state that promotes cell sorting, possibly via upregulation of Gata4. After completing cell sorting, PrE precursors become fully committed upon downregulation of Oct4.

Cell fate in the pre-implantation embryo can thus be considered a balance between levels of Nanog, Erk-induced Gata6 and polarity signals (Fig. 7). Trophoblast fate is driven by polarity signals in cells with low-to-moderate levels of Nanog and low Gata6. PrE fate can be driven by polarity signals in cells with low Nanog and high Gata6. Epiblast fate is ultimately acquired in cells with high Nanog.

Our observations define distinct and novel properties of subpopulations of cells within the ICM. Cells display considerably greater plasticity when allowed to develop in an appropriate environment. ES cells have conventionally been considered to lack the ability to contribute to extra-embryonic lineages in chimeras. We propose that by analogy with their embryo counterpart, the naïve epiblast, this is probably due to a relative insensitivity to growth factor signalling. By contrast, PrE precursors are more plastic because they can more readily respond to growth factor signalling, as well as revert to a naïve state. In summary, our observations reveal that the embryo has evolved sequential hierarchical mechanisms to ensure the production of sufficient numbers of epiblast cells.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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