Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst

Berenika Plusa1,2,*,‡, Anna Piliszek1,*, Stephen Frankenberg1,3,*,†, Jérôme Artus1 and Anna-Katerina Hadjantonakis1,‡

The first two lineages to differentiate from a pluripotent cell population during mammalian development are the extraembryonic trophoderm (TE) and the primitive endoderm (PrE). Whereas the mechanisms of TE specification have been extensively studied, segregation of PrE and the pluripotent epiblast (EPI) has received comparatively little attention. A current model of PrE specification suggests PrE precursors exhibit an apparently random distribution within the inner cell mass of the early blastocyst and then segregate to their final position lining the cavity by the late blastocyst. We have identified platelet-derived growth factor receptor alpha (Pdgfra) as an early-expressed protein that is also a marker of the later PrE lineage. By combining live imaging of embryos expressing a histone H2B-GFP fusion protein reporter under the control of Pdgfra regulatory elements with the analysis of lineage-specific markers, we investigated the events leading to PrE and EPI lineage segregation in the mouse, and correlated our findings using an embryo staging system based on total cell number. Before blastocyst formation, lineage-specific factors are expressed in an overlapping manner. Subsequently, a gradual progression towards a mutually exclusive expression of PrE- and EPI-specific markers occurs. Finally, cell sorting is achieved by a variety of cell behaviours and by selective apoptosis.

KEY WORDS: Lineage specification, Mouse blastocyst, ICM, Primitive endoderm, Epiblast, Pdgfra, Histone H2B-GFP fusion, Live imaging

INTRODUCTION

Eutherian development from the morula to the late blastocyst is largely devoted to the allocation of two extraembryonic lineages, the trophoderm (TE) and the primitive endoderm (PrE), in preparation for implantation. The TE forms as an epithelium enclosing the blastocyst cavity and the inner cell mass (ICM). By the late blastocyst, the PrE appears as a conspicuous layer of cells on the surface of the ICM lining the cavity, with deeper cells comprising the pluripotent epiblast (EPI), which gives rise to the embryo proper.

The initial differentiation of the TE depends on asymmetric positional signals that induce cell polarisation, followed by epithelialisation (reviewed by Yamanaka et al., 2006). Only the maintenance of TE, rather than its initial differentiation, is dependent on known TE-specific transcription factors, such as Cdx2 and Eomes (Niwa et al., 2005; Ralston and Rossant, 2008). Similarly, specification of PrE was previously believed to occur in response to positional signals at the interface between the ICM and the blastocyst cavity, reflecting the ultimate position of the tissue (reviewed by Yamanaka et al., 2006). This view was supported by the observation that when embryonic stem (ES) cells are allowed to form embryoid bodies, the outer layer of cells differentiates to form PrE-derived tissue (Becker et al., 1992; Martin and Evans, 1975; Murray and Edgar, 2001).

Recently, an alternative model has been proposed from the observation that two early markers of EPI (Nanog) and PrE (Gata6) lineages exhibit an apparently random and mutually exclusive (‘salt-and-pepper’) distribution within the ICM at the mid-blastocyst stage, prior to PrE formation (Chazaud et al., 2006; Rossant et al., 2003). Moreover, individually labelled mid-blastocyst ICM cells exhibited a propensity to contribute to either the EPI or PrE, but not both. These data suggest that EPI and PrE cells are fated at a relatively early stage, and only later sort into their respective layers (Chazaud et al., 2006). We reasoned that live imaging of PrE formation would provide validation of the model, and that a fluorescent reporter of PrE precursors would provide the necessary tool.

A microarray analysis of gene expression in single cells isolated from the mouse blastocyst revealed that expression profiles largely fall within two cohorts: one characterised by PrE-specific genes, the other by EPI-specific genes (Kurimoto et al., 2006). The gene encoding platelet-derived growth factor receptor alpha (Pdgfra) stood out within the PrE-specific cohort, as it was also identified in expression profiles of extraembryonic endoderm (XEN) cells (Kunath et al., 2005). A functional role for Pdgfra in PrE specification was also plausible because of evidence suggesting a role for receptor tyrosine kinase signalling in this process (Chazaud et al., 2006). Activation of the PI3K pathway by PDGFRs promotes actin reorganisation, directs cell movements and inhibits apoptosis. PDGFRs have also been implicated in the integrin-induced enhancement of cell migration and cell survival (reviewed by Andrae et al., 2008; Hoch and Soriano, 2003).

We used the Pdgfra<sup>H2B-GFP</sup> mouse strain (Hamilton et al., 2003), in which a cassette containing human histone H2B fused to enhanced green fluorescent protein (H2B-GFP) was targeted to the Pdgfra locus, to image PrE formation. The localisation of H2B-GFP to active chromatin facilitated the identification and tracking of individual reporter-expressing cells in vivo (Hadjantonakis and Papaioannou, 2004; Kanda et al., 1998).
Our live imaging experiments combined with an immunohistochemical analysis of lineage-specific factors revealed a series of sequential and distinct phases in the process of PrE formation. Our data lead us to propose a model in which the early overlapping expression of transcription factors precedes the maturation of inhibitory regulatory pathways and lineage-restricted expression after around the 64-cell stage. Cell sorting then occurs via a combination of behaviours, including cell movement, adhesion and selective apoptosis.

MATERIALS AND METHODS

Embryo collection and culture
Mice were maintained under a 12-hour light cycle. Embryos were collected from ICR or PdgfraH2B-GFP+/− (Hamilton et al., 2003) females mated with ICR or PdgfraH2B-GFP+/− males. PdgfraH2B-GFP+/+ males were indistinguishable from PdgfraH2B-GFP+/− embryos at all stages examined. Preimplantation embryos were flushed from uteri or oviducts in M2 (Chemicon) and cultured in KSOM (Chemicon) under mineral oil (Sigma) at 37°C and 5% CO2 in air. Postimplantation embryos were dissected in PB-1 (Papazianow and West, 1981). For live imaging, embryos were cultured in agarose-coated glass-bottom dishes (MatTek) in an environmental chamber (Solent Scientific).

Detection of Pdgfra protein and transcripts in early embryos
Oocytes/embryos were collected from natural matings of ICR animals. Pool embryos were lysed by three rounds of heat shock. Reverse transcription was performed using Superscript III First-strand Kit (Invitrogen). RT-PCR and nested RT-PCR were performed using an equivalent of 0.5 to 1 oocytes/embryos.

For Hprt detection, conditions of RT-PCR were 95°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minutes; this was followed by 10 minutes at 72°C. For Pdgfra detection, a first round of PCR was performed using the conditions stated above but with 25 amplification cycles. A second round of PCR was performed under similar conditions with 20 amplification cycles, using 5 μl of the first PCR reaction. Hprt primers: 5′-GTGTTGGCCTGACCTGCTGATTAC-3′, 5′-GCTAAAGGGCATATCCACACAACAC-3′. Pdgfra primers (first round of amplification): 5′-AATCTCTGAGACAGAGGACAC-3′, 5′-GCCACAGGAGGGAAAGTTT-3′. Pdgfra primers (second round of amplification): 5′-CACACAGTAATGTCCTCCATTGCT-3′, 5′-GCCATAGGACCAGCGTCACT-3′.

Immunostaining
The zona pellucida was removed using acid Tyrode’s solution (Sigma). Embryos were fixed in 4% paraformaldehyde (PFA) in PBS with 0.1% Tween 20 (Sigma) and 0.01% Triton X-100 (Sigma) overnight (2% PFA for 2 hours for Pdgfrα immunostaining) at 4°C, permeabilised in 0.55% Triton X-100 in PBS for 15 minutes and blocked in 10% fetal bovine serum in PBS for 1 hour. Primary antibodies used were: anti-cleaved caspase-3 (Cell Signalling), anti-Cdx2 (BioGenex), anti-DAB2 (BD Transduction Laboratories), anti-Gata4 (Santa Cruz), anti-Gata6 (R&D Systems), anti-GFP (Invitrogen) and anti-Pdgfrα (Santa Cruz, eBioscience) at 1/100, and anti-Nanog (Chemicon) at 1/150. Secondary Alexa Fluor (Invitrogen)-conjugated antibodies were used at a dilution of 1:500. DNA was visualized using Hoechst 33342 (5 μg/ml, Molecular Probes).

TUNEL analysis
Freshly recovered embryos were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed three times in PBSA and permeabilised for 2 minutes in 0.1% Triton X-100 and 0.1% sodium citrate in PBSA. Embryos were then washed three times in PBSA and incubated in TUNEL reaction mixture (Roche) for 1 hour at 37°C.

Cell number staging system for preimplantation embryos
By convention, mouse developmental stages are defined by the calculated time from which copulation is presumed to occur. However, embryos collected at the same time vary substantially in terms of cell number and developmental stage. Thus, we based our staging system on total cell number and collected embryos at approximately 3-hour intervals between E2.75 and E4.75. Fixed specimens were stained with a nuclear dye (Hoechst 33342). For in vivo observations, we used the nuclear- and plasma membrane-labelling vital dye FM4-64 (Invitrogen) at a dilution of 1/10 in M2 medium for 30 minutes at 37°C. Only non-dividing cells with clearly visible nuclei were included when scoring for nuclear-localised transcription factors by immunostaining.

Embryo transfer
Laser live-imaged embryos were transferred to pseudopregnant females and allowed to develop to term (Nagy, 2003). Four to six CAG::mRFP1Tg+ (Long et al., 2005) ‘carrier’ age-matched embryos were co-transferred.

Image acquisition
Laser scanning confocal images were acquired on a Zeiss LSM 510 META. Immunostained embryos were mounted in Vectashield (Vector Laboratories). Fluorescence was excited with a 405-nm laser diode (Hoechst), a 488-nm Argon laser (GFP), a 543-nm HeNe laser (Alexa Fluor 543, 555, FM4-64 and mRFP1) and a 633-nm HeNe laser (Alexa Fluor 633 and 647). Images were acquired using a Plan-apochromat 20×/NA 0.75 objective. Optical section thickness ranged from 1 μm to 4 μm. For 3D time-lapse imaging, 10-20 μm planes were acquired, separated by 3-4 μm. Time intervals between z-stacks were 7-15 minutes, for a total of 6-17 hours.

Image processing and analysis
Raw data were processed using Zeiss AIM software (Carl Zeiss Microsystems). Movies of time-lapse sequences were compiled and annotated using QuickTime (Apple Computer) and ImageJ (NIH). For fluorescence quantification measurements, images were analysed using IMARIS 6.0.1 software (Bitplane AG). Nuclei were identified using the ‘spot’ option with an estimated diameter of 7-10 μm in the Hoechst channel. The number of nuclei identified by the software was confirmed manually. Protein levels were analysed as mean fluorescence intensities inside ‘spot’ regions of interest (ROI), and were normalised by dividing by mean fluorescence intensity in the Hoechst channel.

RESULTS

A strain harbouring a histone H2B-GFP reporter knock-in at the Pdgfra locus is a high-resolution live imaging reporter of PrE
Using whole-mount immunofluorescence staining at E4.0, a time when the PrE is established, we confirmed that Pdgfrα protein is localised within ICM cells adjacent to the blastocyst cavity, consistent with the PrE. At E4.0, Pdgfrα was co-expressed with Gata4, a zinc-finger-containing transcriptional regulator and known PrE marker (Fig. 1A, see also Movie 1 in the supplementary material).

We next investigated whether a pre-existing PdgfraH2B-GFP line (Hamilton et al., 2003) could serve as a reporter of PrE. In E4.0 PdgfraH2B-GFP embryos, GFP was colocalised in all cells with detectable endogenous Pdgfrα, which localised to the plasma membrane (Fig. 1B). We were able to detect GFP fluorescence earlier than Pdgfrα protein. In embryos with 16-32 cells (n=9), in which endogenous Pdgfrα was undetectable, some cells were weakly GFP-positive (see Fig. S1A in the supplementary material), whereas in 33- to 64-cell embryos (n=6; see Fig. S1B in the supplementary material), a subset of GFP-positive cells was negative for Pdgfrα (endogenous protein was always localised to GFP-positive cells). This was not unexpected, because a lower level of total fluorescence is required for detection in a volumetrically constrained structure, such a nucleus, than in a volumetrically dispersed structure, such as the plasma membrane. By the 64-cell stage, all GFP-positive cells were positive for Pdgfrα protein (n=10; see Fig. S1C in the supplementary material). These observations were supported by RT-PCR (see Fig. S1D in the supplementary material).
We next demonstrated the colocalisation of GFP with markers of the PrE, including Gata4, Gata6 and the cell adhesion protein Dab2 (Fig. 1C-F). GFP fluorescence was restricted to cells of the parietal (arrowheads) and visceral endoderm, the two derivatives of the PrE (Fig. 2F,G). The co-expression of the Pdgfr\textsubscript{α}H2B-GFP reporter and endogenous Pdgfr\textsubscript{α} protein with established PrE markers confirmed that Pdgfr\textsubscript{α} could be used as a marker of PrE formation.

**Changes in Pdgfr\textsubscript{α}H2B-GFP reporter expression correlate with specific developmental stages**

We used the Pdgfr\textsubscript{α}H2B-GFP line to investigate PrE formation with a high degree of spatial and temporal resolution. First, we examined changes in GFP localisation in live embryos collected every 2-3 hours between E2.75 and E4.75. We detected a few GFP-positive cells as early as the morula stage (~70-84 hours post coitum (hpc), 16-34 cells), but none in 8-cell embryos (n=7). In the majority of GFP-positive morulae, we detected GFP expression in some outer cells destined to form the TE, as well as in some inner cells destined to form the ICM (15/20; Fig. 2A). By the early blastocyst stage (~87-96 hpc, 64 to ~79 cells), GFP was almost always restricted to the ICM and was distributed in an apparently random, heterogeneous manner, resembling the distribution of other known PrE markers (Chazaud et al., 2006; Gerbe et al., 2008). Although the level of fluorescence varied among cells, the majority of ICM cells (68.2% of 151 cells scored) were GFP positive (Fig. 2C,D; n=7 embryos). Interestingly, as total cell number increased, the strongest GFP expression became progressively more restricted to cells lining the cavity, whereas cells exhibiting reduced levels of GFP fluorescence were located deeper within the ICM (Fig. 2D). In the majority of late blastocysts (~96-114 hpc, >80 cells), GFP-positive cells were found exclusively in the nascent PrE layer (8/12; Fig. 2E,F).

Our observation of Pdgfr\textsubscript{α}H2B-GFP reporter expression between the early morula and the late blastocyst revealed distinct phases during PrE formation that correlated with developmental stage as defined by total cell number.
Expression of lineage-specific transcription factors is not mutually exclusive in the morula (16-33 cells) and early blastocyst (<32 cells)

To elucidate the significance of early Pdgfra<sup>H2B-GFP</sup> expression, we examined the distribution of the EPI-specific homeobox transcription factor Nanog and the PrE-specific factors Gata4 and Gata6 during this period (reviewed by Yamanaka et al., 2006). Expression of Gata6 has previously been reported in blastocysts (Chazaud et al., 2006; Koutsourakis et al., 1999), and in ‘late morulae/early blastocysts’ (Rossant et al., 2003), whereas the expression of Nanog has been reported as early as the 8-cell stage (Dietrich and Hiiragi 2007). We used total cell number for staging embryos, as we noted that embryos collected at the calculated same time after mating varied in cell number (see Fig. S2A in the supplementary material), even within the same litter (see Fig. S2B in the supplementary material). Interestingly, although total cell
number appeared to increase approximately linearly with time (see Fig. S2C in the supplementary material), embryos with 30-36 cells and 58-72 cells were disproportionately over-represented (see Fig. S2D in the supplementary material), suggesting that some degree of synchrony in the cell cycle may exist until at least the 64-cell stage. This also appeared to be supported by a relative ‘levelling out’ in the rate of increase in cell number at these stages (see Fig. S2C in the supplementary material).

We detected Gata6 in the majority of embryos as early as the 8-cell stage (9/11; Fig. 3B), in some cells in 4-cell embryos (2/9), but not at the 2-cell stage (0/17). This is the earliest onset of expression reported for any PrE-specific marker. In agreement with a previous study (Dietrich and Hiiragi, 2007), we detected Nanog in most 8-cell stage embryos (10/11). GFP expression was detected from the 16-cell stage in a subset of cells (Fig. 2A) in PdgfraH2B-GFP embryos, and from its onset overlapped with expression of Gata6 (Fig. 3A). At the morula stage, Nanog (n=33), and Gata6 (n=12) were detectable in almost all non-dividing inner and outer cells (88.8% of 770 cells were Nanog positive and 92.2% of 380 cells were Gata6 positive), in agreement with previous observations (Strumpf et al., 2005). Strikingly, the localisation of these two factors was not mutually exclusive and overlapped at these early stages (see Table S1 in the supplementary material). Moreover, we observed strong Gata6- and/or Nanog-positive staining in both inside and outside cells (Fig. 3C). This observation mirrors a recent report for the expression of Nanog relative to that of the TE-associated transcription factor Cdx2 (Dietrich and Hiiragi, 2007).

A comparable expression pattern was observed in very early blastocysts of up to 33 cells (n=43 for Nanog, n=7 for Gata6), with both factors detected in both ICM and TE cells (83.3% of 1314 cells analysed were Nanog positive and 91% of 221 cells were Gata6 positive; Fig. 3D). Similar to at earlier stages, Nanog and Gata6 expression were not mutually exclusive at this stage (n=19; Fig. 3C,D; see also Table S1 in the supplementary material). This is in contrast to previous reports describing the localisation of these factors in later blastocysts (Chazaud et al., 2006; Gerbe et al., 2008).

To investigate further the relationship between Nanog and Gata6 expression, we measured signal intensity within each cell nucleus for these factors and calculated correlation coefficients (see Table S2 in the supplementary material). Embryos of less than 30 cells generally showed no correlation between Nanog and Gata6 expression. With increasing total cell number, an increased frequency of positive correlations was noted when calculated across all cells. When calculated across only the 25% of cells exhibiting the highest Nanog and Gata6 expression (defined as the product of normalised Nanog and Gata6 signal intensities), we recorded strong negative correlations, with the frequency increasing with total cell number (see Table S2 in the supplementary material). Thus, positive correlations in low-expressing cells may reflect a variation amongst cells in general levels of transcription, whereas negative correlations observed in high-expressing cells may suggest that the effect of mutually inhibitory pathways becomes significant with the increased expression of these factors.

At around the 32-cell stage, the pattern of PdgfraH2B-GFP expression showed no consistent relation to Nanog distribution (n=7; see Table S3 in the supplementary material). We were unable to detect expression of the PrE-specific factor Gata4 during these stages (Fig. 4A).

We also noted that strongly Nanog-positive cells in the TE did not exhibit reduced levels of Cdx2 (see Fig. S3 in the supplementary material), in agreement with a recent report (Dietrich and Hiiragi, 2007) revealing no reciprocal relationship between Nanog and Cdx2 at these stages. Thus, in embryos with up to 32 cells, PrE and EPI markers are not restricted to the ICM, nor, together with Cdx2, are they mutually exclusive.

**Changes in lineage-specific gene expression during the 32- to 64-cell transition**

We observed a decreasing proportion of Nanog-positive and Gata6-positive cells (63.3% of 2303 cells scored in n=54 embryos and 62.7% of 346 cells in n=8 embryos, respectively) in blastocysts that had entered the next round of cell divisions (between 33 and 63 cells). This was more pronounced in TE cells. During this transition, the expression patterns of Gata6 and Nanog became increasingly mutually exclusive (n=8; Fig. 3E; see also Table S1 in the supplementary material). Ubiquitous or near-ubiquitous Nanog expression was not observed in blastocysts of more than 36 cells (Fig. 3E, Fig. 4B). We speculate that this...
period reflects a transition to a transcription factor-dependent phase of TE maintenance, following an earlier phase of Cdx2-independent TE differentiation (Niwa et al., 2005; Ralston and Rossant, 2008).

The onset of Gata4 expression occurred in embryos of at least 58 cells, with detectable staining in 60% (n=15) of embryos containing 58-63 cells (Fig. 4B,E,F). The number of Gata4-positive cells per embryo during this phase was variable and ranged from two to 13. Interestingly, we also observed that, in contrast to Gata6 staining, Gata4-positive cells were rarely Nanog positive (see Movie 4 in the supplementary material); only 15% of Gata4-positive cells were also Nanog positive (n=45), whereas 48.5% of Gata6-positive cells (n=268; see Table S1 in the supplementary material) were also Nanog positive at this stage.

Fig. 4. Localisation of Nanog, Gata4 and GFP in Pdgfra<sup>H2B-GFP<sup>+/−</sup> blastocysts. (A) In a 32-cell blastocyst, Nanog and GFP show broad, overlapping expression; Gata4 is undetectable. (B) Salt-and-pepper distribution of Nanog, Gata4 and GFP in a 64-cell blastocyst. Gata4 is expressed only in a subset of Nanog-negative and GFP-positive cells. (C) Partial segregation of Nanog-positive and Gata4-/GFP-positive cells in a 115-cell blastocyst. Gata4 and GFP are co-expressed in cells mostly localised to the nascent PrE layer. Nanog is expressed in the EPI layer and no longer colocalises with Gata4 or GFP. (D) In a 118-cell blastocyst, PrE markers (Gata4 and GFP) are co-expressed and mutually exclusive from the EPI marker Nanog; cells expressing PrE and EPI markers are fully restricted to their respective layers. Each row represents a single optical section of one embryo. bf, bright field; green, GFP; white, Nanog; red, Gata4 (A-D); blue, Hoechst. Scale bar: 20 μm. (E) Comparison of the number of Gata4- and Nanog-positive cells versus total cell number. An abrupt decrease in the number of Nanog-expressing cells at around the 64-cell stage coincides with the emergence of Gata4-expressing cells. Thereafter, the number of Nanog-expressing cells appears to increase only slightly, whereas the number of Gata4-expressing cells increases approximately linearly. (F) Comparison of the number of Gata4- and GFP-expressing cells with respect to total cell number.
**PrE formation is preceded by the downregulation of Nanog and upregulation of Gata4 in presumptive PrE precursors at around the 64-cell stage**

At around the 64-cell stage, the localisation of lineage-specific markers underwent a dramatic transition. The number of embryos with Gata4-positive cells increased significantly between the stages of 64 to 80 cells (81.5%, n=27). Consistent with previous observations of other PrE markers (Chazaud et al., 2006; Gerbe et al., 2008), Gata4-positive cells were distributed in an apparently random heterogeneous pattern within the ICM (Fig. 4B, Fig. 5A,B). Similar to during the 32- to 64-cell transition, we observed very little overlap between Gata4 and Nanog staining. In 40 embryos of 58-79 cells, only 7.8% (n=204) of Gata4-positive cells were also Nanog positive. This is in agreement with a single-cell microarray analysis (Kurimoto et al., 2006), in which Gata4 was more consistently expressed than Gata6 in cells exhibiting a PrE-like expression profile. Together, these results indicate that Gata4 is a more specific marker of PrE fate than Gata6, and for this reason it was favoured in our further analyses of PrE formation.

We also observed a decreased number of Nanog-positive cells in the TE (n=25 embryos). The proportion of cells positive for both GFP and Nanog decreased significantly to only 20.8% (n=101 cells), in the nine embryos analysed for both factors.

It is interesting to note that the decrease in both the proportion and the absolute number of Nanog-positive cells correlated with the appearance of Gata4-positive cells, whose number increased approximately linearly thereafter (correlation coefficient: +0.78). Meanwhile, the number of Nanog-positive cells remained constant with increasing total cell number (correlation coefficient: +0.025; Fig. 4E). Considering their mutual exclusiveness during this period, it is likely that the divergence in relative numbers of Nanog- and Gata4-expressing cells was largely due to differences in proliferation rates.

**Establishment of the nascent primitive endoderm layer occurs in embryos of more than ~80 cells**

Approximately one half of embryos with 80-100 cells exhibited partial or complete segregation of Gata4-positive cells to the PrE layer and Nanog-positive cells to the EPI layer (53.1%; n=32; Fig. 5I), whereas the remainder still exhibited a salt-and-pepper distribution. In the latter, Gata4 and GFP expression were almost invariably exclusive from Nanog expression. In embryos with partial segregation of the EPI and the PrE, which we defined as an essentially formed PrE with EPI and the PrE, which we defined as an essentially formed PrE with one or two Gata4-positive cells persisting in deeper layers of the ICM (Fig. 5C), those cells usually exhibited lower levels of fluorescence than did GFP-positive/Gata4-positive cells in the PrE layer (see Fig. S4 in the supplementary material).

In 80.2% (n=96) of embryos of >100 cells, Gata4-positive cells were restricted to the PrE layer (Fig. 4D, Fig. 5I). PrE formation was invariably complete in embryos with more than 155 cells (n=8). At no stage was Gata4 observed in TE cells. We found that the number of GFP-positive cells increased approximately linearly (Fig. 4F).

Significantly, in Pdgfra<sup>H2B-GFP</sup> embryos of >64-cells, Gata4 was expressed only in GFP-expressing cells (n=80), although in 64- to 100-cell embryos GFP-positive/Gata4-negative cells were frequently encountered. These data indicate that Gata4-expressing PrE precursors are recruited from the population of Pdgfra<sup>H2B-GFP</sup>-expressing cells at around the 64-cell stage. Subsequently, they become segregated to the layer of cells lining the cavity. This process starts at around the 80-cell stage and is almost complete in embryos of >100 cells.

**Live-imaged Pdgfra<sup>H2B-GFP</sup> embryos progress through stages comparable with in vivo development**

To document the dynamics of sorting of PrE and EPI precursors, we performed a 3D time-lapse analysis. Live-imaged Pdgfra<sup>H2B-GFP</sup> embryos progressed through stages comparable with development.
in utero (Fig. 5E-H). The earliest cells expressing GFP were distributed in a random heterogeneous manner in morulae and early blastocysts (see Movie 5 in the supplementary material). By the mid-blastocyst stage, the majority of ICM cells expressed GFP at variable levels. Subsequently, GFP-positive cells comprised the layer of cells lining the cavity, which we interpreted as the nascent PrE (Fig. 5; see also Movie 6 in the supplementary material). As an additional criterion, we were also able to identify the PrE morphologically under bright-field illumination (arrowheads, Fig. 5I’).

Although the transition from an apparently random distribution of GFP-positive cells to a nascent PrE layer is usually a gradual process taking several hours, our data revealed that this transition can occur very rapidly – within only 15 minutes – in some embryos (see Movie 7 in the supplementary material). After the PrE was formed, in some cases we were still able to distinguish cells with weak GFP signal deeper within the ICM. However, from movies of embryos that had been cultured beyond the 128-cell stage, it could be seen that these cells either downregulated GFP until it was no longer detectable or underwent cell death, evident by nuclear fragmentation. Concomitantly, the GFP signal intensity in cells lining the cavity increased (see Fig. S4 and Movie 8 in the supplementary material).

PrE- and EPI-specific markers showed normal (similar to in utero) segregation in embryos fixed after 17 hours of live imaging (n=24; see Fig. S5 in the supplementary material). Embryos transferred to recipient females after >14 hours of imaging developed to term, were viable and exhibited normal fertility in adult life. Thus, live imaging did not compromise either the viability of embryos or the process of lineage segregation. The latter was similar to that observed in stage-matched freshly flushed embryos.

**Directionally biased relocation and apoptosis of GFP-positive cells contribute to cell sorting during PrE formation**

It was recently suggested that cell sorting of PrE and EPI precursors leads to the segregation of these two lineages (Rossant et al., 2003; Chazaud et al., 2006). To explore this process, we analysed the behaviour of 90 GFP-positive cells in six embryos developing through the 32- to 64-cell transition (early-stage movies; see Movie 5 in the supplementary material) and 150 GFP-positive cells in six embryos developing through the 64- to 128-cell transition (late-stage movies; see Movies 6 and 7 in the supplementary material). We scored the original position of each GFP-positive ICM cell within the embryo as being either ‘cavity’ (in contact with the cavity) – or ‘inner’ (lying within the deeper layers of the ICM). We then scored the final position at the end of the movie; see Table S4 in the supplementary material) of the original cell, or of its daughter cells if the cell had divided.

We observed that almost half of GFP-positive cells in the early-stage movies (42/90) and a third in the late-stage movies (52/150) acquired a position within a layer different from that of its original position (or that of its parental cell in cases where division had occurred). Some of the cells analysed exhibited downregulation of GFP to undetectable levels or underwent apoptosis (see Table S4 in the supplementary material). These cells were not included in the calculation of the final position of cells within an embryo, as this could not be reliably determined.

In the early stage movies, the vast majority (72.5%, n=40) of GFP-positive cells originating in the cavity layers maintained their position there, whereas only 34% of GFP-positive cells (n=47) originating in the inner layers maintained their position. This indicated that although relocation of GFP-positive cells could occur in any direction, it was strongly biased towards the cavity. During the 64- to 128-cell stage transition, this tendency became even more pronounced, as 100% of GFP-positive cells (n=64) originating in the cavity layer maintained their position, whereas only 15.8% of GFP-positive cells originating in the inner layers maintained theirs (n=57). GFP-positive cells that remained in the inner layers once the PrE layer had formed were observed to be undergoing apoptosis or downregulating GFP (see Movie 8 and Fig. S4 in the supplementary material).

It has previously been reported that a small proportion of cells in mid-to late blastocysts undergo apoptosis (Copp, 1978). As H2B-GFP localises to chromatin, it served as a live imaging reporter of the nuclear fragmentation associated with cell death. Indeed, we observed apoptosis during the 64- to 128-cell transition (see Table S4 in the supplementary material), but not during the 32- to 64-cell stage transition (n=90). We detected a steadily increasing rate of apoptosis in embryos of more than 64 cells. The number of GFP-positive cells lining the cavity, observed in late-stage movies, that underwent apoptosis (8.1%, n=123 cells) was similar to that reported previously for ICM cells (Copp, 1978). Surprisingly, in the subset of GFP-positive cells that remained deeper within the ICM and isolated from other GFP-positive cells, the rate of apoptosis was considerably higher (48.2%, n=27). GFP-positive cells that maintained an inside position during the 64- to 128-cell transition yet did not apoptose were more likely to downregulate reporter expression (915 cells). These observations were confirmed by TUNEL analysis and activated-caspase staining (see Fig. S6 in the supplementary material).

These results are consistent with the recent model of PrE formation (Rossant et al., 2003; Chazaud et al., 2006), whereby cells expressing PrE markers relocate unidirectionally towards the cavity, whereas those already lining the cavity are likely to maintain their position. In addition, our data point to apoptosis as being an additional mechanism that ensures appropriate segregation of the PrE and EPI layers. GFP-positive cells that do not contribute to PrE or downregulate Pdgfrα are eliminated from the EPI layer by apoptosis.

**Multiple cell behaviours contribute to cell sorting during PrE formation**

We distinguished several modes by which GFP-positive cells could contribute to the cavity layer (Fig. 6; see also Movie 9 in the supplementary material). ‘Intercalation due to cavity expansion’ occurred when a cell intercalated into the layer of cells lining the cavity due to expansion of the cavity and the accompanying ‘stretching’ of cell layers. ‘Intercalation by cell division’ occurred when a cell either acquired a position different from that of its parent cell because of the orientation of division of that parent cell, or acquired a new position because of cell rearrangements resulting from the division of a neighbouring cell. In another category, termed ‘cell allocation due to other reasons’, a cell changed its original position for reasons that could not be distinguished as being either a ‘passive’ mechanism (such as intercalation by cell division or cavity expansion) or active migration. The frequencies of each mode of cell behaviour during early- and late-stage movies are shown in Fig. 6B.

During the 32- to 64-cell transition, the majority of GFP-positive cells that were positioned in the cavity layer by the end of the time-lapse sequence originated in the cavity layer and/or were passively incorporated into it as a result of cavity expansion (75.4%, n=61, Fig. 6), similar to during the 64- to 128-cell transition (67.3%, n=113, Fig. 6). Only a minority of GFP-positive cells acquired their final position in the cavity layer by cell allocation due to other reasons in both early-stage (18%) and late-stage (31.2%) movies.
heterogeneous expression of \( \text{Pdgfr}^{\text{H2B-GFP}} \) is reminiscent of the recently reported salt-and-pepper expression of Nanog and Gata6 in the ICM of the E3.5 blastocyst (Chazaud et al., 2006; Rossant et al., 2003). This, along with the lineage tracing studies, has led to a model for lineage specification in which EPI and PrE precursors are specified in a possibly random manner within the ICM and later segregate to their respective layers. This is in contrast to the previously assumed model that cell position with respect to the blastocyst cavity is the primary determinant of PrE fate (reviewed in Yamanaka et al., 2006).

Our results revealed a multi-step process of PrE formation that shares features of both of these models. As also shown by Dietrich and Hiiragi (Dietrich and Hiiragi, 2007), lineage-specific factors initially exhibit widespread and overlapping expression in early blastocysts. This is followed by progression towards the mutually exclusive expression of Cdx2 in the TE (Dietrich and Hiiragi, 2007) (this study) and Nanog, Gata6 and \( \text{Pdgfr}^{\alpha} \) in the ICM (this study, see Fig. 7) and, subsequently, by the mutually exclusive expression of EPI- and PrE-specific markers in the ICM during the 32- to 64-cell transition.

We also noticed that at early stages (morula and early blastocyst), the levels of Gata6 and Nanog vary among cells throughout the embryo and generally appear to be mutually independent. A similar observation was described by Dietrich and Hiiragi for TE and ICM markers (Dietrich and Hiiragi, 2007). There are two possible explanations for this type of expression. First, the relative levels of different factors, while varying among cells, may remain fairly constant within individual cells. Differences would then become amplified by the maturation of mutually inhibitory pathways at later stages. Alternatively, it is possible that the relative levels of factors fluctuate with time. Interestingly, it was reported recently that Nanog expression fluctuates in ES cells (Chambers et al., 2007). Low levels of Nanog predisposed cells towards differentiation, but did not mark commitment. In another study, cells were flow-sorted according to their level of Nanog expression and, when subsequently cultured, they reverted to their original heterogeneous state. An association between stochastic gene expression and differentiation has been reported in other systems (Hu et al., 1997), but evidence during embryo development, especially in the mouse, has previously been lacking (reviewed by Laforge et al., 2005; Martinez-Arias and Hayward, 2006).

It seems plausible that such a mechanism may operate within the ICM. We propose that before blastocyst formation, a large number of genes exhibit fluctuating and mutually independent expression at low levels. As transcription factor levels increase, mutually inhibitory regulatory pathways begin to take effect. The latter is supported by our observation that mutually exclusive expression is typically observed only in the subset of cells with the highest expression levels. Heterogeneous expression of Nanog that correlated negatively with Gata6 expression has recently been reported in mouse ES cells (Singh et al., 2007). Nanog was shown to repress Gata6 directly, through binding of its promoter. Conversely, the Grb/Mek pathway, which regulates Gata6 expression, has been shown to repress Nanog expression (Hamazaki et al., 2006).

Our data suggest that downregulation of Nanog and Gata6 occurs in two phases. The first reflects downregulation in TE cells, which was often evident immediately after blastocyst formation but had invariably commenced in all blastocysts of at least 37 cells. The second phase initiates around the 64-cell stage, when the number of Nanog-positive cells drops dramatically and thereafter remains relatively constant. This phase reflects the maturation of the

**DISCUSSION**

We have investigated the events leading to formation of the primitive endoderm in the preimplantation mouse embryo. Our results indicate that, during early stages of development, cells co-express markers for different lineages. Subsequently PrE fate results from a progressive restriction of expression of PrE-specific markers followed by cell sorting.

Pdgfra is a novel marker for PrE. A mouse line expressing histone H2B-EGFP reporter from the Pdgfra locus served as a live imaging reporter of PrE. Pdgfra\(^{\text{H2B-GFP}}\) expression is initially distributed in an apparently random, heterogeneous manner until approximately the 64-cell stage, or after six rounds of divisions. The early
presumed inhibitory mechanisms between Nanog- and Gata4/Gata6-expressing pathways, analogous to the state of ES cells.

We propose that upregulation of Gata4 and downregulation of Nanog in a subset of ICM cells at around the 64-cell stage reflects a crucial stage of stabilisation, or fixing, of a previously fluctuating expression state. Lineage tracing data (Perea-Gomez et al., 2007) suggests that 30% of ICM cells from early blastocysts have a dual fate, with daughter cells occupying both EPI and PrE layers. This is in contrast to the restricted fate of ICM cells found in what were likely to be later stage blastocysts (Chazaud et al., 2006). This would suggest that fate becomes restricted around the mid-blastocyst (~64-cell) stage, and is coincident with changes in marker expression.

Our data support the restriction of ICM cell fate preceding cell sorting. Despite this, our live imaging data revealed an upregulation of Pdgfrcα expression in cells lining the blastocyst cavity and downregulation in deeper-lying cells, supporting a role for positional signals. The variety of behaviours of GFP-positive cells we observed in our live imaging studies suggests a more complex model of cell sorting than a simple segregation of precursors to the respective EPI and PrE layers. The failure of some GFP-positive cells to contribute to the PrE suggests that, although the mutually exclusive expression of Nanog and Gata6 biases cells towards a particular fate, positional signals are required to complete or reinforce specification. Cell polarisation is likely to be involved, consistent with the observation that, prior to PrE formation, the sub-cellular localisation of the PrE markers Lrp2 (megalin) and Dab2 becomes polarised only in cells lining the cavity (Gerbe et al., 2008).

We observed that GFP-positive cells lining the cavity rarely changed their position. By contrast, deeper-lying cells tended to be more migratory, but lost this property upon reaching the cavity. Thus, even a randomly directed migration of deeper-lying cells would suffice to drive the majority of cell sorting because PrE precursors, once in contact with the cavity, will remain there. The probability of PrE precursors reaching the cavity would also be enhanced by adhesive contacts with any neighbouring PrE precursors that already line the cavity. The adhesive properties of PrE precursors have previously been implicated in PrE formation (reviewed by Yamanaka et al., 2006).

Once a PrE precursor reaches the cavity, its position may be maintained by establishing cell polarity and epithelial properties, to the competitive exclusion of non-polarised cells. In a study of mouse blastocyst formation, it was shown that differences in epithelial properties between subsets of cells were sufficient to influence their propensity to occupy an outer position and to contribute to the TE (Plusa et al., 2005). Thus, PrE precursors occupying the cavity surface may have a propensity to flatten, due to cell polarisation. Increased flattening would cause cells to occupy a greater surface area of the cavity, to the competitive exclusion of non-polarised EPI precursors. Deeper-lying PrE precursors that maintain contact with such cells would thus be able to maintain proximity to the cavity.

An additional selective mechanism, using apoptosis, appears to account for the minority of PrE precursors that fail to come into contact with the cavity. The prevalence of apoptosis in deeper-lying GFP-positive cells after the 64-cell stage suggests that the stabilisation of PrE-specific transcription factor expression in strongly GFP-positive cells excludes EPI potential, and leaves the cell with either of two possible outcomes: contributing to the PrE or defaulting to apoptosis. More weakly GFP-expressing cells showed a tendency to downregulate expression, suggesting that they may retain the potential to form EPI. In agreement with our observations, a surge in the frequency of cell death in the ICM of embryos of 60-110 cells was previously reported (Copp, 1978). We suggest that one of the primary functions of apoptosis within the ICM is the elimination of inappropriately positioned PrE precursors. This is supported by our observation that the probability of GFP-positive cells undergoing apoptosis is much greater for inner cells than for those in the PrE layer.

In summary, our results reconcile the two apparently disparate models of PrE lineage specification within the ICM of the mouse blastocyst. Our observations lead us to propose a three-step model in which stochastic expression of lineage-specific transcription factors, at the 16- to 32-cell stage, precedes the maturation of mutually inhibitory regulatory pathways, leading to a salt-and-pepper distribution of EPI and PrE precursors in the ICM after the 64-cell stage. This is followed by cell sorting, which may be largely, if not completely, explained by a passive, selective mechanism involving cell movement, cell adhesion and apoptosis.

Fig. 7. Multi-step model of EPI/PrE lineage formation. Initial (16- to 32-cell stage) overlapping expression of lineage-specific transcription factors is followed by a progression towards a stabilised salt-and-pepper pattern of PrE (Gata6) and EPI (Nanog) expression (32- to 64-cell stage). Subsequently, stabilised Nanog or Gata6 expression biases cells towards a particular fate (~64-cell stage) and cell sorting proceeds, although positional signals (arrows) are still required to complete specification.
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Supplementary material
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