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Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse

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ABSTRACT

Early mammalian embryogenesis is controlled by mechanisms governing the balance between pluripotency and differentiation. The expression of early lineage-specific genes can vary significantly between species, with implications for developmental control and stem cell derivation. However, the mechanisms involved in patterning the human embryo are still unclear. We analyzed the appearance and localization of lineage-specific transcription factors in staged preimplantation human embryos from the zygote until the blastocyst. We observed that the pluripotency-associated transcription factor OCT4 was initially expressed in 8-cell embryos at 3 days post-fertilization (dpf), and restricted to the inner cell mass (ICM) in 128–256 cell blastocysts (6 dpf), approximately 2 days later than the mouse. The trophoblast-associated transcription factor CDX2 was upregulated in 5 dpf blastocysts and initially coincident with OCT4, indicating a lag in CDX2 initiation in the TE lineage, relative to the mouse. Once established, the TE expressed intracellular and cell-surface proteins cytokeratin-7 (CK7) and fibroblast growth factor receptor-1 (FGFR1), which are thought to be specific to post-implantation human trophoblast progenitor cells. The primitive endoderm (PE)-associated transcription factor SOX17 was initially heterogeneously expressed in the ICM where it co-localized with a sub-set of OCT4 expressing cells at 4–5 dpf. SOX17 was progressively restricted to the PE adjacent to the blastocoel cavity together with the transcription factor GATA6 by 6 dpf. We observed low levels of Laminin expression in the human PE, though this basement membrane component is thought to play an important role in mouse PE cell sorting, suggesting divergence in differentiation mechanisms between species. Additionally, while stem cell lines representing the three distinct cell types that comprise a mouse blastocyst have been established, the identity of cell types that emerge during early human embryonic stem cell derivation is unclear. We observed that derivation from plating intact human blastocysts resulted predominantly in the outgrowth of TE-like cells, which impairs human embryonic stem cell derivation. Altogether, our findings provide important insight into developmental patterning of preimplantation human embryos with potential consequences for stem cell derivation.

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Introduction

Human and mouse embryo development begins in transcriptional silence with an oocyte to zygotic transition. This encompasses fertilization and karyogamy, epigenetic reprogramming, a series of cleavage divisions and embryonic genome activation

(EGA). The first molecular difference is the timing of EGA, which initiates between the 1- and 2-cell stage for mice (Flach et al., 1982; Wang et al., 2004), and the 4- and 8-cell stage for humans (Braude et al., 1988; Tesarik et al., 1988). A compacted morula forms following EGA marking the first morphological indication of a break in radial symmetry.

Subsequent cell divisions and cavitation lead to the formation of the blastocyst comprised of a fluid-filled blastocoel cavity and an inner cell mass (ICM), surrounded by trophoblast (TE) cells. Humans lag behind mice in the timing of blastocyst formation, which corresponds to 3–3.5 days post-fertilization (dpf) in mice in contrast to 4–5 dpf in humans (Brinster, 1963; Hertig et al., 1959; Steptoe et al., 1971). At the time of implantation into the maternal uterus, the ICM is comprised of epiblast progenitor and

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primitive endoderm (PE) cells. Mouse blastocysts implant at approximately 4–4.5 dpf, whereas human embryos undergo at least one additional cell division and implant between 6 and 8 dpf (Cockburn and Rossant, 2010; Finn and McLaren, 1967; Hertig et al., 1959; Niakan et al., 2012; Norwitz et al., 2001). Lineage studies in mice indicate that after implantation TE cells contribute to the placenta, PE cells contribute principally to the yolk sac and epiblast progenitor cells contribute to the fetus and extraembryonic mesoderm (Gardner, 1985; Gardner et al., 1973; Gardner and Rossant, 1979; Papaioannou et al., 1975).

In mice, the octamer-binding transcription factor Oct4 (also known as *Pou5f1*) is required for the formation of the ICM, while the caudal-like transcription factor *Cdx2* is required for TE development (Chawengsaksophak et al., 1997; Nichols et al., 1998; Strumpf et al., 2005). Zygotic Oct4 mRNA and protein are initially expressed in all cells of mouse embryos from the 4-cell (Nichols et al., 1998) and 8-cell stage, respectively (Liu et al., 2004; Palmieri et al., 1994). Similarly, Oct4 protein was detected in 8-cell bovine embryos (Khan et al., 2012). Variable (mosaic, detectable and undetectable) *Cdx2* protein expression has been noted in mouse embryos at the 8-cell stage (Ralston and Rossant, 2008) and *Cdx2* is detectable in the nucleus of 16-cell morulae at 2.5 dpf (Dietrich and Hiiragi, 2007; Jedrusik et al., 2010; Ralston and Rossant, 2008; Strumpf et al., 2005). *CDX2* expression in the cow follows blastocyst formation at approximately 5 dpf (Berg et al., 2011). However, it is unclear when embryonic OCT4 or *CDX2* protein expression is initiated in the human embryo and whether their expression resembles the mouse or the cow. Later in development, Oct4/OCT4 co-localizes with *Cdx2/CDX2* in the TE of mouse, the cow, pig and human embryos (Berg et al., 2011; Chen et al., 2009; Dietrich and Hiiragi, 2007; Kuijk et al., 2008; Pant and Keefer, 2009; Ralston and Rossant, 2008). Species-specific differences in the timing of the restriction of these key developmental regulators to the respective ICM and TE have been noted. For example, in contrast to mouse embryos where Oct4 expression is downregulated in the TE of blastocysts by 4.5 dpf (Dietrich and Hiiragi, 2007; Grabarek et al., 2012; Palmieri et al., 1994) human, cow, pig and rhesus monkey blastocysts have persistent OCT4 protein expression in the TE until 6–10 dpf, depending on the species (Berg et al., 2011; Cauffman et al., 2006; Chen et al., 2009; Harvey et al., 2009; Kirchhof et al., 2000; Kuijk et al., 2008; Mitalipov et al., 2003; Pant and Keefer, 2009; van Eijk et al., 1999). Altogether, this suggests that the segregation of the TE and ICM may be initiated just prior to implantation in many species, including humans, and that there may be species-specific differences in the appearance and localization of lineage specifying factors.

The earliest known event influencing differentiation of the mouse ICM into its two distinct components, the epiblast and PE, is signaling through the fibroblast growth factor (FGF)/Erk pathway that leads to expression of the GATA transcription factors, *Gata6* and *Gata4*, which are eventually restricted to the PE (Arman et al., 1998; Chazaud et al., 2006; Cheng et al., 1998; Feldman et al., 1995). More recently, we and others have demonstrated that the SRY HMG homeobox transcription factor, *Sox17* also functions in specifying PE cells from epiblast cells within the ICM (Artus et al., 2011; Morris et al., 2010; Niakan et al., 2010). It is thought that the inhibitory interactions between PE transcription factors *Gata6/Gata4/Sox17* oppose the expression of the epiblast-associated transcription factor *Nanog* that eventually compartmentalizes the ICM into distinct domains (Artus et al., 2011; Chazaud et al., 2006; Morris et al., 2010; Niakan et al., 2010; Plusa et al., 2008). These mechanisms are thought in part to be facilitated by expression of a cell-surface extracellular matrix protein *Laminin* and the receptor tyrosine kinase *Pdgfr α* (Chazaud et al., 2006; Niakan et al., 2010; Plusa et al., 2008; Yamanaka et al., 2006). Consistent with studies in the mouse (Chazaud et al., 2006; Plusa et al., 2008), *GATA6* is expressed broadly in the human embryo (Kimber et al., 2008; Kuijk et al., 2012; Roode et al., 2012). In late-blastocysts *GATA4*, *GATA6* and

SOX17 are restricted to a subset of putative PE cells in human embryos that lack *NANOG* expression (Kuijk et al., 2012; Roode et al., 2012). While mosaic expression of *GATA6* and *NANOG* has been shown in human mid-blastocysts (Roode et al., 2012), PE-associated protein expression has not been reported prior to the mid- to late-blastocyst and it remains unclear whether additional PE-associated genes, such as *SOX17*, are also heterogeneously expressed in the early human ICM.

Human embryonic stem cell (hESC) lines established from the ICM of preimplantation embryos (Thomson et al., 1998) are a tractable system for understanding molecular mechanisms of early embryogenesis and have tremendous potential in medical applications. Typically, hESC derivation involves isolating the human ICM either by chemical-based immunosurgery (Reubinoff et al., 2000; Solter and Knowles, 1975; Thomson et al., 1998) or laser-mediated TE ablation (Chen et al., 2009; Turetsky et al., 2008). hESCs have also been derived, although with lower efficiency, from intact blastocysts by isolating adherent ICM outgrowths prior to differentiation (Baharvand et al., 2004; Chen et al., 2009; Genbacev et al., 2005; Heins et al., 2004). Although ICM isolation significantly improves hESC derivation efficiency, it remains unclear what cell types emerge during early stem cell derivation from plated whole blastocysts and whether these cells may be promoted to self-renew as either hESCs or extraembryonic stem cells. We investigated the implication of the presence of extraembryonic cells in blastocyst outgrowths during hESC derivation. In contrast to stem cell derivation from plated mouse blastocysts, we found that although human extraembryonic cells initially proliferate, they cannot be maintained and negatively affect hESC derivation, underscoring the importance for ICM isolation.

To further understand lineage specification in the human preimplantation embryo we analyzed OCT4, *CDX2* and *SOX17* protein distribution from the zygote until the blastocyst. We observed that OCT4 was initially detectable at the 7 or 8-cell stage following EGA and was persistently expressed in the TE of blastocysts. *SOX17* and *CDX2* were detectable in the blastocyst at approximately 5 dpf, where they initially co-localize with OCT4 in the PE and TE, respectively. OCT4 expression in human embryos was primarily restricted to the ICM in late-blastocysts and downregulated in a subset of PE cells, which is consistent with recent observations (Roode et al., 2012) and similar to the mouse late-blastocyst (Grabarek et al., 2012). To understand the dynamics of pluripotency-associated gene expression we analyzed *NANOG* expression, which we confirm was restricted to the ICM prior to OCT4 (Kimber et al., 2008; Roode et al., 2012). To further understand human TE cell identity, we analyzed the expression of proteins identified in the post-implantation trophoblast such as cytokeratin-7 (CK7) and fibroblast growth factor receptor-1 (FGFR1), which were expressed in preimplantation human embryos. In all, our study significantly expands the characterization of protein expression patterns throughout human preimplantation development revealing unique properties of human embryogenesis that have important implications for stem cell derivation.

Materials and methods

All experiments were performed using human embryos that were excess to infertility treatment and donated for research following informed consent in accordance with the guidelines established by the Harvard University Embryonic Stem Cell Research Oversight (ESCRO) Committee and the Committee on the Use of Human Subjects Institutional Review Board (IRB).

Embryo culture

Cleavage or blastocyst stage human embryos were thawed using Quinn's Advantage Thaw Kit (Sage) according to the manufacturer's instructions. Embryos were cultured in Global Media (LifeGlobal) supplemented with 15% plasminase (Telacris) under mineral oil (Fertipro). Embryos were cultured in a humidified atmosphere at 37 °C and 5% CO₂ in air. Embryos that were of good quality with no significant fragmentation or lysis were used in subsequent experiments.

Immunohistochemistry of human preimplantation embryos

Embryos were placed directly in 4% paraformaldehyde and fixed for either 30 min at room temperature or overnight at 4 °C on a rotating shaker. Embryos were then transferred through several washes of 1X PBS plus 0.1% Tween-20 (PBS/0.1% T) to remove residual paraformaldehyde. Embryos were placed in PBS/1% T overnight on a rotating shaker at 4 °C for permeabilization. A longer permeabilization step was necessary to facilitate the detection of nuclear antigens; given the thickness of the human zona pellucida compared to the mouse. Embryos were blocked for 1 h at room temperature in blocking solution (10% serum diluted in PBS/0.1–0.5% T). Embryos were placed in primary antibodies at a concentration of 1:500 in blocking solution overnight at 4 °C on a rotating shaker (antibodies used in this study are listed in Supplementary Table 1). On the following day, embryos were transferred through several washes of PBS/0.1% T then placed in a last wash for 30 min. Secondary antibodies (Cy3, FITC or Cy5 donkey anti-rabbit, mouse or goat, Invitrogen) were diluted in blocking solution at 1:300 concentration. Embryos were placed in secondary antibody for 1 h at room temperature on a rotating shaker, transferred through several washes of PBS/0.1% T and placed in a last wash for 30 min. Embryos were placed in a 50 µl 1:3 dilution of Vectashield containing DAPI (Vector Labs): PBS/0.1% T on a coverslip bottom dish (MatTek) for confocal imaging.

Imaging and analysis

Laser scanning microscopy was performed using a Zeiss LSM 510 confocal microscope with META software and optical sections obtained every 1–3 µm with a pinhole diameter of < 2 Airy units. 25X and 40X oil-immersion objectives and 405, 488, 543 and 633 nm lasers were used. Cell numbers were counted using the IMARIS cell imaging software (Bitplane AG, Versions 5.7 and 6.0) and ImageJ using the Cell Counter Plugin. DAPI or transcription factor-stained nuclei were determined either visually or by segmentation using 3D reconstructions with isosurface areas of 5–10 µm (depending on the nucleus size) with background subtraction. Unmodified images were used for cell counting and nuclei were considered positive if the intensity was above cytoplasmic staining. Co-staining was determined by marking spots in one channel that co-localized with a DAPI spot. The images in some of the figures were modified by performing contrast and brightness enhancement using Adobe Photoshop CS2.

Blastocyst outgrowths

Human embryos cultured to 6 dpf were used for blastocyst outgrowth experiments. The zona pellucida was dissolved using Acidic Tyrode's Solution (Sigma) and embryos were washed in Global Media then plated on a feeder layer of gamma-irradiated mouse embryonic fibroblasts (MEFs) isolated from 12.5 dpf embryos and passaged three times before irradiation, in HUES cell derivation media. HUES cell derivation media consisted of 75% KO-

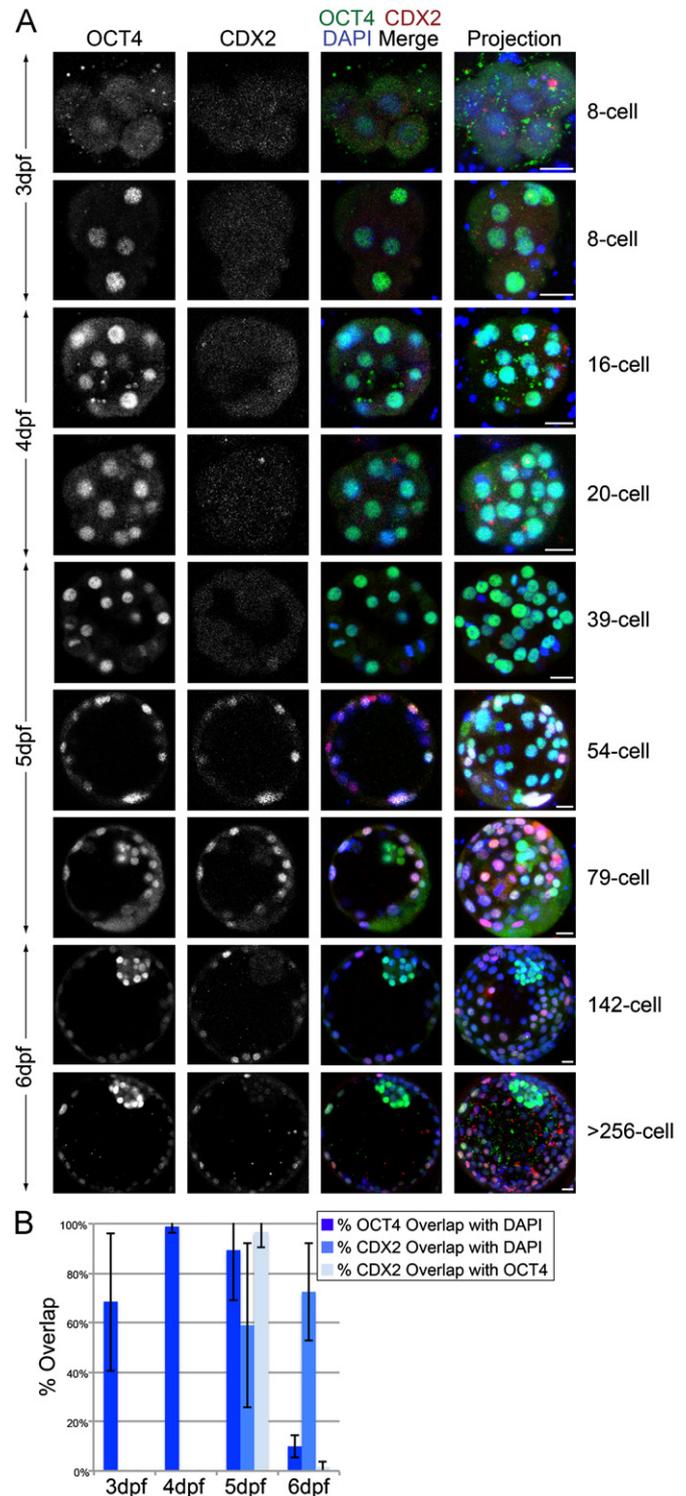


Fig. 1. Human blastocyst formation precedes CDX2 expression in the trophectoderm, where OCT4 persists. (A) Optical sections and 3D projections of immunostained human preimplantation embryos at defined stages of development. The number of days post-fertilization (dpf) and the approximate cell number is indicated for each embryo. OCT4 (green), CDX2 (red) and DAPI (blue) nuclear staining. Scale bar is 100 µm. (B) Quantification of the total number of OCT4 (dark blue bar) and CDX2 (medium blue bar) expressing cells as a percentage of total DAPI-expression. The percentage of CDX2 expressing cells that overlap with the expression of OCT4 is indicated by the light blue bar. Data are the mean ± standard deviation of biological replicates.

DMEM (Invitrogen), 10% KO-SR replacement (Invitrogen), 10% Plasmanate (Talecris), 2.5% embryonic stem cell tested Fetal Bovine Serum (FBS) (Hyclone), 2 mM Glutamax-1, 1% non-essential amino

acids, 50 units/ml penicillin and 50 μ g/ml streptomycin (Invitrogen), 0.1 mM β -mercaptoethanol (Invitrogen), 5 ng/ml bFGF (Invitrogen), as described previously (Cowan et al., 2004).

Results

Embryonic OCT4 expression was initiated at the 8-cell stage and CDX2 expression followed blastocyst formation

To gain further insight into the mechanism of lineage specification in human preimplantation development, we assessed the expression of key developmental regulators OCT4 and CDX2, which are functionally required for early mouse development (Chawengsaksophak et al., 1997; Nichols et al., 1998; Strumpf et al., 2005). As previously published (Chen et al., 2009), we thawed donated human in vitro fertilized embryos and selected those of good-quality (without significant lysis or fragmentation) for analysis of OCT4 and CDX2 expression at defined stages from the zygote to the blastocyst.

Similar to immunofluorescence results from mouse cleavage stage embryos (Dietrich and Hiiragi, 2007; Strumpf et al., 2005), it was unclear whether cytoplasmic staining in human embryos prior to the 8-cell stage represents specific signal or background. Consistent with these previous studies, we considered positive expression only when nuclear staining was clearly distinguishable from cytoplasmic staining. OCT4 protein expression was absent or below the level of detection in human embryos prior to 3 dpf with less than 7-cells (data not shown). OCT4 protein expression was first detectable in the nuclei of nearly all cells at the 7–8-cell stage (3 dpf), consistent with the initiation of human EGA (Braude et al., 1988; Tesarik et al., 1988) ($68 \pm 28\%$, $n=6$, Fig. 1A, B, see Table S2). We observed that OCT4 was nearly uniformly expressed in the nuclei of all cells up to 4 dpf ($99 \pm 3\%$, $n=11$, Fig. 1A, B, see Table S2). OCT4 expression remained high in both the ICM and TE in 5 dpf early-blastocysts between 33 and 128 cells and was downregulated in a subset of TE cells in mid-blastocysts ($90 \pm 20\%$, $n=21$, Fig. 1A, B, see Table S2). By 6 dpf, human blastocysts were comprised of approximately 129–256 cells and nuclear OCT4-high expression was primarily restricted to the ICM ($10 \pm 5\%$, $n=17$, Fig. 1A, B, see Table S2). Thus, initiation of OCT4 expression is consistent with the timing of human EGA and its restriction to the ICM is consistent with our previous study suggesting a correlation with the optimal time for hESC derivation at 6 dpf (Chen et al., 2009).

We also performed a detailed time-course analysis of the expression of CDX2, which is initiated in mouse embryos prior to the formation of a blastocyst (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Strumpf et al., 2005) and has been suggested to specify the TE lineage (Jedrusik et al., 2008, 2010). In contrast to the mouse, we observed that CDX2 protein was not appreciably expressed in human 8-cell and morula stage embryos at 3–4 dpf ($n=13$, Fig. 1A, B, see Table S2) and was first detectable in embryos at 5 dpf where its

expression was localized to the TE of blastocysts with a discernable blastocoel cavity ($59 \pm 33\%$, $n=10$, Fig. 1A, B, Table S1). CDX2 expression overlapped with the expression of OCT4 in nearly all TE cells at 5 dpf ($97 \pm 6\%$, $n=6$, Fig. 1A, B, see Table S2) and by 6 dpf, the human blastocyst was comprised largely of CDX2 expressing TE cells ($79 \pm 14\%$, $n=12$, Fig. 1A, B, see Table S2). It was unclear whether CDX2 expression between 5 and 6 dpf was lost, reduced or apparently reduced due to confocal optical sectioning. We used IMARIS cell imaging software to quantify the intensity of CDX2 expression in trophectoderm cells at 5 versus 6 dpf in all optical sections. This analysis suggests that while the proportion of CDX2 expressing cells per DAPI stained nuclei was not significantly different (Fig. 1B) and that CDX2-high cells were present at these two stages, there were more cells with moderate or weak CDX2 expression at 6 dpf (Fig. 1A). We estimate that there were between 1 and 2 cell divisions from the 35-cell embryo, when CDX2 expression was first observed, to the 130-cell embryo, when we first observed OCT4-high restriction to the ICM (see Table S2). This suggests that the restriction of OCT4-high expression to the ICM is likely to be the result of factors downstream of CDX2 expression, which is consistent with observations made in mouse, pig and cow embryos (Dietrich and Hiiragi, 2007; Kuijk et al., 2008; Pant and Keefer, 2009; Ralston and Rossant, 2008).

NANOG expression has been previously shown to be restricted to epiblast progenitor cells within the ICM prior to OCT4 in mouse, cow, pig and rhesus monkey embryos (Dietrich and Hiiragi, 2007; Harvey et al., 2009; Kuijk et al., 2008; Pant and Keefer, 2009). To further investigate pluripotency gene expression patterns we analyzed early blastocysts for the expression of NANOG (Fig. 2). As expected, at 4 dpf in a 44-cell embryo we found that OCT4 is uniformly expressed in all DAPI-positive cells (Fig. 2). We observed that NANOG was indeed expressed in human preimplantation embryos and its expression was restricted to the ICM earlier than OCT4 (Fig. 2). We did not find evidence of NANOG localization in the TE at this cell stage (Fig. 2) or later in 6 dpf embryos (data not shown). The restriction of NANOG expression within the ICM, prior to OCT4, is consistent with previous reports (Cauffman et al., 2009; Kimber et al., 2008; Roode et al., 2012), suggesting a conserved expression pattern for these functionally important pluripotency-associated genes.

SOX17 was heterogeneously expressed in the early human ICM and was eventually restricted to putative PE cells, where it largely co-localized with GATA6

In the mouse ICM, mosaic expression of Nanog and Gata6/Sox17 eventually resolves in the restriction of Gata6 and Sox17 expression to the PE, underlying Nanog expressing epiblast progenitor cells (Artus et al., 2011; Chazaud et al., 2006; Morris et al., 2010; Niakan et al., 2010; Plusa et al., 2008). GATA6, GATA4 and SOX17 protein expression has been identified in putative PE cells in human mid- to late-blastocysts (Kuijk et al., 2012; Roode et al., 2012). However, it is unclear whether PE-associate

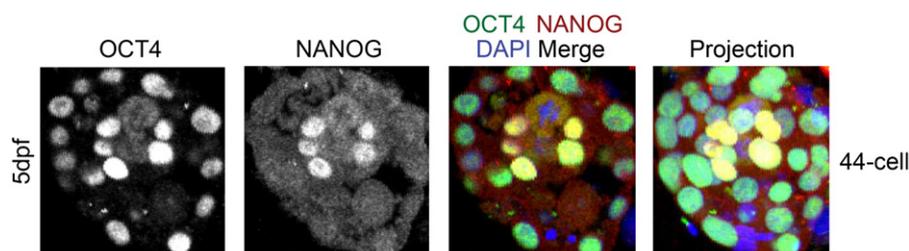


Fig. 2. NANOG expression is restricted to the human inner cell mass prior to OCT4. Optical section and 3D projection of an immunostained human preimplantation embryo at 5 days post-fertilization (dpf). OCT4 (green), NANOG (red) and DAPI (blue) nuclear staining. Scale bar is 100 μ m.

genes are expressed at earlier stages in humans, are heterogeneously expressed within the early human ICM and are coincidentally expressed within all or a subset of putative human PE cells.

We assessed the emergence of human PE cells by performing a detailed time-course immunofluorescence analysis of SOX17 expression from the zygote to the blastocyst. SOX17 was initially detectable in a 32-cell early-blastocyst ($n=1$) (Fig. 3A, B, see Table S2), but not in human embryos prior to this stage (Fig. 3A, B). At 5 dpf, SOX17 was highly expressed in the nuclei of a subset of cells that line the blastocoel cavity as well as cells deeper within the ICM ($8 \pm 7\%$, $n=10$, Fig. 3A, B, see Table S2) consistent with the heterogeneous pattern previously observed for PE-associated genes during mouse preimplantation development (Artus et al., 2011; Chazaud et al., 2006; Morris et al., 2010; Niakan et al., 2010; Plusa et al., 2008). SOX17 expression at 5 dpf largely overlapped with OCT4 expression in the ICM, though we observed that an embryo at this stage had begun to down-regulate OCT4 expression in a subset of cells (SOX17+/OCT4+ overlap: $98 \pm 6\%$, $n=10$, Fig. 3A, B, see Table S2). In fully expanded 6 dpf blastocysts, SOX17 expressing cells were restricted to the putative PE within the ICM ($4 \pm 2\%$, $n=13$, Fig. 3A, B, see Table S2). While the majority of SOX17 expressing cells also expressed high levels of OCT4 at 6 dpf, a subset of SOX17 expressing putative PE cells had downregulated OCT4 (SOX17+/OCT4+ overlap: $90 \pm 23\%$, $n=9$, Fig. 3A white arrows, B, see Table S2). Intriguingly, loss of OCT4 expression in the ICM has been observed in mouse embryos at the late-blastocyst and peri-implantation stage (4.5 dpf), which correlates with commitment of PE cells (Grabarek et al., 2012). Our observation that human preimplantation embryos downregulate OCT4 expression in the PE (Fig. 3A, white arrows), similar to late- or peri-implantation mouse blastocysts, suggests that the human PE is committed at this stage.

While Gata6 expression is initially more broadly expressed in the mouse embryo, in the late-blastocyst Gata6 and Sox17 expressing cells are restricted to in the PE adjacent to the blastocoel cavity (Artus et al., 2011; Morris et al., 2010; Niakan et al., 2010). To assess the overlap of PE-associated protein expression we performed immunofluorescence analysis of GATA6 and SOX17 in human 6 dpf blastocysts. Surprisingly, we found that while GATA6 expression was detectable in the majority of SOX17 expressing cells in the PE, we also observed a few cells within the human ICM that expressed SOX17, but not GATA6 (Fig. 4, white arrows). This suggests that PE lineage specification may be distinct in human embryos compared to the mouse.

The inhibitory interactions between Sox17/Gata6 and Nanog that initiate a cell-sorting process compartmentalizing the ICM into distinct PE and epiblast domains is thought to be facilitated by specific expression of cell-surface proteins, including Laminin, which has been hypothesized to confer an adherence difference to cells fated for the PE (Chazaud et al., 2006; Niakan et al., 2010; Yamanaka et al., 2006). To determine whether this mechanism is conserved in humans we performed immunofluorescence analysis of Laminin expression in human blastocysts. We observed that Laminin protein is most highly expressed in the human TE (Fig. 5), consistent with Laminin localization in the mouse TE (Niakan et al., 2010). However, unlike the mouse, human ICM cells lacked appreciable Laminin expression in the PE, suggesting that there may be fundamental differences in the mechanism of PE lineage specification.

Human blastocysts expressed CK7 and FGFR1 in the TE

It has been demonstrated that proteins such as Cdx2 and the ETS-related transcription factor, Elf5 are initially expressed in the mouse TE and maintain their expression in later post-implantation

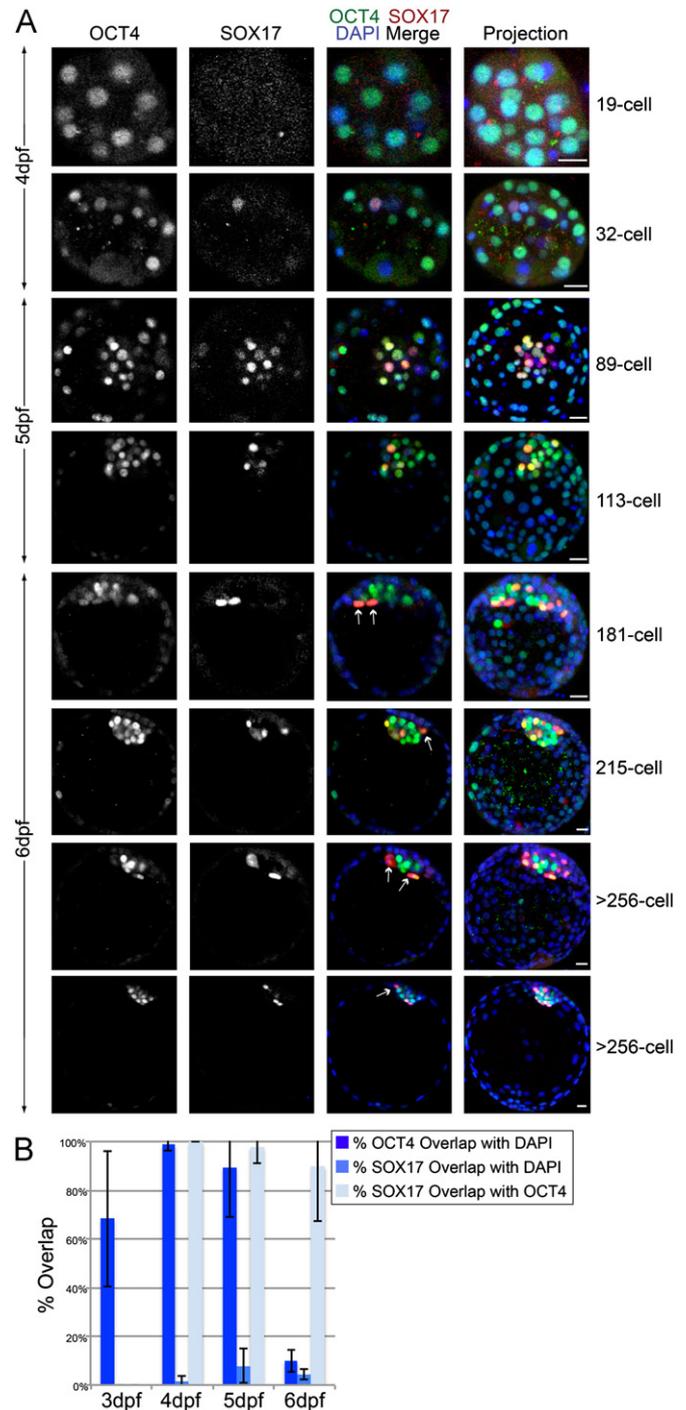


Fig. 3. SOX17 is initially heterogeneously expressed and eventually restricted to human primitive endoderm cells where OCT4 is downregulated. (A) Optical sections and 3D projections of immunostained human preimplantation embryos at defined stages of development. The number of days post-fertilization (dpf) and the approximate cell number is indicated for each embryo. OCT4 (green), SOX17 (red) and DAPI (blue) nuclear staining. Scale bar is 100 μ m. White arrows indicate SOX17-high expressing cells that have down-regulated OCT4 expression within the inner cell mass. (B) Quantification of the total number of OCT4 (dark blue bar) and SOX17 (medium blue bar) expressing cells as a percentage of total DAPI-expression. The percentage of SOX17 expressing cells that overlap with the expression of OCT4 is indicated by the light blue bar. Data are the mean \pm standard deviation of biological replicates.

trophoblast progenitor cells (Beck et al., 1995; Ng et al., 2008). Recently, it has been demonstrated that these factors are conserved in their expression in human first trimester trophoblast cells (Hemberger et al., 2010). However, some proteins that are expressed

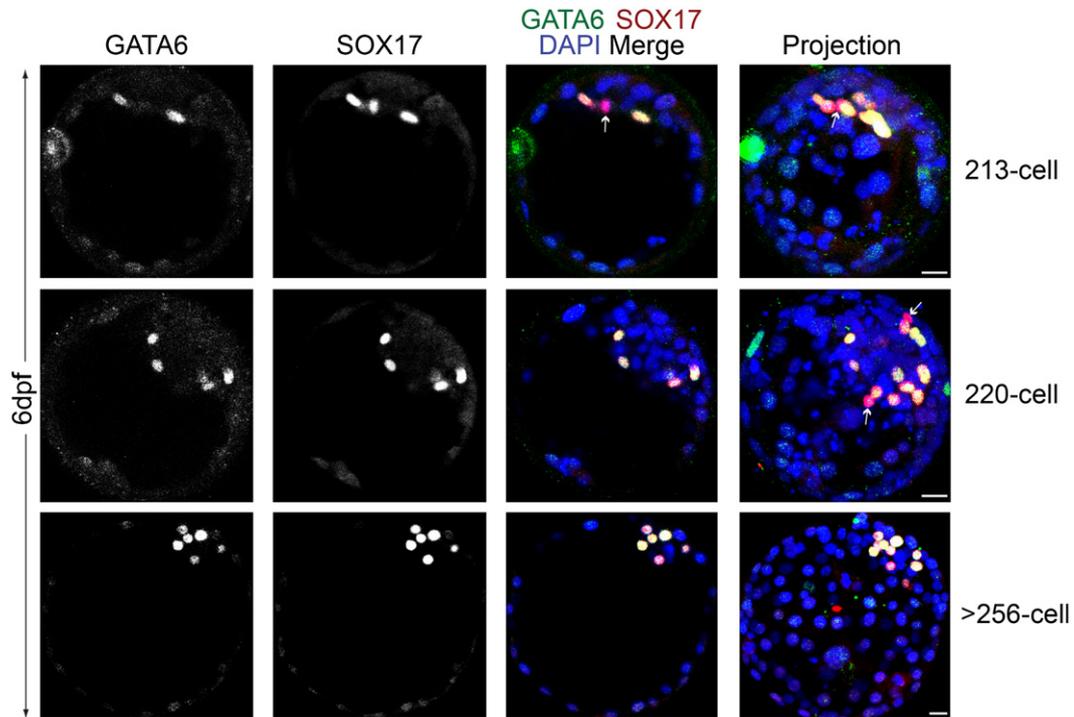


Fig. 4. Overlap of GATA6 and SOX17 expression in the majority of human primitive endoderm cells. Optical sections and 3D projections of immunostained human preimplantation embryos at defined stages of development. The number of days post-fertilization (dpf) and the approximate cell number is indicated for each embryo. GATA6 (green), SOX17 (red) and DAPI (blue) nuclear staining. White arrows indicate SOX17-high expressing cells that have down-regulated GATA6 expression within the primitive endoderm. Scale bar is 100 μ m.

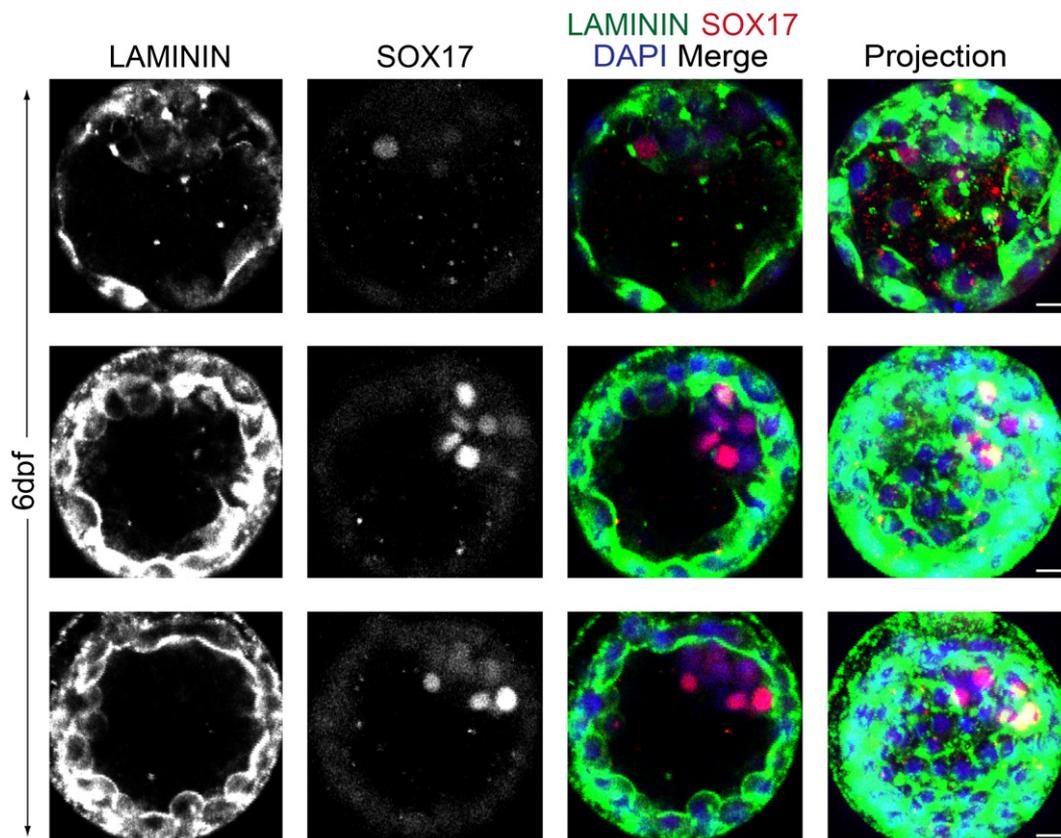


Fig. 5. Laminin is highly expressed in human trophoderm, but not in primitive endoderm cells. Optical sections and 3D projections of immunostained human preimplantation embryos at defined stages of development. The number of days post-fertilization (dpf) and the approximate cell number is indicated for each embryo. Cell surface Laminin (green) and SOX17 (red) and DAPI (blue) nuclear staining. Scale bar is 100 μ m.

in a subset of trophoblast cells such as the non-classical MHC class I allele HLA-G, are not expressed in preimplantation human embryos (Hiby et al., 1999). This suggests that while there is some overlap in the expression of pre- and post-implantation extraembryonic-associated proteins, there are notable differences. We were interested in determining the preimplantation expression of cytokeratin 7 (CK7), an intermediate filament protein whose expression is a basic requirement used to identify post-implantation human trophoblast progenitor cells (King et al., 2000).

As expected, we observed that CK7 was highly expressed in the TE of human blastocysts prior to implantation (Fig. 6A). CK7 expression is consistent with the utility of this protein as a marker of TE progenitor cells. Together with the Laminin expression described above, this suggests that human TE cells have a cell surface and cytoplasmic composition that is distinct from ICM cells. Fibroblast growth factor receptor-1 (FGFR1) is also enriched in human first-trimester trophoblast progenitor cells (Anteby et al., 2005), however, its expression has not been reported in preimplantation human embryos. Using a well-characterized antibody with no cross-reactivity to FGFR2, FGFR3 or FGFR4 (Larocca et al., 1998), we observed that FGFR1 was initially expressed in the majority of TE cells in human blastocysts. Interestingly, we also observed that FGFR1 expression was eventually restricted to polar TE cells directly adjacent to the ICM in human embryos with greater than 164-cells (Fig. 6B). This expression pattern suggests that there are distinct populations of FGFR1-high polar TE cells compared to FGFR1-low mural TE cells.

Human blastocyst outgrowths predominantly expressed extraembryonic-associated proteins, which negatively influenced hESC derivation efficiency

The derivation of hESCs from preimplantation blastocysts is most commonly performed by isolating the ICM away from surrounding TE cells either by immunosurgery or laser-mediated ablation (Chen et al., 2009). This has been estimated to improve derivation efficiency of hESCs from 8.5 to 10% when intact blastocysts were used versus 14–58% following ICM isolation (Chen et al., 2009). The consequence of leaving the TE intact and plating the whole blastocyst has not been fully investigated, including the identity of cells that can emerge at the earliest time-points during derivation. Moreover, it remains unclear whether extraembryonic stem cell lines may be established from plating whole blastocysts in these conditions.

To determine the identity of cells that initially proliferated in the outgrowth of intact blastocysts, we performed immunofluorescence analysis of OCT4 as well as extraembryonic-associated proteins following 4–5 days of in vitro culture under conventional hESC conditions ($n=5$, Fig. 7) (Chen et al., 2009; Cowan et al., 2004; Thomson et al., 1998). Strikingly, we found that the majority of cells in the outgrowths robustly expressed CK7 (Fig. 7) suggesting that TE cells from human blastocysts do have the ability to proliferate somewhat during in vitro culture, although these cells could not be maintained (data not shown).

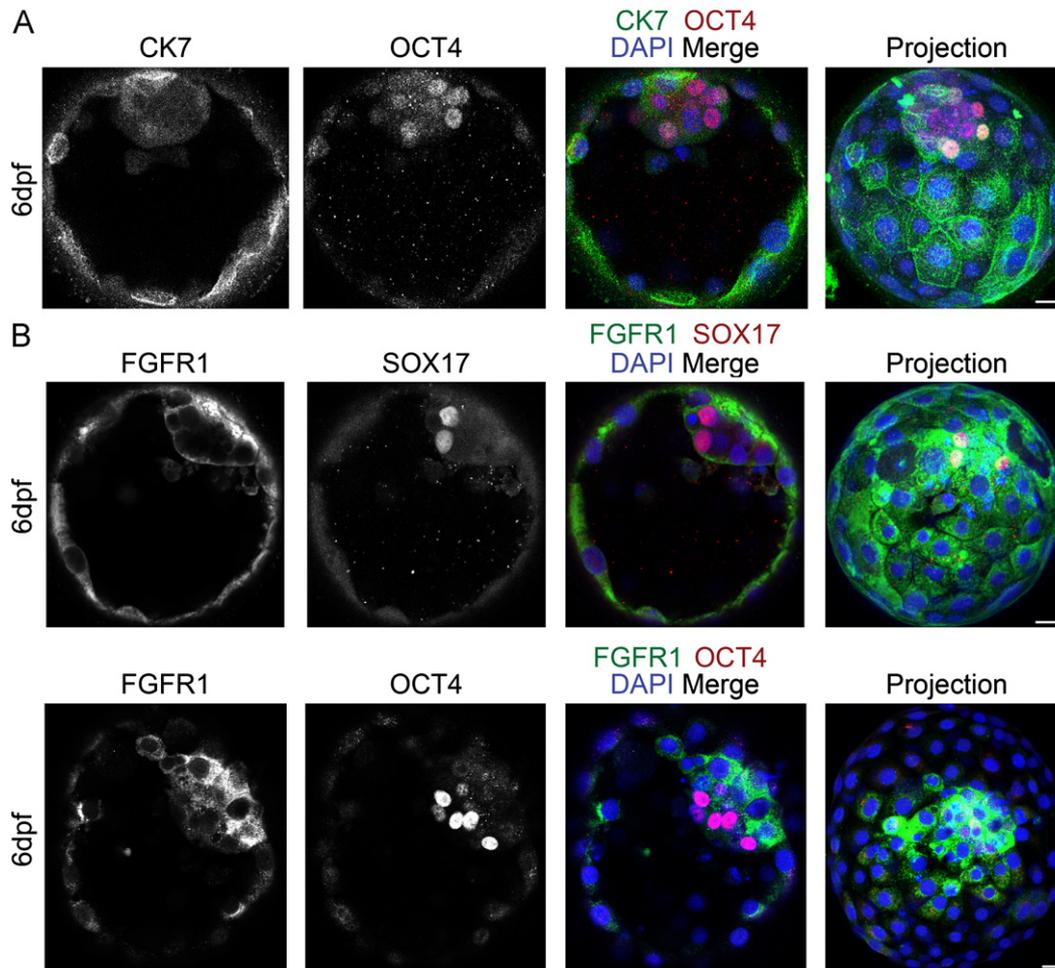


Fig. 6. Markers of post-implantation trophoblast progenitor cells are expressed in human preimplantation embryos. Optical sections and 3D projections of immunostained human preimplantation embryos at defined stages of development. The number of days post-fertilization (dpf) and the approximate cell number is indicated for each embryo: (A) Intracellular Cytokeratin 7 (CK7) (green) and SOX17 (red) and DAPI nuclear staining. (B) Cell surface and intracellular FGFR1 (green) and either OCT4 or SOX17 (red) and DAPI nuclear staining. Scale bar is 100 μm.

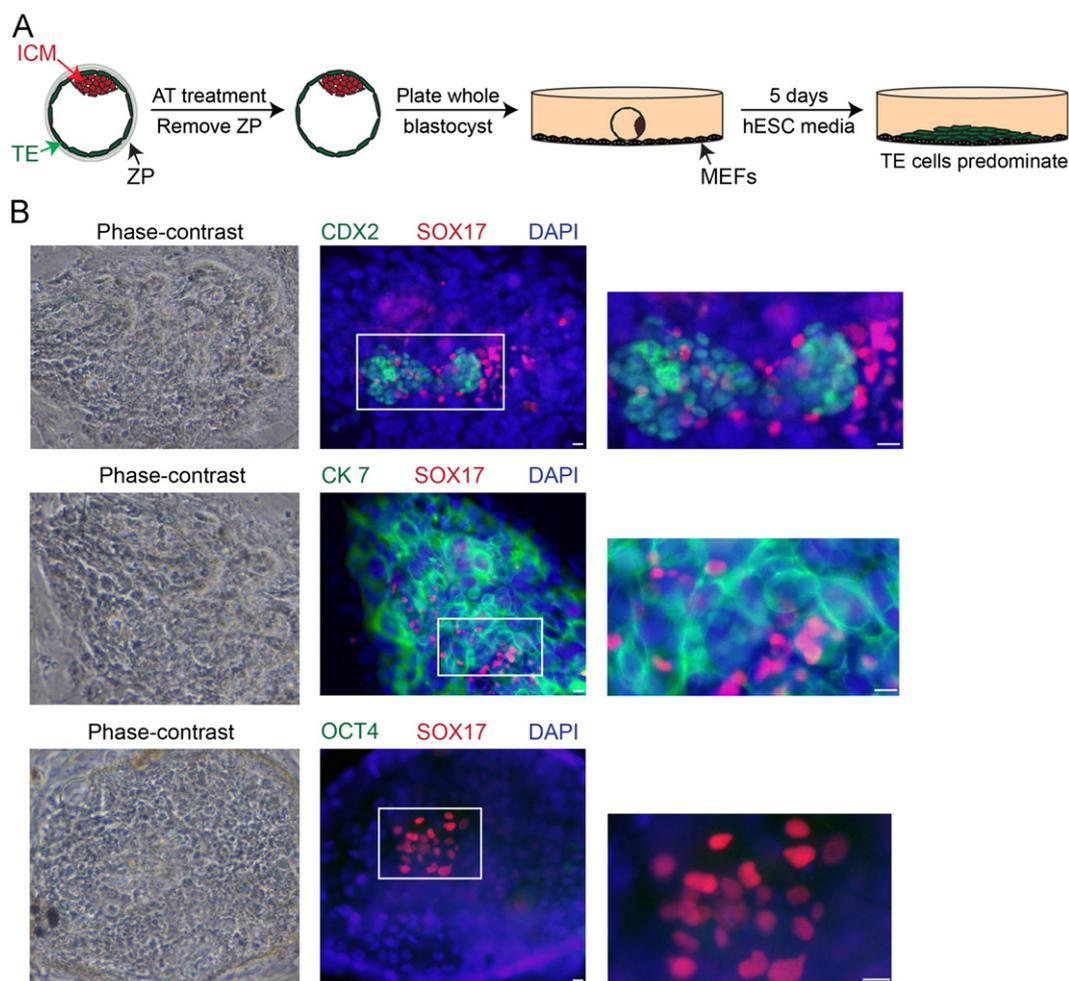


Fig. 7. A subset of human blastocyst outgrowths express proteins associated with primitive endoderm and trophectoderm cells. (A) Experimental design: the zona pelucida (ZP) of human blastocysts were removed by treatment with Acidic Tyrode's (AT) solution. Whole blastocysts with both the inner cell mass (ICM) and trophectoderm (TE) were plated under human embryonic stem cell (hESC) derivation conditions for 5 days (on mouse embryonic fibroblasts (MEFs) in the presence of bFGF). The majority of cells in the outgrowths were CK7-expressing TE cells (green). (B) Outgrowths following 4–5 days of in vitro culture were fixed and immunofluorescently analyzed for the expression of SOX17 (red) and CDX2 (green) or OCT4 (green) and Cytokeratin-7 (CK7) (green) with DAPI nuclear staining (blue). Scale bar is 100 μ m.

We found that the expression of OCT4 was very rarely observed in the outgrowth of blastocysts and most outgrowths lacked OCT4 expression altogether (Fig. 7). These observations confirmed the previous suggestions that derivation from intact blastocysts is detrimental to hESC derivation efficiency.

We also observed that under these conditions very few cells expressed SOX17, which presumably originated from the PE (Fig. 7). Similarly, only a few of the cells in the outgrowths expressed CDX2, which suggests that the majority of TE cells under these conditions have lost the capacity to retain a key gene associated with TE progenitor cells (Fig. 7). The lack of sustained SOX17 and CDX2 expression in these early outgrowths suggests that these culture conditions are not suitable for the establishment of extraembryonic stem cell lines.

Discussion

We have undertaken a detailed immunofluorescence analysis of human preimplantation embryos from the zygote to the blastocyst for the appearance and localization of lineage-specific transcription factors OCT4, CDX2 and SOX17 (summarized in Fig. 8). Some of our observations confirmed the expression pattern suggested from investigation of other

mammalian embryos, including cows (Berg et al., 2011) where CDX2 was detectable in the TE following cavitation. By contrast, Cdx2 expression was detectable in the mouse morula prior to cavitation (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Strumpf et al., 2005), suggesting that TE lineage segregation may be distinct in humans and cows compared to mice. As regionalized activity of Tead4 in cleavage-stage embryos is thought to function upstream of Cdx2 in the mouse embryo (Nishioka et al., 2009; Yagi et al., 2007) it will be important to determine whether human TE specification requires the same pathway and whether additional lineage associated genes are conserved or divergent in their expression in the developing TE (i.e. GATA3, EOMES and FGFR2) or PE (i.e. GATA4, PDGFR α) by analysis throughout human preimplantation development.

We confirmed the previous studies demonstrating that SOX17 was restricted to the putative PE by 7 dpf (Roode et al., 2012). Significantly, we showed for the first time that, similar to mouse embryos, human embryos initially exhibited heterogeneous SOX17 expression within the early ICM with eventual restriction to the PE lining the blastocoel cavity. Human embryos, like the mouse, also initially expressed GATA6 and OCT4 more broadly throughout the embryo (Chen et al., 2009; Kimber et al., 2008; Roode et al., 2012) and this investigation. However, lack of appreciable Laminin expression in

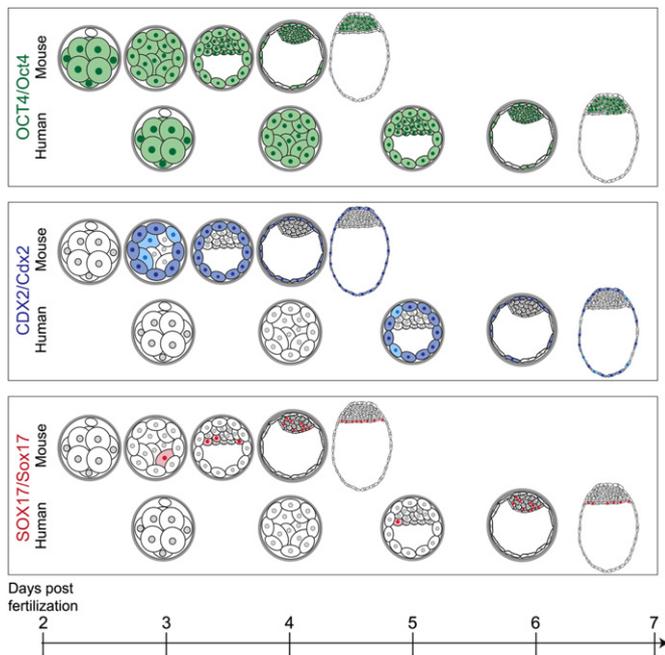


Fig. 8. Schematic summary of the distribution of lineage-specific transcription factors in mouse versus human embryos. Although maternal Oct4 transcript and protein was expressed from the zygote stage (Palmieri et al., 1994), zygotic Oct4 (green) protein was initially detectable above background in the 8-cell mouse embryos (data based on Palmieri et al., 1994; Liu et al., 2004). Similarly human embryos initiate OCT4 (green) expression at the 8-cell stage following embryo genome activation. In both humans and mice, Oct4/OCT4 was persistently expressed in the trophoblast (TE) and eventually restricted to the ICM in mid-blastocysts. Oct4/OCT4 protein was further restricted to epiblast progenitor cells within the ICM of human and mouse late-blastocysts, just prior to implantation into the maternal uterus. In mice, Cdx2 initially exhibits mosaic expression at the 16-cell stage (shades of blue represent strong (dark blue) or absent (gray) expression) and was eventually uniformly expressed in all TE cells (data based on Dietrich and Hiiragi, 2007; Strumpf et al., 2005). CDX2 expression in human embryos was initiated after blastocyst formation and in the late-blastocyst there was variable CDX2 expression in the TE. Sox17 was initially detectable in the mouse morula, heterogeneously expressed in the early- to mid-blastocyst and eventually restricted to the PE (data based on Niakan et al., 2010; Morris et al., 2010; Artus et al., 2011). In humans, SOX17 expression was initially detectable in the early-blastocysts, heterogeneously expressed in mid-blastocysts and restricted to most putative PE cells.

the presumptive PE and downregulation of GATA6 in a subset of SOX17 expressing cells suggest that the mechanism of human PE specification may be distinct in humans compared to mice. Moreover, recent analyses of human embryos treated with FGF/Erk signaling inhibitors suggest that the PE may segregate by mechanisms distinct from mouse, rat and cow embryos (Kuijk et al., 2012; Nichols et al., 2009; Roode et al., 2012; Yamanaka et al., 2010). Therefore, despite conserved expression of some lineage-specific transcription factors in early human embryogenesis, the mechanism for PE and TE specification is unknown and may be distinct compared to other species.

We observed downregulation of OCT4 expression in a subset of SOX17-high expressing human ICM cells at 6 dpf, suggesting that these are committed PE cells. OCT4-low expression in SOX17- and GATA4-high putative PE cells has also been noted in human embryos at 7 dpf (Roode et al., 2012). In the mouse, while *Oct4* transcripts are reduced in the PE, Oct4 protein has been suggested to be more abundantly expressed in early migrating PE cells (Palmieri et al., 1994). By 4.5 dpf late-blastocyst, peri-implantation or implantation-delayed mouse embryos no longer express Oct4 in the PE, which correlates with a restriction in potency (Grabarek et al., 2012). Altogether, this suggests that human mid- to late-blastocysts exhibit an expression pattern that is more analogous to late-blastocyst or peri-implantation mouse embryos, when the

three embryonic lineages have diverged. It is possible that further restriction of OCT4 to putative epiblast progenitor cells within the human ICM restricts the nature of the pluripotent stem cell lines that can be derived. It will be important to determine whether by 6 dpf epiblast cells within the human ICM are molecularly similar to pre- or post-implantation mouse epiblast cells or are distinct and whether this has consequences for the nature of pluripotent stem cells that can be derived.

In the mouse, Nanog expression is downregulated at the time of implantation (Chambers et al., 2003; Grabarek et al., 2012). It will therefore be important to assess whether NANOG expression is also downregulated in the human late-blastocyst as this would further suggest that human embryos share molecular characteristics with the late-blastocyst or peri-implantation mouse embryo. Significantly, in contrast to mouse, rat and cow, NANOG and GATA4 expression within the human ICM was unaffected by FGF, MEK and/or Erk signaling inhibition (Kuijk et al., 2012; Roode et al., 2012), suggesting that this signaling pathway may not be required for the derivation of pluripotent stem cells from human embryos and that alternative pathways may be responsible for epiblast and PE specification. Our observation that human embryos at 6 dpf resemble late-blastocyst, peri-implantation or implantation-delayed mouse embryos suggests that deriving hESCs similar to mESCs may require both novel culture conditions and derivation from embryos at an earlier developmental stage. Alternatively, the type of stem cells that can be derived from human versus mouse embryos may reflect intrinsic genetic differences between these species, as is suggested by the type of induced pluripotent stem cells that can be derived from mouse versus human fibroblasts (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Currently only one type of hESC has been derived which more closely resembles post-implantation mouse epiblast (Brons et al., 2007; Tesar et al., 2007). Further understanding human embryogenesis will significantly improve the utility of human embryos or adult cells by expanding the repertoire of pluripotent stem cells which may be distinct in their differentiation efficiency or potential, as recently demonstrated for pre- versus post-implantation derived mouse stem cells (Cho et al., 2012).

We investigated the consequence of leaving extraembryonic cells intact during conventional hESC derivation by plating whole blastocysts. Our results suggest that the negative correlation with hESC derivation efficiency is likely due to the presence of primarily CK7-expressing TE-like cells, which we are presently unable to maintain indefinitely in culture. It will be interesting to determine whether human embryos cultured in alternative conditions or at earlier stages of development prior to TE specification could facilitate the establishment of extraembryonic stem cell lines. Our study could therefore also facilitate future attempts to establish models of human placental cells, which would be of significant importance for further elucidating mechanisms of early human extraembryonic development and to model human placental defects. In all, there is a need to further understand the molecular basis of cell fate specification in human embryogenesis compared with other species as these investigations have importance for improving assistant reproduction technology and stem cell based regenerative medicine (Rossant, 2011).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2012.12.008>.

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