



Human blastocyst outgrowths recapitulate primordial germ cell specification events

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ABSTRACT: Our current knowledge of the mechanisms leading to human primordial germ cell (PGC) specification stems solely from differentiation experiments starting from human pluripotent stem cells. However, information regarding the origin of PGCs *in vivo* remains obscure. Here we apply an improved system for extended *in vitro* culture of human embryos to investigate the presence of PGC-like cells (PGCLCs) 12 days post fertilization (dpf). Good quality blastocysts ($n = 141$) were plated at 6 dpf and maintained in hypoxia, in medium supplemented with Activin A until 12 dpf. We primarily reveal that 12 dpf outgrowths recapitulate human peri-implantation events and demonstrate that blastocyst quality significantly impacts both embryo viability at 12 dpf, as well as the presence of POU5F1⁺ cells within viable outgrowths. Moreover, detailed examination of 12 dpf blastocyst outgrowths revealed a population of POU5F1⁺, SOX2⁻ and SOX17⁺ cells that may correspond to PGCLCs, alongside POU5F1⁺ epiblast-like cells and GATA6⁺ endoderm-like cells. Our findings suggest that, in human, PGC precursors may become specified within the epiblast and migrate either transiently to the extra-embryonic mesoderm or directly to the dorsal part of the yolk sac endoderm around 12 dpf. This is a descriptive analysis and as such the conclusion that POU5F1⁺ and SOX17⁺ cells represent *bona fide* PGCs can only be considered as preliminary. In the future, other PGC markers may be used to further validate the observed cell populations. Overall, our findings provide insights into the origin of the human germline and may serve as a foundation to further unravel the molecular mechanisms governing PGC specification in human.

Key words: primordial germ cells / peri-implantation development / human blastocyst outgrowths / human embryo quality / lineage specification / *in vitro* implantation model

Introduction

Primordial germ cells (PGCs), the precursors of sperm and oocytes, are the only cells capable of passing (epi)genetic information to the next generation (Chuva de Sousa Lopes and McLaren, 2013; Saitou and Miyauchi, 2016; Tang *et al.*, 2016). Given their importance, specification of mammalian PGCs has been extensively studied *in vivo* and *in vitro* (Bertocchini and Chuva de Sousa Lopes, 2016). Studies in monkey (Sasaki *et al.*, 2016) and pig (du Puy *et al.*, 2011; Kobayashi *et al.*, 2017) have added to the widely accepted view that the molecular mechanisms that regulate PGC specification during mammalian early development *in vivo* are highly species specific. However, due to the

inherent inaccessibility of human peri-implantation embryos, there are no reports tracing the origin of human PGCs.

Studies investigating human PGC-like cell (hPGCLC) specification *in vitro*, starting from human pluripotent stem cells (hPSCs), have revealed that although some transcriptional signals are evolutionary conserved (induction by BMP4 and WNT3, dependence on PRDM1 and TFAP2C expression), other signals such as the expression of SOX2 and SOX17 differ considerably (Irie *et al.*, 2015; Saitou and Miyauchi, 2016). It has been shown that the *in vitro* specification of hPGCLCs traverses through incipient mesoderm-like cells (Sasaki *et al.*, 2016) or mesendoderm precursors (Kobayashi *et al.*, 2017). Extraembryonic mesoderm (ExM) (or reticulated mesenchyme) emerges 10–12 days

post fertilization (dpf) in human, several days prior to gastrulation and the formation of intraembryonic mesoderm at 15–16 dpf (Bianchi et al., 1993; Dobрева et al., 2010; Schoenwolf et al., 2014). Therefore, it is assumed that human PGCs are most likely specified between 10 and 16 dpf.

A unique and invaluable glimpse of human peri-implantation development *in vivo* has been obtained from embryos from the Carnegie collection (Hill, 2017; Schoenwolf et al., 2014). Our current knowledge of implantation, cavitation and early lineage specification events thus largely originates from the detailed morphological descriptions of the few human peri-implantation embryos available (Carnegie Stage, CS 5; Hertig et al., 1956).

In vitro, early signs of human peri-implantation development were initially captured during human embryonic stem cell (hESC) derivation. During the formation of blastocyst outgrowths around 12 dpf, the post-inner cell mass intermediate (PICMI), a compact flattened structure of densely packed epithelial-like cells, proved imperative for the derivation process (O'Leary et al., 2012, 2013). The PICMI contains around 30 POU5F1⁺ epiblast-like cells (EPILCs), alongside a population of GATA6⁺ endoderm-like cells (ENDOLCs) and is often surrounded by a crescent-shaped cavity, reminiscent of the yolk sac cavity (O'Leary et al., 2012, 2013). Several studies have further characterised the self-organising properties of human blastocyst outgrowths containing a PICMI, focusing on EPILC expansion, trophoctoderm (TE) diversification and cavity formation (both amniotic and yolk sac; Deglincerti et al., 2016; Shahbazi et al., 2016, 2017). The unique transcriptional signature of the PICMI encompassing germ cell marker genes, such as KIT, ALPL and IFITM1 (O'Leary et al., 2012), prompted us to investigate the presence of PGCLCs in 12 dpf human blastocyst outgrowths.

Materials and methods

Ethical permission

Approval for the use of human preimplantation embryos was obtained from the Local Ethical Committee of Ghent University (EC2013/822; EC2017/584) and the Belgian Federal Commission for medical and scientific research on embryos *in vitro* (ADV_052_UZ Gent; ADV_075_UZ Gent). Approval for the collection and use of human foetal material was obtained from the Medical Ethical Committee of the Leiden University Medical Centre (P08.087) in The Netherlands. All materials were donated for research with written informed consent.

Preimplantation embryo culture and generation of blastocyst outgrowths

Preimplantation embryos were thawed either at 2 or 3 dpf. Those thawed at 2 dpf were cultured for 24 h in Cook Cleavage Medium (COOK, Ireland) and transferred to Cook Blastocyst Medium (COOK), while embryos thawed at 3 dpf were cultured directly in Cook Blastocyst Medium (COOK) in 25 µl drops under mineral oil (Irvine Scientific, The Netherlands) until 6 dpf, at 37°C, 6% CO₂ and 5% O₂. Blastocyst quality was evaluated at 6 dpf using the Gardner and Schoolcraft grading system (Gardner and Schoolcraft, 1999; Supplementary Figure S1). Embryos with an expansion grade of 3 or higher (N = 141) were briefly exposed to pre-warmed Acidic Tyrode's Solution (Sigma-Aldrich, Belgium) for removal of

the zona pellucida. After washing, individual blastocysts were plated per well, in 8-well IbiTreat µ-plates (Ibidi, GmbH, Belgium) and cultured as described (Popovic et al., 2019); in particular, (i) culture was performed in hypoxic conditions (5% O₂) and (ii) culture medium contained 100 ng/ml of Activin A (PeproTech, United Kingdom). The 12 dpf blastocyst outgrowths were fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS0) for 20 min at room temperature (RT).

Based on conditions shown to induce differentiation of hPSCs to PGCLCs (Irie et al., 2015), blastocyst outgrowths (N = 7) were supplemented from 8 to 12 dpf with BMP4 (500 ng/ml, Gibco, Life Technologies, Belgium), leukemia inhibiting factor (LIF, 1000 U/ml, Peprotech), stem cell factor (100 ng/ml, Gibco), epidermal growth factor (50 ng/ml, Gibco) and Activin A (100 ng/ml, Peprotech) and compared with blastocyst outgrowths grown without supplements (N = 7).

Immunofluorescence

Fixed blastocyst outgrowths and CS20 gonadal slices were permeabilised with 0.2% (v/v) Triton X-100 (Sigma-Aldrich) in PBS0 for 20 min at RT. HESCs, line UGent I 1-2 (Van der Jeught et al., 2013), were fixed as above and permeabilised with 0.1% (v/v) Triton X-100/PBS0 for 8 min at RT. Samples were then blocked in 10% foetal calf serum (Gibco) and 1% bovine serum albumin (BSA, Sigma-Aldrich) solution for 1 h at RT, incubated with primary antibodies (Supplementary Table S1) in 0.1% BSA/PBS0 overnight (o/n) at 4°C and washed in 0.1% Tween-20 solution (Merck, Germany). Samples were incubated with secondary antibodies (Supplementary Table S1) in 0.1% BSA/PBS0 o/n at 4°C, washed three times in 0.1% Tween-20 solution and counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, The Netherlands). HESCs on coverslips and CS20 gonadal slices were mounted on glass slides with ProLongGold antifade reagent (Thermo Fisher Scientific).

Imaging and cell counting

Bright field images of blastocysts and embryo outgrowths were taken on an Olympus IX73 inverted microscope (Olympus, Belgium). Fluorescence images were obtained on a Leica TCS SP8 inverted confocal microscope (Leica, Germany) equipped with a white light laser and LAS X software (Leica). The outer limits of the outgrowths were the bottom of the dish and the last visible DAPI⁺ nucleus. Z-stack images were acquired with 2 µm spacing, at Airy 1 unit pinhole, at 12 bits in 1024 × 1024 pixels and at 200 Hz laser frequency. For image analysis, z-stack images were saved as Tiff files and processed using Fiji (version 2.0.0-rc43/1.5k; Schindelin et al., 2012). Cell counting was performed manually using the Cell Counter plug-in. A cell was considered positive when it was labelled in the channel of interest and contained a DAPI⁺ nucleus. 3D views were constructed using 3D viewer in Fiji.

Statistical analysis

The association between embryo quality, outgrowth viability and the efficiency of generating POU5F1⁺ outgrowths was investigated using Fisher's Exact Test (two-sided). *P*-values of <0.05 were considered significant.

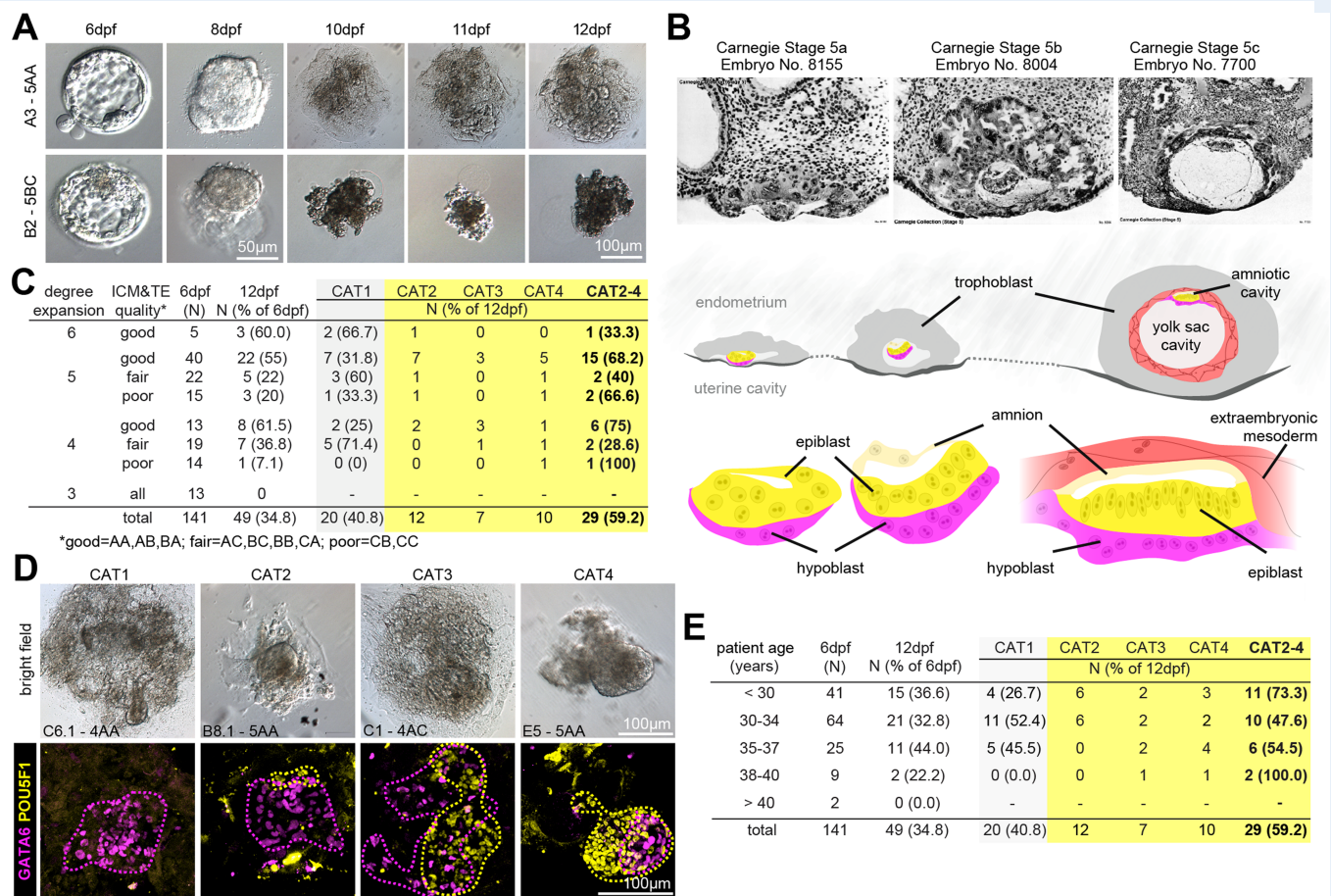


Figure 1 Embryo quality affects viability and development of 12 dpf blastocyst outgrowths. (A) Culture progression of good quality (top) and poor quality (bottom) human blastocyst from 6 to 12 dpf. Scale bar is 50 µm in 6–8 dpf and 100 µm in 10–12 dpf. (B) Schematic representation of three different stages of development based on three figures from Hertig et al. (1956), reproduced with permission from Wiley & Sons, Inc. Copyright © 2005, John Wiley and Sons. (C) Impact of blastocyst quality at 6 dpf on viability and presence of POU5F1⁺ cells in 12 dpf outgrowths. (D) Four categories (CAT) of 12 dpf blastocyst outgrowths based on POU5F1 expression. Bright field images of blastocyst outgrowths (top row) and corresponding immunostaining for POU5F1 (yellow) and GATA6 (magenta; bottom row). Scale bar is 100 µm. (E) Effect of maternal age on viability and presence of POU5F1⁺ cells in 12 dpf outgrowths.

Results

Blastocyst quality affects the survival of embryo outgrowths containing POU5F1⁺ cells

Blastocysts with a discernible inner cell mass (ICM), expansion grade 3 or higher ($N = 141$) were plated at 6 dpf. Embryos were cultured *in vitro* in hypoxic conditions until 12 dpf (Fig. 1A) to mimic *in vivo* human peri-implantation conditions more closely (Fig. 1B). A total of 49 out of 128 (38.3%) plated blastocysts, expansion grade 4–6 were viable at 12 dpf, while none of the plated blastocysts with expansion grade 3 ($N = 13$) survived to 12 dpf (Fig. 1C). Moreover, good quality blastocysts were significantly more likely to develop to 12 dpf compared to fair and poor quality embryos ($P = 0.01$; Fig. 1A and C).

Next we immunostained the viable 12 dpf blastocyst outgrowths for POU5F1 and GATA6 to analyse the formation of EPILCs and ENDOLCs, respectively. POU5F1⁺ outgrowths ($N = 20$) were class-

fied as category I (CAT1) and were not studied further. The remaining outgrowths ($n = 29$) were divided into CAT2 (1–10 POU5F1⁺ cells), CAT3 (>10 POU5F1⁺ cells; flat outgrowth) and CAT4 (>10 POU5F1⁺ cells; spherical outgrowth; Fig. 1C and D). Good quality embryos were significantly more likely to generate POU5F1⁺ outgrowths (CAT2–4) compared to both fair and poor quality embryos ($P = 0.0203$ and $P = 0.048$, respectively; Fig. 1A and C). However, we did not observe an effect of advanced maternal age (≥ 38) on outgrowth viability ($P = 0.5203$) nor the number of POU5F1⁺ outgrowths ($P = 1.000$; Fig. 1E). Nevertheless, the limited number of analysed embryos/outgrowths per age category warrants careful interpretation.

12 dpf blastocyst outgrowths recapitulate early human peri-implantation

We observed a wide variety of 12 dpf blastocyst outgrowth phenotypes, ranging from those comparable to early stages of cavitation with EPI cells rearranged as a rosette (CS5a; Fig. 2A and B) to bilaminar

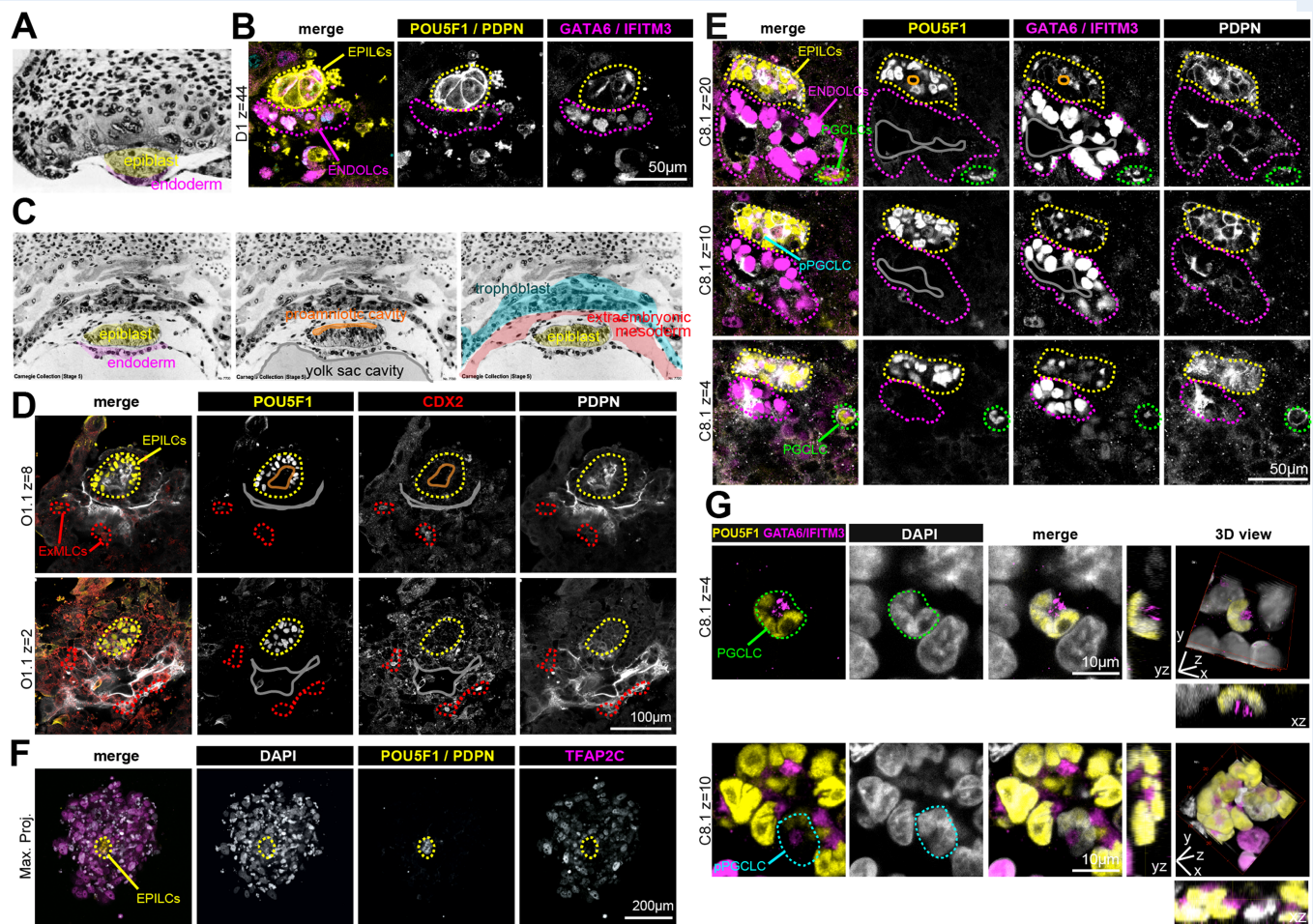


Figure 2 Blastocyst outgrowths mimic human peri-implantation. **(A)** Positioning of epiblast (yellow) and endoderm (magenta) in images of a CS5a human embryo (Hertig et al., 1956), reproduced with permission from Wiley and Sons Inc. Copyright © 2005, John Wiley and Sons. **(B)** Single z-plane of 12 dpf outgrowth immunostained for POU5F1 and PDPN (yellow); and GATA6 and IFITM3 (magenta), as merge and single channels. Yellow dashed line depicts EPILCs and magenta dashed line ENDOLCs. Scale bar is 50 μ m. **(C)** Positioning of the epiblast (yellow), endoderm (magenta), amniotic cavity (orange), yolk sac cavity (grey), ExM (red) and trophoblast (cyan) in images of a CS5c human embryo (Hertig et al., 1956), reproduced with permission from Wiley and Sons Inc. Copyright © 2005, John Wiley and Sons. **(D)** Single z-planes of 12 dpf outgrowth immunostained for POU5F1 (yellow), CDX2 (red) and PDPN (white), as merge and single channels. Yellow dashed line depicts EPILCs, red dashed line extra embryonic mesoderm-like cells (ExMLCs) and grey line the yolk sac cavity. Scale bar is 100 μ m. **(E)** Single z-planes of 12 dpf outgrowth immunostained for POU5F1 (yellow), GATA6 and IFITM3 (magenta) and PDPN (white), as merge and single channels. Yellow dashed line depicts EPILCs, magenta dashed line ENDOLCs, green dashed line PGCLCs, grey line the yolk sac cavity, orange line the amniotic cavity and cyan line points to precursors of PGCLCs (pPGCLCs). Scale bar is 50 μ m. **(F)** Maximal projection (Max. proj.) of 12 dpf outgrowth immunostained for POU5F1 and PDPN (yellow), TFAP2C (magenta) and DAPI (white), as merge and single channels. Scale bar is 200 μ m. **(G)** 3D view of cell marked as PGCLC (top) and pPGCLC (bottom) in (E). Scale bar is 10 μ m.

embryos (CS5c; Fig. 2C–E). In culture, the ICM epithelialised/polarised and cavitated, giving rise to a central (pro)amniotic cavity (Fig. 2A–E) with cells showing prominent expression of (nuclear) POU5F1, (membrane/Golgi) IFITM3 and (membrane) PDPN (Fig. 2A–E). The EPILCs (POU5F1⁺ cells in contact with ENDOLCs) and the (future) amniotic ectoderm (POU5F1⁺ cells not facing the ENDOLCs) expressed the same set of markers analysed and had comparable cell morphology, suggesting that lineage restriction into squamous amniotic ectoderm and columnar epiblast had not yet occurred (Fig. 2A–E).

The ENDOLCs, expressing (nuclear) GATA6, either appeared as a disc facing the EPILCs (Fig. 2A and B) or formed a closed cavity,

presumably the (first) yolk sac cavity (Fig. 2E). However, there was no morphological distinction between ENDOLCs (facing the EPILCs) and ENDOLCs forming the yolk sac. Interestingly, PDPN was useful to mark not only the formation of the amniotic cavity, but also the apical surface of the developing yolk sac cavity (Fig. 2D and E).

Furthermore, we assessed the expression of CDX2, a known (Ex)M determinant (Bernardo et al., 2011). ExM emerges prior to gastrulation (and the formation of the primitive streak) and envelops both the amniotic ectoderm and yolk sac ENDO (Figs 1B and 2C); however, the origin of ExM *in vivo* remains obscure (Bianchi et al., 1993; Dobreva et al., 2010). We observed scattered CDX2⁺ cells often near the

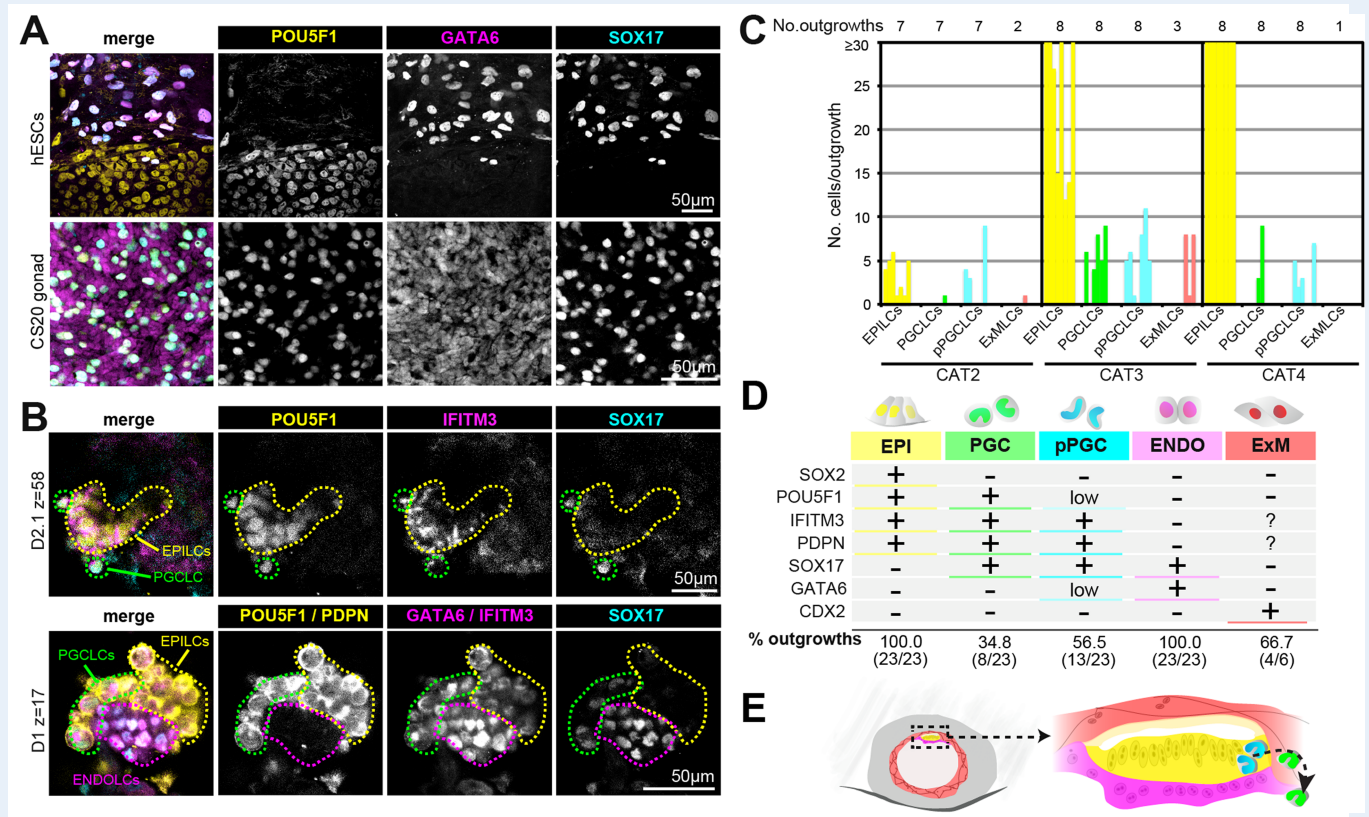


Figure 3 PGCLCs emerged by 12 dpf in human blastocyst outgrowths. (A) Expression of POU5F1 (yellow), GATA6 (magenta) and SOX17 (cyan) in hESCs and a CS20 gonad, as merge and single channels. Scale bar is 50 μ m. (B) Single z-planes of 12 dpf outgrowths immunostained for POU5F1 (and PDPN) (yellow), IFITM3 (and GATA6) (magenta) and SOX17 (cyan) as merge and single channels. Yellow dashed line depicts EPILCs, magenta dashed line ENDOLCs and green dashed line PGCLCs. Scale bar is 50 μ m. (C) Quantification of EPILCs, PGCLCs, pPGCLCs and ExMLCs in 12 dpf outgrowths from different categories (CAT2-4). (D) Expression of different antibodies in inner cell mass-derived cell types (EPI, ExM, ENDO, PGCs, pPGCs) in 12 dpf blastocyst outgrowths and the percentage of outgrowths containing that specific cell type. (E) Model for PGC specification in humans.

presumptive yolk sac cavity (Fig. 2D) in agreement with previous observations by Deglincerti *et al.* (2016). Moreover, TFAP2C, a marker of trophoblast cells (Okoe *et al.*, 2018), marked the large trophoblast cells surrounding the tight cluster of presumably EPILCs (Fig. 2F).

12 dpf blastocyst outgrowths contain POU5F1⁺/SOX17⁺ PGCLCs

In addition to the (cavating) sphere of POU5F1⁺ cells we noted the presence of isolated POU5F1⁺/IFITM3⁺/PDPN⁺ cells, sometimes distant from the EPILC cluster, with a more mesenchymal-like morphology (Fig. 2E). These isolated cells had kidney-shaped nuclei engulfing a prominent IFITM3⁺ Golgi (Fig. 2G). This morphology is typically observed in early mouse PGCs cultured *in vitro* for a short period of time (Chuva de Sousa Lopes *et al.*, 2008; Bialecka *et al.*, 2012) and thus may represent *bona fide* hPGCLCs. Moreover, cells with similar kidney-shaped nuclei, but less positive for POU5F1, were observed delaminating from the EPILC cluster (Fig. 2E and G) and may correspond to precursors of pPGCLCs.

SOX17 has been described as a determinant of hPGCLCs (Irie *et al.*, 2015). To utilise SOX17 as a marker specific for hPGCLCs, we first

evaluated its expression in primed hESCs (similar to the EPI) and early hPGCs in gonads of CS20 embryos. Indeed, SOX17 was expressed in POU5F1⁺ hPGCs, but not in POU5F1⁺ hESCs (Fig. 3A). Rather, SOX17 co-localised with GATA6 in ENDO-differentiating hESCs, at the border of POU5F1⁺ hESC colonies (Fig. 3A). Using this combination of antibodies, we were able to identify a population of SOX17⁺/POU5F1⁺ cells, distinct from the SOX17⁻/POU5F1⁺ EPILCs and the SOX17⁺/GATA6⁺ ENDOLCs in 12 dpf blastocyst outgrowths (Fig. 3B), further suggesting the presence of hPGCLCs. These cells had a discernible Golgi accumulation of IFITM3 and often a distinct kidney-shaped nucleus (Fig. 3B). From the 12 dpf blastocyst outgrowths with POU5F1⁺ cells (CAT2-4) 14 out of 23 (60.9%) contained on average 5 (p)PGCLCs (Fig. 3C and D).

In an effort to further validate the hPGCLC population within the blastocyst outgrowths and potentially improve efficiency, we cultured a subset of embryo outgrowths in medium supplemented with cytokines previously shown to induce PGCLC formation (Irie *et al.*, 2015) from 8 to 12 dpf (Supplementary Figure S2). However, following exposure to PGCLC-inducing conditions for 4 days, all outgrowths ($N=7$) degenerated and detached, in contrast to control outgrowths ($N=7$), which predominately remained attached on 12 dpf (57.1%;

Supplementary Figure S2). This suggests that the cytokines may have a negative impact on embryo outgrowth viability.

Overall, based on our results, we propose a model suggesting that (p)PGCs in humans become specified from cells of the EPI and migrate either transiently to the ExM or directly to the dorsal part of the yolk sac endoderm around 12 dpf (Fig. 3E).

Discussion

Here we apply a culture system combining features of hESC derivation and extended *in vitro* blastocyst culture, providing novel insights into the origin of the human germ line. Markedly, examination of 12 dpf blastocyst outgrowths revealed a population of POU5F1⁺/SOX17⁺/SOX2⁻ cells, which resemble PGCLCs.

Our *in vitro* culture results suggest that human PGCs may delaminate from the POU5F1⁺ EPI facing the GATA6⁺ ENDO around 12 dpf. This would be more comparable to the pig, where PGCs have been shown to originate from the posterior pre-primitive streak EPI (Kobayashi et al., 2017) and different from the mechanism described in cynomolgus monkey, whereby PGCs appear to be specified in the amnion with POU5F1⁺ cells facing away from the ENDO (Sasaki et al., 2016). Future experiments will reveal whether human (p)PGCLCs progress via a mesendoderm intermediate, as observed from hPSC-derived PGCLCs (Sasaki et al., 2015; Kobayashi et al., 2017).

As hESCs derived in the presence of Activin A show increased germ cell derivation efficiency (Duggal et al., 2013, 2015), we supplemented extended blastocyst cultures with Activin A following plating. Interestingly, isolated POU5F1⁺ cells, distant from the main epithelial POU5F1⁺ cluster, were also visible in the previous study by Deglincerti et al. (2016). However, the authors did not use SOX17, IFITM3 and PDPN to identify (p)PGCLCs, hence these cells were not analysed further. In agreement with our results, Deglincerti et al. (2016) also observed CDX2 expression in cells surrounding the yolk sac cavity. However, the authors propose that this population corresponds to the previously undescribed 'yolk sac TE', while we suggest that CDX2 marks cells of the ExM, not TE cells. Caution should be taken when using TFAP2C to mark nascent PGCLCs (Irie et al., 2015), as it also clearly marked TE cells in the 12 dpf blastocyst outgrowths. To further validate the PGCLC population within the blastocyst outgrowths, we cultured a small subset of embryos in PGCLC induction medium (Irie et al., 2015). Although these conditions have been shown to promote the derivation of PGCLCs from hPSCs *in vitro*, they did not support further embryonic developmental progression. As such, their effect cannot be evaluated in this context.

It is well established that embryo quality traits influence IVF outcomes (Baird et al., 2005). Accordingly, we provide evidence that blastocyst quality significantly affects both the survival rate of embryo outgrowths and the presence of POU5F1⁺ cells at 12 dpf. It is thus imperative to use embryos of sufficient quality ($\geq 4AB$, 4BA) for *in vitro* extended culture to avoid confounding effects. As such, variability in embryo quality may explain the results of Shahbazi et al. (2016), who did not observe POU5F1⁺ blastocyst outgrowths in hypoxic culture conditions. Hypoxia was previously shown to be more beneficial for human embryo development in terms of embryo expansion rates (Waldenstrom et al., 2009) and EPI maintenance (Roode et al., 2012) compared to high oxygen.

Here we illustrate that 12 dpf blastocyst outgrowths serve as a valuable model for studying early germ cell specification events. Nevertheless, as this is a descriptive study, current findings should be considered only as preliminary. In the future, additional markers of human PGC specification (Gomes Fernandes et al., 2018) as well as culture adaptations to improve embryo development may serve to further characterise the observed cell population. While additional high-throughput analyses may also be applied to validate the PGCLCs, the set of antibodies used highlights the high degree of lineage heterogeneity in 12 dpf blastocyst outgrowths, which will inherently limit efficiency and cell recovery. Studying the implantation period of early human embryos and the events governing peri-implantation development certainly remains challenging. However, as *in vivo* studies are ethically not feasible, *in vitro* systems for extended embryo culture provide a promising way forward. Future work will not only continue to shed light on the underlying mechanisms of human embryogenesis but may also support PGC derivation efforts, enhancing both stem cell and reproductive technologies.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

M.P. and M.B. designed the study, performed experiments, acquired data, interpreted data and wrote the manuscript, contributing equally to the study. M.G.F., J.T. and M.V.D.J. performed experiments and assisted in acquiring and interpreting data. P.D.S., S.M.C.S.L. and B.H. designed the study, interpreted data and wrote the manuscript. All authors reviewed the manuscript and accepted the final version.

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Conflict of interest

None declared.

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