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Human germline differentiation charts a new course

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Understanding the molecular events of reproduction requires a system to differentiate human pluripotent stem cells to germline cells (gametes) *in vitro*. Such a system is not only critical to unlock the secrets of germline development; it may also allow screening for environmental agents that affect gametogenesis. Two recent papers, one in this issue of *The EMBO Journal*, have developed complementary approaches for generating human germline cells with unprecedented efficiency from pluripotent stem cells (Sugawa *et al*, 2015; Irie *et al*, 2015). This work illustrates the power and limitations of extrapolating molecular pathways for lineage differentiation from mice to humans and illuminates the importance of using human cell-based models to study reproductive health.

See also: **F Sugawa *et al*** (April 2015) and **N Irie *et al*** (January 2015)

The germline has a singular purpose, and that is to transmit genetic material from one generation to the next. As a result, the correct differentiation of eggs and sperm (gametes) is essential for human fertility and reproductive health. In the human population, about 10% of couples of reproductive age (15–44 years) have trouble getting pregnant or carrying a baby to term. Although there are many causes of infertility, abnormal differentiation of the germline has the greatest potential to affect reproduction and child health outcomes.

Human germline differentiation is traditionally modeled on mouse research, and it is assumed that both share an inductive strategy for gametogenesis. In contrast, model organisms such as flies, worms, fish, and frogs do not use an inductive strategy and instead use a preformation mechanism, where cells of the embryo become endowed with RNAs and proteins inherited from the egg following fertilization. Even though the inductive strategy is not used by the non-mammalian model organisms, the signaling principle for germline induction in mice is well understood, and involves bone morphogenetic protein 4 (BMP4) signaling to the WNT3 primed-proximal epiblast, a pluripotent epithelial cell layer found in the peri-implantation embryo. This induces the formation of precursor primordial germ cells (PGCs) in the epiblast that transition through a BRACHYURY-positive mesodermal-like intermediate to activate expression of the DNA binding proteins *Prdm1, Prdm14*, and *Tcfap2c* (Ohinata *et al*, 2005; Yamaji *et al*, 2008; Weber *et al*, 2010; Aramaki *et al*, 2013). Once established, the definitive PGCs will migrate from their site of origin to the developing testis or ovary of the embryo during gestation. At this point, the PGCs will differentiate into male or female germline cells and follow sex-specific instructions for differentiation into male or female gametes, respectively. Definitive PGCs are the only embryonic cell types that have the ability to differentiate into gametes. This is why the creation of this progenitor cell type in the laboratory is so crucial to understanding reproductive health and germline differentiation.

In order to differentiate human germline cells based on the principles of the elegant and orderly sequence of PGC formation in mice, Sugawa *et al* started by inducing primed human embryonic stem cell (hESC) and induced pluripotent stem cell (hiPSC) lines into primitive streak/mesodermal-like precursor cells (Fig 1). This approach efficiently generates *BRACHYURY*-positive cells, which, analogous to mouse, could function as an intermediate in human PGC formation. Next, the primitive streak/mesodermal-like precursors were cultured as aggregates in PGC-like cell (PGCLC) differentiation media containing BMP4, leukemia inhibitory factor (LIF), and ROCK inhibitor. Using a cell surface marker sorting strategy ($cKIT^+$/$TRA\text{-}1\text{-}81^+$) developed previously for isolating human germline cells (Gkountela *et al*, 2013), Sugawa and colleagues acquired a high fraction of PGCLCs at days 4 and 6 of differentiation, which decreased significantly by day 8. RNA-Seq of the PGCLCs revealed some transcriptional overlap with 16-week prenatal human germline cells, indicating PGCLCs generated within the first 6 days of differentiation are most likely immature progenitors much younger than 16 weeks of human development. Furthermore, *NANOS3* mRNA (a specific marker of newly specified germline cells) increases specifically between days 4 and 6 of differentiation, suggesting that germline identity progresses in an orderly fashion similar to the mouse. However, unlike the mouse, which expresses high levels of *Prdm14* in the newly specified germline (Yamaji *et al*, 2008), the *in vitro* differentiated human PGCLCs, as well as the *in vivo* 16-week human germline cells, had very low levels of *PRDM14* mRNA. Based on this result, the authors proposed a provocative hypothesis that unlike mice, PRDM14 will not be required for human germline development (Sugawa *et al*, 2015).

To address this, a short hairpin RNA strategy was used to knockdown *PRDM14* by around 50%. This resulted in no effect on PGCLC differentiation at day 4. In contrast, a knockdown of *PRDM1* caused a significant reduction in PGCLCs. In future studies,

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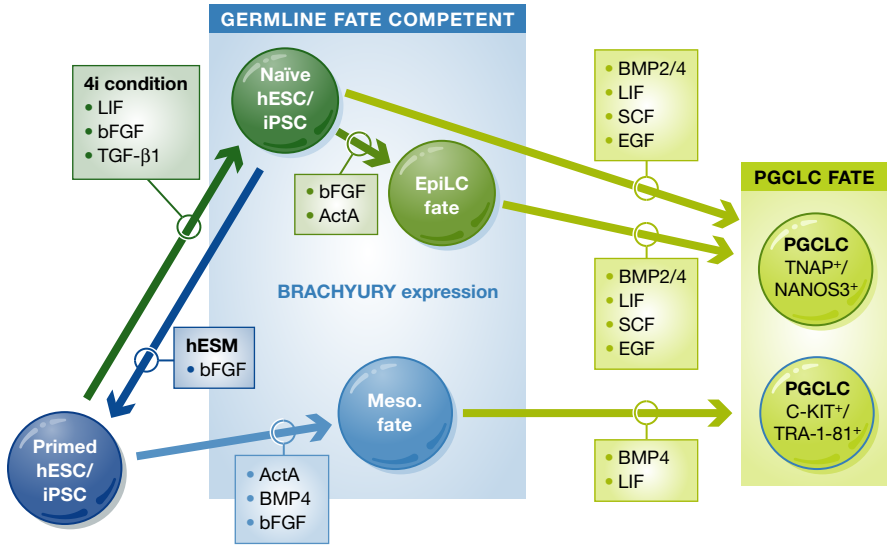

Figure 1. Inducing germline cells from human pluripotent stem cells.
Germline differentiation *in vitro* can be achieved by different routes. Irie *et al* started with hESCs/hiPSCs cultured in 4i media (naïve). Sugawa *et al* used conventional hESM (primed). Starting from the naïve state, PGCLCs were induced through either EpiLCs or directly as aggregates in PGCLC media. Starting from the primed state, PGCLCs were induced via a primitive streak/mesodermal intermediate stage. PGCLCs were identified using either TNAP/NANOS3-mCherry (Irie *et al*, 2015) or c-KIT/TRA-1-81 (Sugawa *et al*, 2015). EpiLC: epiblast-like cell; Meso.: mesodermal; hESM: human embryonic stem cell media; and ActA: activin A.

complete deletion of PRDM14 will be required to determine whether small amounts of PRDM14 are sufficient to enable human PGC development. Nonetheless, the low levels of *PRDM14* in human germline cells are suggestive that direct extrapolation of molecular pathways from mice to humans is not always possible.

The work by Irie *et al* (2015) used a different strategy to create human germline cells *in vitro*. Rather than differentiating through a primitive steak/mesoderm intermediate (Sugawa *et al*, 2015), Irie *et al* generated epiblast-like cells (EpiLC), followed by differentiation of PGCLCs in floating aggregates reminiscent of the protocol first established with mouse pluripotent stem cells (Hayashi *et al*, 2011). A second major difference was the starting pluripotent stem cell population. Irie and colleagues started with pluripotent stem cells cultured in naïve human stem cell media called 4i (Gafni *et al*, 2013). This media includes LIF, basic fibroblast growth factor (bFGF), TGF-β1, and four protein kinase inhibitors (hence the name 4i), which inhibit the GSK-3, MEK, MAPK, and JNK signaling pathways, respectively (Fig 1). It is interesting to note that cells cultured in the 4i media express *BRACHYURY*, and the authors suggest that this is the mechanism for priming human germline formation. However, additional studies are still needed to test this.

In order to differentiate human germline cells, the 4i-cultured pluripotent stem cells were either pre-induced for 2 days in bFGF, TGF-β, and 1% knockout serum replacer (KSR) or differentiated directly as aggregates in BMP4 or BMP2, LIF, epidermal growth factor (EGC), and stem cell factor (SCF). Similar to Sugawa *et al* (2015), Irie *et al* (2015) discovered that human PGCLC induction peaked at days 4–5 of differentiation (with PGCLCs defined as being *NANOS3*-mCherry/TNAP double positive), and in the one cell line used for the majority of analyzes (WIS2), the PGCLCs were mostly sustained over the next 3 days of culture with around 20% of the culture still corresponding to PGCLCs at day 7 (from a peak of 27% at days 4–5). The reason for this difference in PGC survival and/or proliferation between the two approaches warrants further investigation. Although the percentage of PGCLCs in Irie *et al* could still be detected with extended culture, the percentage of germline cells in each experiment was highly variable ranging from 10 to 47% at day 4 of differentiation (Irie *et al*, 2015). The source of this experimental variability, despite starting with the same cell line, is unclear. Using elegant CRISPR/Cas9 approaches to mutate the PRDM1 locus, Irie *et al* demonstrated that *PRDM1* is required for human PGCLC differentiation. Therefore, regardless of whether PGC formation was induced through a primitive streak/mesoderm-like pathway (Sugawa *et al*, 2015) or directly from 4i-cultured/pre-induced hESCs (Irie *et al*, 2015), these studies show that PRDM1 is a conserved regulator of germline differentiation in mice and humans.

In order to identify novel pathways for human germline development, Irie *et al* pursued the role of *SOX17*, which they discovered was expressed in human PGCs from the embryo as well as PGCLCs generated *in vitro*. A deletion of *Sox17* in mice has no effect on PGC formation (Hara *et al*, 2009). Using CRISPR/Cas9 to mutate the *SOX17* locus, Irie *et al* revealed quite unexpectedly that SOX17 (a classic endoderm marker) is required to generate *PRDM1*-positive PGCLCs *in vitro*. Similarly, Sugawa *et al* also discovered that *SOX17* was induced from as early as day 2 of *in vitro* culture. In their final model, Irie *et al* suggest that SOX17 is upstream of PRDM1 in germline differentiation. However, it is possible that this transcription factor might have a less straightforward link to PRDM1 given that we do not know the affect of deleting SOX17 on the intermediates between the naïve 4i state and PGCLCs differentiated at day 4. Further studies will be required to address this.

In summary, the studies of Sugawa and Irie and colleagues lay the foundation for robust human cell-based models beyond *in vitro* PGC differentiation such as increasing the likelihood of generating functional gametes for research and possibly for the clinic. However at a basic science level, both papers have opened up new areas of investigation focused on both improving the *in vitro* germline differentiation model and understanding the logic of human germ cell formation from different states of pluripotency. Now that the efficiency of germline formation from human pluripotent stem cells is robust, epigenetic reprogramming of the PGCLCs at single base resolution must be assessed, and new approaches for coaxing differentiation into gametes are required. Even though it is difficult to predict the time frame for when human gametes will be generated *in vitro*, the time for discussing policy on generating embryos for research purposes (not reproductive purposes) is now. Without the ability to test fertilization and pre-implantation embryo development

from *in vitro* differentiated gametes for research only, the quality of any *in vitro* gamete made from stem cells will never be truly known.

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