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The many dimensions of germline competence

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Abstract

Primordial germ cell (PGC) specification is the first step in the development of the germline. Recent work has elucidated human-mouse differences in PGC differentiation and identified cell states with enhanced competency for PGC-like cell (PGCLC) differentiation *in vitro* in both species. However, it remains a subject of debate how different PGC competent states *in vitro* relate to each other, to embryonic development, and to the origin of PGCs *in vivo*. Here we review recent literature on human PGCLC differentiation in the context of mouse and non-human primate models. In contrast to what was previously thought, recent work suggests human pluripotent stem cells (hPSCs) are highly germline competent. We argue that para-doxical observations regarding the origin and signaling requirements of hPGCLCs may be due to local cell interactions. These confound assays of competence by generating endogenous signaling gradients and spatially modulating the ability to receive exogenous inductive signals. Furthermore, combinatorial signaling suggests that there is no unique germline competent state: rather than a one-dimensional spectrum of developmental progression, competence should be considered in a higher dimensional landscape of cell states.

Introduction

Primordial germ cell (PGC) specification is the first step in the development of the germline that occurs around the onset of gastrulation in mammals. Efficient differentiation of human PGCs *in vitro* is required for *in vitro* gametogenesis: a technology with the potential to transform reproductive medicine [1]. In addition, understanding PGC differentiation is essential for understanding pluripotency, as germline competence is a defining feature of

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pluripotent stem cells [2]. Difficulties with efficient differentiation of human pluripotent stem cell (hPSCs) into PGC-like cells (hPGCLCs) have been attributed to the limited germline competence of hPSCs [3–5]. This prompted a search for pluripotent states with increased germ cell competence. Here we provide a concise review of the generation of PGCLCs from pluripotent cells. We first discuss signals required for PGC differentiation followed by PGC-competent states. We then describe recent work achieving efficient PGCLC differentiation from hPSCs, suggesting that there is no inherent limitation in germline competence of hPSCs. We propose two explanations for discrepancies in the literature describing germline competent states.

First, to address apparent discrepancies in hPSC to hPGCLC differentiation efficiency, we argue that assays of PGCLC differentiation efficiency in response to growth factors in the media do not measure competence as often presumed. Competence has been defined as "the ability [of a tissue] to respond to a specific inductive signal" [6,7]. At face value, this definition encompasses issues with the inductive signal reaching its receptors [8]. However, what is often not explicitly stated but implicitly assumed is that competence is a cell-intrinsic, epigenetic property. Here, we define competence as "the ability to differentiate upon appropriate activation of the inductive signaling pathways," where "appropriate" means that signaling is activated at the right level, for the right duration, etc. By this definition, PGC differentiation efficiency in response to an inductive signal (in this case exogenous BMP) does not actually reflect competence of individual cells. Instead, it reflects tissue level properties due to local cell-cell interactions. Cell interactions spatially modulate hPSC responses to exogenous BMPs and give rise to additional endogenous cell signaling gradients that play an essential but underappreciated role in PGC differentiation. Thus, PGCLC differentiation is limited, not because cells are not competent, but instead because most cells do not receive the appropriate inductive signals to begin with.

As a second cause for a lack of a consensus regarding PGC(LC) competence we propose that there is not a unique competent state. Combinatorial action of exogenous BMPs with multiple endogenous signals in controlling germ cell fate suggests PGCLC progenitor states cannot be simply placed on a one-dimensional spectrum from less to more developmentally advanced. Instead, germline competency may be multidimensional, forming a plateau in the Waddington landscape through which multiple paths can lead to PGCLCs. For example, Wnt signaling followed by BMP signaling may take cells along one path, while BMP followed by Wnt takes a different path. From this perspective there is neither a unique competent state, nor a unique inductive signal: different states may give rise to identical PGCLCs in response to different signals. Our discussion focuses on the relationship between cell signaling and germline competence; for a detailed discussion of the transcription factor network and epigenetic changes underlying PGC differentiation, we refer to several excellent recent reviews [9–13].

Signals for germline differentiation in the mouse

PGCs originate in the posterior embryonic epiblast of the pre-primitive streak and early primitive streak mouse embryo [14] where they are marked by expression of the PGC marker PRDM1 starting ~E6.25 [15]. Early gene knockout studies revealed that BMP4 and

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BMP8b from the extra-embryonic ectoderm (ExE; placenta precursor) are required for PGC induction, while loss of BMP2 from the visceral endoderm (VE; yolk-sac precursor) leads to a reduction in PGCs. Furthermore, grafting experiments suggested the entire embryonic epiblast is germline competent [16–21]. A landmark study by Ohinata et al. [22] further dissected PGC induction. It found that BMP4 from the ExE acts directly on the epiblast and that exogenous BMP4 is sufficient to induce PGC fate across the entire dissected epiblast between E5.5 and E6.5. In contrast, BMP8b from the ExE restricts induction of the anterior visceral endoderm, which in turn secretes inhibitors that restrict the primitive streak and PGC induction to the posterior epiblast. Exogenous BMP8b therefore has little impact on dissected epiblasts to generate PGCs. Exogenous BMP2 is also sufficient to induce PRDM1 expressing cells in epiblast explants, but PRDM1 expression is lower than with BMP4. Importantly, BMPs could not induce PRDM1 expression in *Wnt3*−/− epiblasts, which could be rescued by supplying exogenous WNT3A, showing that BMPs and WNTs may act combinatorially. WNT3 in the epiblast induces Nodal expression in the mouse epiblast during mouse gastrulation [23–25]. However, a Nodal signaling inhibitor had little impact on PRDM1 expression in BMP-treated epiblast explants. In summary, BMP4 and WNT3 are required for mouse PGC differentiation.

While exogenous BMP4 is sufficient for PGC induction in the epiblast, several factors that do not impact induction efficiency were found to positively impact survival and proliferation [22,26,27]. The protocol for PGCLC induction from mouse epiblasts thus included BMP4, BMP8b, SCF, LIF, and EGF and has remained largely unchanged for *in vitro* induction of PGCLCs from PSCs from various species, including human and mouse.

The roles of Nodal and BMP signaling in mouse PGCs have been re-investigated recently. BMP signaling in PGCs was found to be reduced relative to the neighboring extraembryonic mesoderm [28,29], indicating that PGCs may downregulate BMP signaling after they are induced. Surprisingly, BMPR1A−/− mESCs could be differentiated to PGCLCs, and BMP activation or inhibition on day 2 in 3D mouse gastruloids, respectively decreased or increased the number of TFAP2C positive cells on day 5 [28,30]. Therefore, it was suggested that BMP signaling may not be directly required for mPGCLC induction. However, a quantitative evaluation of signaling levels and a full panel of PGC markers over time was not performed, nor were BMP manipulations at different times. This leaves open the possibility that PGCLC induction is in fact compromised, or that BMP signaling is required at lower levels and a different time course than assumed. Recent reinvestigation also found that although *Nodal*$^{-/-}$ embryos generate PRDM1-expressing cells, these do not express other PGC markers like TFAP2C, SOX2, and DPPA3, which were not evaluated in earlier work. This suggests that PGC induction may have also been compromised by Nodal inhibition in the earlier studies. However, genetic experiments with Nodal are difficult to interpret because Nodal is part of multiple feedback loops within and between the epiblast and extraembryonic tissues. Moreover, Nodal is involved in both maintenance of pluripotency in the epiblast and its differentiation into a diverse spectrum of fates [25] (Figure 1). Importantly, BMP4 expression is also lost in Nodal-mutants as Nodal is both downstream of BMP signaling and part of a feedback loop that maintains BMP4 expression. Thus, the lack of sustained BMP signaling may be responsible for the loss of PGC markers

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in the absence of Nodal, although reduced PGCLC differentiation from *Nodal*$^{-/-}$ ESCs suggests a direct requirement for Nodal signaling in the generation of PGCLCs [31].

Mouse germline competence and pluripotency *in vitro*

When the protocol to generate PGCLCs from epiblast cells was first developed [22], two types of mouse pluripotent stem cells had been established in culture: embryonic stem cells (mESCs) and epiblast stem cells (mEpiSCs) which represent distinct "naïve" and "primed" pluripotent epiblast states [32]. mESCs are derived from and correspond to the pre-implantation epiblast progenitor cells in the ~E4 embryo [33,34]. mESC colonies have a dome shaped morphology and are maintained in culture media supplemented with MEK and GSK3 inhibitors and leukemia inhibitory factor (LIF) (2i + LIF) [3,35]. In contrast, EpiSCs are derived from embryos up to E7.5 and represent post-implantation epiblast, likely E6.5-E7.5, although variations in the derivation and culture conditions confound precise staging [36–38]. They are maintained in media supplemented with Activin and FGF2, and form an epithelial monolayer. mESCs and EpiSCs also differ in their ability to contribute to chimeras, X-inactivation status, gene expression, and chromatin modification profiles, matching the pre- and post-implantation embryonic epiblasts they represent.

Although WNT3 and BMP4 act combinatorially in PGC differentiation *in vivo* [22,39], the extent to which germline competence *in vitro* is mediated by endogenous Wnt is unknown and competence is evaluated as the fraction of cells expressing PGC marker genes after treatment with only BMP4 and maintenance factors. By this measure, it was found that neither mESCs nor EpiSCs efficiently differentiate into PGC-like cells [4]. It was reasoned that since these correspond to the E4-E4.5 pre-implantation and E6.5-E7.5 post-implantation epiblast, both are outside the epiblast competence window for PGC induction of E5.5-E6.5. By switching mESCs to EpiSC maintenance conditions, cells were found to pass through a transient intermediate state resembling the E5.5 epiblast named mouse epiblast-like cells (mEpiLCs) from which they efficiently differentiate into PGCLCs [4].

The discovery of intermediate but transient cell types like EpiLCs motivated a search for protocols to stabilize these distinct intermediate or "formative" pluripotent states, which can be functionally defined by germline competence [3,5]. Formative pluripotent cells (FPCs) have a flattened epithelial morphology and, unlike primed pluripotent cells, maintain the ability to contribute to chimeras and express intermediate levels of naïve and primed pluripotency genes. Several recent studies have identified conditions to stably maintain FPCs in culture [40–45]. A common theme in these protocols is an emphasis on the importance of controlling Wnt, FGF, and TGF-beta signaling. However, these FPCs differ in their culture conditions and properties, and consequently all go by different names (Table 1). To earn the designation of FPCs, cells should efficiently differentiate into PGCLCs upon BMP treatment, although this property has not been demonstrated in each case.

PGC differentiation of human cells

hPSCs resemble EpiSCs more closely than mESCs in many aspects including maintenance conditions, morphology, X-inactivation status, and gene expression profiles. After the

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discovery of EpiSCs, hPSCs were quickly equated with mEpiSCs [48]. Subsequently, conditions that capture varying degrees of naïve pluripotency in human cells were discovered [49]. Accordingly, the limited success in differentiating hPSCs to hPGCLCs [50–53] with exogenous BMPs was interpreted as low germline competence of primed cells. Attempts to improve hPGCLC induction efficiency have therefore focused on first achieving a competent state, followed by PGC induction with BMPs.

In one approach mimicking the mouse EpiLC protocol, human pluripotent cells maintained under conditions which induce naïve-like features ("4i" or NHSM [54]) were converted to an epi-like state with FGF and Activin after which they differentiated to hPGCLCs more efficiently (from <5% to 27%) [52]. More recently, a similar strategy was pursued with improved naïve human pluripotency protocols, leading to "reset" hPGCLCs with comparable efficiency [55]. A different approach [53,56] exposed cells to primitive streak inducing signals to create "incipient mesoderm-like cells" (iMeLC) or "precursors of mesendoderm" (PreME). PreME differentiation also enhanced subsequent hPGCLC induction by BMP treatment, in line with the earlier finding that mPGC induction requires the gene brachyury (TBXT) which marks mesoderm precursors [39]. Several of the conditions that maintain mouse FPC states were also found suitable for human cells but hPGCLC competence was not compared to other approaches.

It is counter-intuitive that both developmental progression towards mesoderm (by iMeLC differentiation) and developmental reversal (by resetting to formative or naïve pluripotency) would each enhance hPGCLC competence. One attempt to reconcile this discrepancy defined iMeLCs as an intermediate between primed and naïve pluripotency; however, this approach projected a high-dimensional gene expression profile on two points without evaluating the alternative hypothesis that iMeLC is an intermediate state between pluripotency and primitive streak [57]. Further work showed 4i-derived precursors and iMeLC to be similar to each other and distinct from the resetting naïve cells [55].

To begin to understand this, it is important to consider several differences between human and mouse in both the germline competent progenitors and signaling requirements. PGCs are first observed in the amnion [58–60] in monkey embryos, in contrast to mouse, rabbit, and pig PGCs, which all originate from the pre-streak posterior epiblast. This raises the question if monkey/human PGCs derive from amnion. However, the fact that amnion and PGC differentiation occur simultaneously suggest that rather than PGCs deriving from the amnion, both the PGCs and the amnion derive from pluripotent cells in overlapping signaling environments. Consistent with this, and in contrast to mouse cells, hPSCs treated with BMP4 in the absence of Wnt differentiate to what are thought to be amnionic ectoderm-like cells (hAELCs), although this remains subject to debate [61–66]. Furthermore, TFAP2A-positive progenitors give rise to both hAELCs and hPGCLCs [57,67]. On the other hand, in spatially organized stem cell models for the human embryo [68–70], hPGCLCs appear scattered among both the amnion-like and primitive streak-like cells [63,71,72]. Moreover, PGCs appear transcriptionally between the epiblast and primitive streak in rare single cell RNA-sequencing data of a gastrulating human embryo [73]. Altogether, this suggests that primate PGCs arise in signaling conditions that are intermediate between those giving rise to primitive streak and amnion, in line with the fact

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that iMeLC differentiation followed by BMP4, i.e., a combination of primitive streak and amnion induction signals, improves PGCLC differentiation.

Furthermore, a comparison between hPSCs, mouse embryos, cynomolgus monkey embryos, and cynomolgus monkey PSCs (cmPSCs) revealed that hPSCs and cmPSCs are transcriptionally very similar to each other and the post-implantation monkey epiblast, but that the latter is transcriptionally stable for a week and resembles the E5.5 mouse epiblast [74]. This is consistent with earlier work transcriptionally placing hPSCs between mESCs and EpiLCs which led to characterization of hPSCs as having an “extended primed pluripotency”, competent to generate all cell types that emerge after implantation including amnion and germ cells [1]. Corroborating this, enhancers for PGC genes are active in hPCSs [75]. Therefore, it is unclear whether hPSCs can be called primed by mouse standards.

PGCLC differentiation efficiency from hPSCs reflects tissue organization, not competence

Consistent with the idea that hPSCs are more like mouse formative PSCs than primed PSCs and therefore highly germline competent, two recent approaches obtained over 50% hPGCLCs from hPSCs using BMP4 without first inducing a competent state.

The first approach [63], by the current authors, used micropatterning to control colony geometry. Disc-shaped colonies treated with exogenous BMP4 form self-organized cell fate patterns that model human gastrulation *in vitro* [76]. With a colony diameter of 700um, PGCLCs constitute 5–10% of the cells and are localized on the interface between the amnion-like and primitive streak-like regions [63] (Figure 2). Crucially, response to exogenous BMP in this system is restricted to the colony edge due to BMP receptors localizing basolaterally [77]. Furthermore, like in mouse gastrulation, the self-organized patterning in this human model depends on a BMP-Wnt-Nodal signaling hierarchy: exogenous BMP4 induces endogenous Wnt3, which in turn induces endogenous Nodal, leading to dynamic and overlapping gradients of these signals [78–80]. Both Wnt inhibition and Nodal knockout block PGCLC differentiation but either can be rescued by exogenous activation of the Nodal pathway, demonstrating a key role for Nodal in hPGCLC induction from hPSCs. Importantly, Nodal signaling duration is a critical parameter and despite the requirement for Nodal, prolonged Nodal signaling inhibits PGCLC differentiation, instead inducing endoderm. There is minimal cell rearrangement in micropatterned colonies [78], suggesting PGCLCs are induced at a fixed distance from the colony edge by the right combination of BMP, Wnt, and Nodal (and possibly other signals). Because in smaller colonies all cells are close enough to the edge to respond to BMP, optimizing micropatterned colony size increased the efficiency of PGCLC differentiation using BMP alone to 50%, with the remaining 50% becoming amniotic ectoderm-like due to higher BMP response on the colony edge.

In a different approach, Overeem [81] et al. added a solution of extracellular matrix (ECM) to form hPSCs spheroids, which upon BMP treatment formed hPGCLCs with over 50% efficiency. They showed that ECM overlay increases BMP response after which PGCLC differentiation requires a much lower BMP4 concentration than in the standard protocol.

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Although this remains to be tested, their results could be explained by the basal localization of BMP receptors and tissue geometry. In contrast to standard tissue culture where the basal surface faces the substrate and is shielded from exogenous BMP, with ECM overlay the cells form spheroids with the basement membrane facing the media so that the receptors are more uniformly exposed to exogenous BMP. Too high a BMP response leads to amnion differentiation, explaining the need to reduce the BMP dose with this approach. It will be important to investigate whether the 50% efficiency can be accounted for by remaining non-uniformity in BMP response and determine how required endogenous Wnt and Nodal signaling downstream of BMP are affected by the ECM overlay. Other signaling pathways may also play important roles, e.g., YAP is likely affected by the tissue organization and has recently been indicated as important for hPGCLC differentiation, in part by modulating Wnt [82].

Overall, a picture emerges wherein differences in PGCLC differentiation efficiency between approaches can be explained by tissue organization, which modulates both the response to exogenous BMP and endogenous cell signaling interactions downstream. Although cell intrinsic differences in competence may also contribute to differentiation efficiency, they cannot account for the recent results. To begin to unravel the relative contributions of tissue organization and competence in determining fate, it will be necessary to both measure how signal reception is affected by tissue organization and directly relate cell signaling activity to fate.

If hPSCs are highly germline competent, it is not clear why strategies using naïve cells improve differentiation. It is worth considering the possibility that these cells are more BMP-responsive due to changes in receptor polarization. Similarly, iMeLC differentiation could improve hPGCLC induction in part by modulating endogenous Wnt and Nodal expression through Wnt and Nodal autoregulation, thereby creating the right combinatorial signaling for PGCLC induction. More generally, some differences in competence in the literature may be accounted for by tissue geometry and endogenous signaling. For example, differences between hPSC lines [57] previously interpreted as a consequence of epigenetic lineage priming, could instead reflect differences that directly modulate responses to inductive signals like receptor polarization or cell junction integrity. In support of this notion, mouse epiblasts efficiently make PGCLCs in floating culture but not on a 2D substrate, where their receptors are likely shielded from the media [22].

Multiple paths through the Waddington landscape

Induction has been defined as involving separate inducing and responding tissues [7]. BMP is produced by the extra-embryonic cells while Wnt and Nodal are produced by the epiblast itself. Nevertheless, it is illuminating to conceptually separate signal interpretation from the signal source and to consider how combinatorial signals are interpreted.

Individually, these signals induce distinct non-PGC fates. For human, BMP alone induces amnion while Wnt and Nodal induce primitive streak. Only the right combination of these signals produces PGCs. Rather than separate them into permissive and instructive signals, we propose to consider their combinatorial action as moving cells around a higher-

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dimensional Waddington landscape. BMP moves cells in the amniotic ectoderm (AELC) direction in this landscape, while Wnt and Nodal move cells towards mesendoderm (a.k.a. primitive-streak, PSLC). PGCs lie in between and to get there, cells can take multiple paths. If they are exposed to BMP first, they will move in the AELC direction, but timely exposure to Nodal and Wnt will turn them in PSLC direction and land them in the PGC basin. This is what happens when hPSCs are differentiated with BMP directly, as endogenous Wnt and Nodal are activated downstream with a delay. Conversely, if cells are first exposed to Wnt and Nodal they move in the PSLC direction, but BMP exposure before they commit may redirect them to become PGCs, which resembles protocols with an iMeLC step.

From this perspective there is no “true” competent state or inductive signal. Nodal and Wnt induce a state that is competent for PGC differentiation in response to BMP, while BMP induces a different state that is competent for PGC differentiation in response to Nodal and Wnt. The right inductive signal, i.e. the right direction in which to move, depends on the current state. Competence does not lie on a line that reflects developmental stage and on which cells can only move forward or backward, but on a plateau on which can move in multiple directions, as Waddington himself described it [6]. Different parts of the tissue at the same developmental time may explore different parts of this plateau. They may also arrive in the same place at different times through different paths, i.e., differentiation is asynchronous if signaling is heterogeneous.

More than a metaphor, this picture can be made precise and falsifiable with mathematical modeling [63,83,84], as illustrated in Figure 3a. It also serves as a reminder that PGCs cannot be understood in isolation: PGC competence is lost when cells commit to alternate fates, therefore understanding commitment to alternate fates is as important as understanding PGC fate itself for improving PGC differentiation [85,86].

This model resolves apparent contradictions between an amnion-like or mesoderm-like origin for PGCLCs: it can be both. That explains the description in the literature of different hPGCLC-competent populations marked by combinations of amnion markers induced by BMP like TFAP2A or mesendoderm markers induced by Nodal and Wnt like TBXT [57,67,87]. It also explains the appearance of PGCLCs in both the amnion-like and PS-like regions of human embryoids [63,71] and the placement of hPGCLCs between those fates in scRNA-sequencing data of BMP treated hPSCs (Figure 3b).

Conclusion and outlook

In contrast to what was previously thought, hPSCs appear broadly competent to differentiate to hPGCLCs in response to BMP4. hPGCLC differentiation efficiency was previously found to be limited not because cells are not competent but because most cells do not receive the appropriate inductive signals: response to exogenous BMPs strongly depends on tissue organization and endogenous signaling gradients. Although cell state and tissue organization are linked – e.g., the polarization of the epithelium reflects gene expression, and conversely modulating epithelial polarization with matrix proteins will affect its state – significant insight can be gained from disentangling their effects. Local cell interactions that organize the tissue may therefore be key to understanding human germline differentiation *in vitro*.

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In this regard, an important open question is whether endogenous signaling in hPGCLC differentiation can be treated as purely autocrine, or whether interactions between distinct populations are required (e.g. amnion-like cells and hPGCLCs).

Germline competence can be considered higher dimensional when endogenous signals are placed on equal footing with exogenous BMPs. There may be distinct competent states along different trajectories through the landscape that are equal in developmental advancement but require distinct signals to reach the same endpoint. This framework may enable a unified understanding of different competent *in vitro* states described in the literature. If multiple trajectories exist, it will be important to determine which trajectories are realized *in vivo*, and whether PGCLCs retain any memory of their trajectory, i.e., whether the result of different protocols is truly equivalent. Transcriptional analysis indicates that the expression profile of hPGCLCs derived with BMP followed by endogenous Wnt and Nodal is equivalent to that of hPGCLCs derived with exogenous Wnt and Nodal activation followed by BMP [63]. On the other hand, hPGCLCs derived from naïve human pluripotent cells may retain epigenetic memory of their origin and mature faster than those derived from standard hPSCs [55]. It therefore merits further investigation to determine if and how different PGCLC differentiation protocols affect the resulting state and subsequent maturation of the cells.

It is interesting to ask why there would be multiple paths towards the same state. One possibility is that it is simply a consequence of controlling three fates with two (sets of) signals. For example, in an oversimplified model for differentiation consisting of two independent switches, where off–off is the pluripotent state, on-off is amnion, off-on is primitive streak, and on–on is PGCLC, one can imagine that it is immaterial which switch is flipped first to get to the on–on state. Another possibility is that flexibility in the relative timing of signals is required by evolution to generate PGCs from divergent sources of BMP across mammalian species. For example, the amnion in primates appears to substitute for the BMP4-producing role of the extraembryonic ectoderm in mice, while in rabbits the margin of the epiblast expresses BMP4, all possibly with different timing relative to Wnt and Nodal expression [65,89]. Insight into how and why multiple progenitor states can give rise to the same final state could also be gained by comparing with other examples, like definitive endoderm, which can arise from both visceral endoderm and epiblast [90,91].

In conclusion, there are many dimensions to germline competence. By breaking down what germline competence means, we will gain deeper insight into human pluripotency and accelerate progress toward *in vitro* gametogenesis.

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Data availability

No data was used for the research described in the article.

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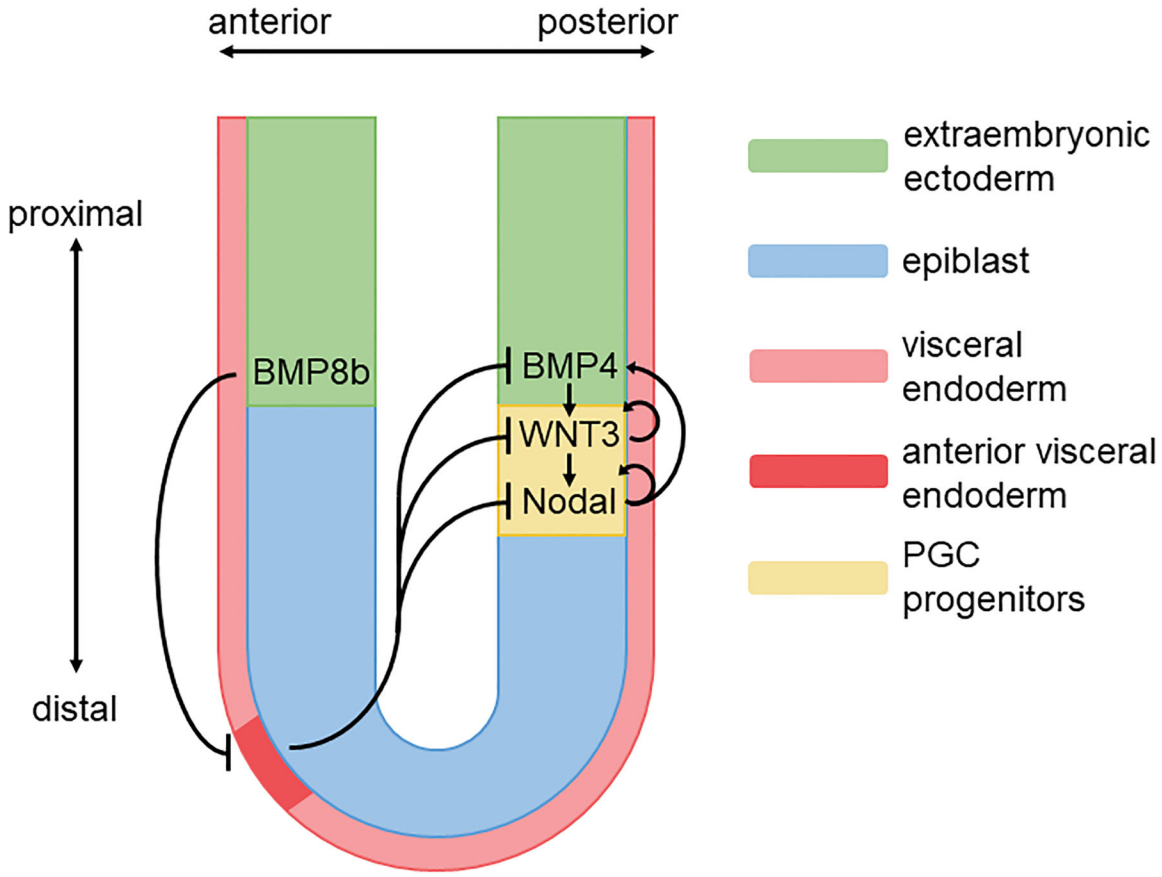

Figure 1.

Signals for PGC specification in the peri-gastrulation mouse embryo. BMP4 from the extraembryonic ectoderm (ExE) induces Wnt3 in the epiblast, which in turn induces Nodal. Nodal signaling forms a positive feedback to maintain BMP expression in the ExE. The ExE also secretes BMP8b, which restricts the anterior visceral endoderm (AVE) to its initial position in the distal part of the visceral endoderm after which it migrates to the anterior border of the epiblast and ExE. Inhibitors from the AVE restrict the signals controlling PGC differentiation to the posterior proximal epiblast.

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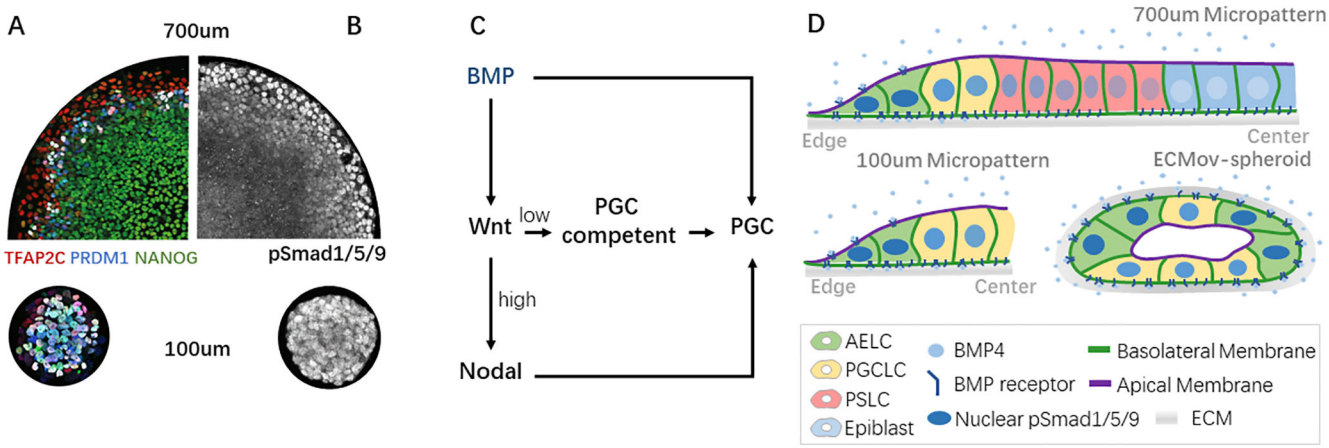

Figure 2. Effect of tissue geometry and endogenous signaling on PGCLC differentiation.

A, B) Immunofluorescence for (**a**) early hPGC marker genes, and **b**) pSmad1/5/9 staining (maximal intensity projection along z) in 700um and 100um micropatterned hPSC colonies treated with BMP4 for 2 days. **c)** Simplified diagram of signaling hierarchy controlling hPGCLC induction in hPSCs. **d)** Cartoon showing how receptor localization and tissue organization impact BMP response in micropatterned colonies and hypothetically in ECMov-spheroids to enable efficient hPGCLC differentiation. AELC: Amniotic Ectoderm-like cell; PGCLC: Primordial Germ Cell-Like cell; PSLC: Primitive Streak-Like cell; ECM: Extracellular Matrix.

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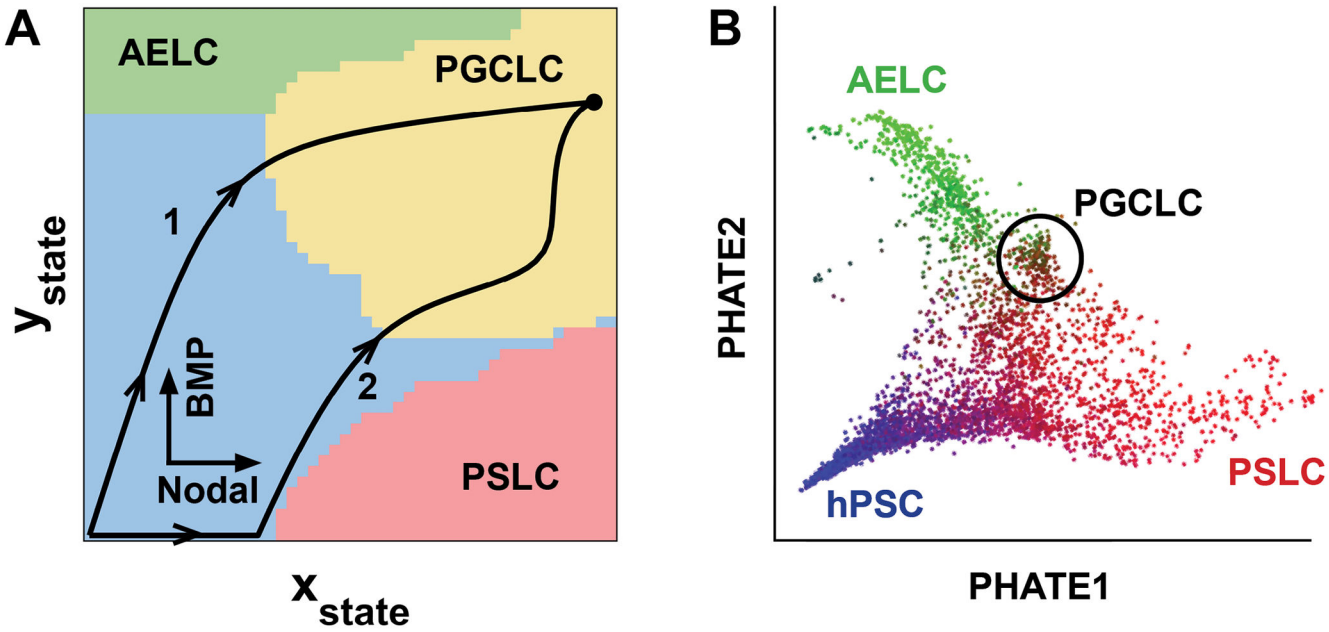

Figure 3. Different routes through the Waddington landscape.

a) A landscape of cell states is shown with basins for amniotic ectoderm-like cells (AELC), primitive streak-like cells (PSLC), and primordial germ cell-like (PGCLC) cells derived from a mathematical model for hPGCLC differentiation [63]. The size of the basins does not reflect the probability of reaching it. Two paths through the landscape driven by distinct signaling dynamics are shown. Path 1 is continuous stimulation with a high level of BMP and low level of Activin/Nodal signaling. Path 2 represents initial treatment with CHIR + a high dose of Activin followed by treatment with a high dose of BMP4. x- and y-axes, respectively correspond to PSLC + SOX17 and AELC + TFAP2C as defined in Jo et al. [63]. The model is a simplification that does not capture many known complications, e.g., it does not separately model the roles of Nodal and Wnt. Nevertheless, it explains the effect of many signaling perturbations on PGCLC differentiation in micropatterned colonies and could be refined without fundamentally changing the picture above. **b)** PHATE visualization [88] of scRNA-sequencing data of BMP treated colonies is structured like the model landscape with primitive streak and amnion branches going in two directions and PGCLCs in the middle. Colors: SOX2 (blue), TBXT (red), ISL1 (green).

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Table 1

Formative pluripotent states.

Name	FGF pathway modulation	Wnt pathway modulation	TGF-b pathway modulation	other characteristics	species examined	PGCLC differentiation?
RSC (rosette-like) [40]	MEKi	Inhibit: IWP2	–	FBS, LIF	Mouse	In chimeras
fPSCs (formative) [41]	FGF2	Inhibit: XAV939	Activin A	spheroids	Mouse	Yes
FSCs (formative) [44]	–	Inhibit: XAV939	Low Activin A	RARi	Mouse, human	Yes
FTW-ESCs/XPSC (X = chimera) [42]	FGF2	Activate: GSK3i or Wnt3a	TGFb1 or Activin A	MEF feeders	Mouse, human, horse	Yes
INTPSCs [45]	FGF2	Activate: GSK3i	Activin A	KSR,BSA	mouse	Yes
PiCs [46]	–	–	–	L-proline, FBS, LIF, feeders	mouse	Yes [47]

MEKi inhibits the MAPK pathway downstream of Fibroblast Growth Factor (FGF). IWP2 is a porcupine inhibitor which blocks Wnt secretion. GSK3 inhibitor (most commonly CHIR99021) acts as a canonical Wnt agonist. RARi: retinoic acid receptor inhibitor. XAV939 is a tankyrase inhibitor, inhibiting canonical Wnt signaling. FBS: fetal bovine serum. LIF: Leukemia Inhibitory Factor. MEF: mouse embryonic fibroblast. BSA: bovine serum albumin. KSR: knockout serum replacement.

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