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The establishment and regulation of human germ cell lineage

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Abstract

The specification of primordial germ cells (PGCs) during early embryogenesis initiates the development of the germ cell lineage that ensures the perpetuation of genetic and epigenetic information from parents to offspring. Defects in germ cell development may lead to infertility or birth defects. Historically, our understanding of human PGCs (hPGCs) regulation has primarily been derived from studies in mice, given the ethical restrictions and practical limitations of human embryos at the stage of PGC specification. However, recent studies have increasingly highlighted significant mechanistic differences for PGC development in humans and mice. The past decade has witnessed the establishment of human pluripotent stem cell (hPSC)-derived hPGC-like cells (hPGCLCs) as new models for studying hPGC fate specification and differentiation. In this review, we systematically summarize the current hPSC-derived models for hPGCLC induction, and how these studies uncover the regulatory machinery for human germ cell fate specification and differentiation, forming the basis for reconstituting gametogenesis in vitro from hPSCs for clinical applications and disease modeling.

Introduction

Germ cells are the sole cell type responsible for transmitting genetic and epigenetic information across generations, forming the foundation for sexual reproduction and species survival [1–3]. There are two modes of germline establishment: preformation and epigenesis [4]. In C. elegans, Drosophila, and many other animals, germ cell fate is determined by the polarized maternal factors known as germ plasm. However, in mammals such as mice and humans, germ cells are specified by epigenesis, in which germ cell fate is induced by extrinsic signals from surrounding somatic tissues during early

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embryogenesis [3, 5–10]. Germ cell development is a precisely controlled, stepwise process that initiates from the specification of primordial germ cells (PGCs), the precursors to the germline [6, 8]. In humans, PGCs are separated from somatic cells at around 2 weeks post fertilization, then migrate to the genital ridge at around week 4, where they interact with somatic cells to form the gonads for further differentiation [7]. Subsequently, gonadal germ cells undergo meiosis, spermatogenesis in males or oogenesis in females to produce mature sperm and eggs for reproduction, constituting the germ cell lineage. Ultimately, fertilization of the egg by the sperm forms a totipotent zygote, initiating the development of a new embryo where a new germ cell cycle commences (Fig. 1). Deciphering how human PGCs (hPGCs) are specified is fundamental to understanding the subsequent events such as PGC migration, gonad formation, gametogenesis, and the pathological mechanisms of germ cell-related diseases [7, 11].

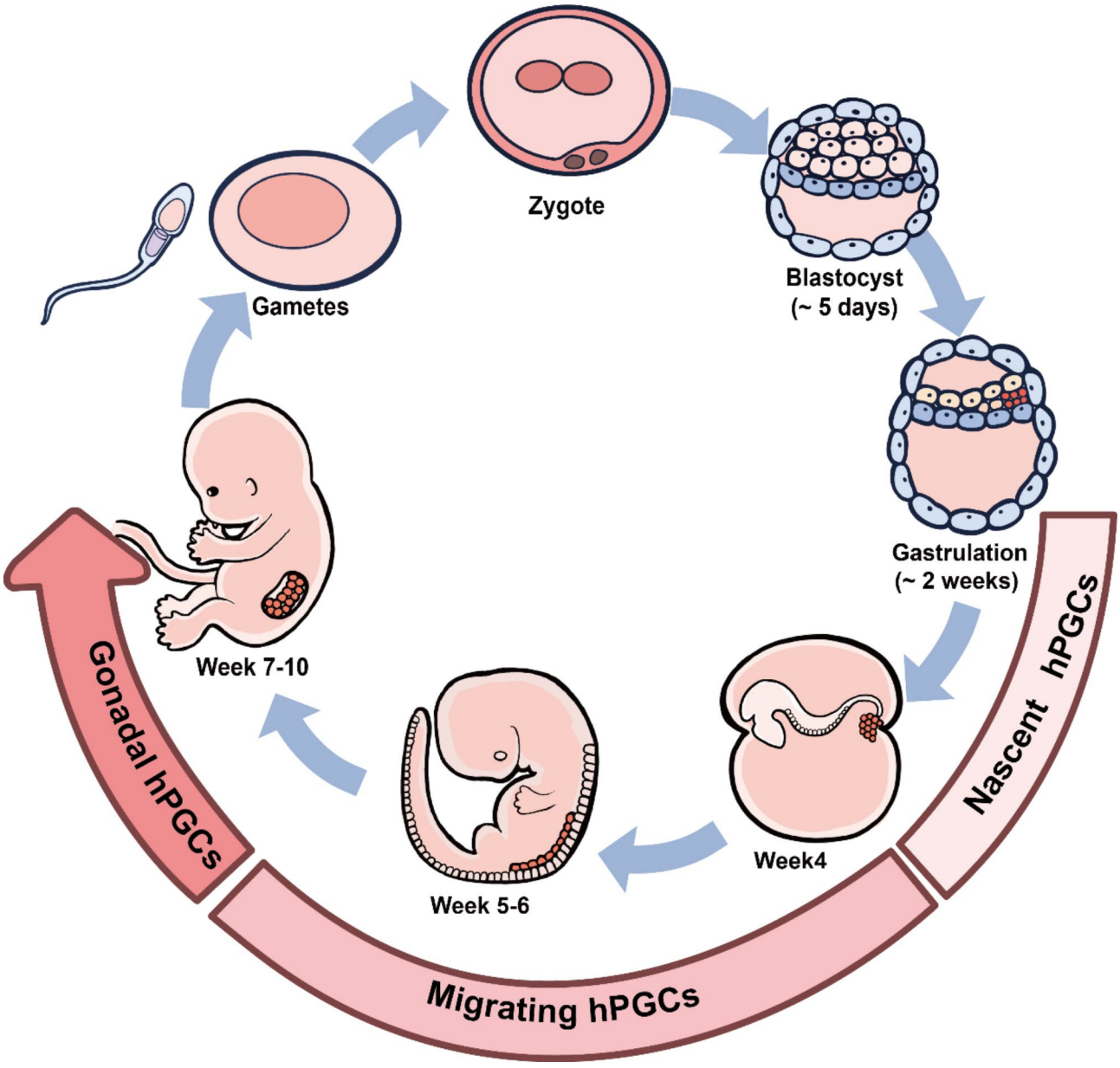

Fig. 1 The cycle of human germ cell lineage

Life begins with the fertilization of the egg by sperm, mature germ cells for reproduction. The fertilized egg develops into a blastocyst with epiblasts that will differentiate into the embryo proper. Human primordial germ cells (hPGCs) (colored red) are specified around the time of gastrulation (nascent hPGCs). After that, these hPGCs migrate along the hindgut (migrating hPGCs) to meet the gonadal somatic precursor cells to form the embryonic gonads (gonadal hPGCs), where they keep differentiation and maturation until they form sperm and eggs in the adult to initiate a new cycle

Approximately 10% of individuals at reproductive age suffer from infertility [12–15]. Defects in PGC specification may lead to infertility, germ cell tumors, and birth defects [11, 16]. However, investigating human embryos between weeks 2 and 4, the key time window for hPGC specification and differentiation, remains practically challenging and ethically unfeasible due to the strict ethical constraints. Thus, understanding the molecular mechanisms underlying human PGC specification relies heavily on the in vitro systems that differentiate germ cells from human pluripotent stem cells (hPSCs). The establishment of PGC-like cells (PGCLCs) provides a critical model system to study the genetic and epigenetic regulation of germ cell specification and maintenance, as well as the potential reconstitution of gametogenesis in vitro for clinical applications for infertility-related diseases [3, 7, 17, 18]. In this review, we summarize the establishment and regulation of human germ cell lineage. We first introduce the establishment of hPGCs in vivo, then describe three types of methods for achieving efficient human PGCLC (hPGCLC) differentiation from hPSCs, and summarize the regulatory network composed of signaling molecules, transcription factors and epigenetic factors, forming the basis for understanding the establishment and regulation of human germ cell lineage and providing insights for investigating the potential pathogenetic mechanisms of infertility-related diseases.

Establishment of human PGCs *in vivo*

Given the scarcity of human embryo resources and ethical restrictions, mouse PGCs (mPGCs) have been the most studied model to understand human PGC development [6, 8, 19–21]. However, increasing evidence suggests that humans and mice diverge in the mechanisms of PGC specification [7, 22–24]. Non-human primate models provide invaluable insights into human germline development due to their closer evolutionary divergence and more similar embryological morphology to humans. In primates, post-implantation embryos develop into a bilaminar disc structure similar to that of humans, rather than the cup-shaped cylinders found in mice [25, 26]. In cynomolgus monkeys, cynomolgus PGCs (cyPGCs) are observed in the early amniotic membrane at embryonic day 11 (E11) before gastrulation [27]. These cyPGCs are identified using immunostaining for markers such as SOX17 and TFAP2C. By E50, cyPGCs persistently express a core set of PGC markers, including SOX17, PRDM1 (also known as BLIMP1), TFAP2C, NANOG, and OCT4 [27]. These observations imply that hPGCs may similarly originate from the early amniotic membrane.

In addition to model organisms, current knowledge about the establishment of hPGCs in vivo mainly comes from in vitro-fertilized embryos and a small number of fetal samples. Specification of hPGCs occurs at around week 2 post-fertilization [7, 28]. Histological observations of embryos have identified small amounts of hPGCs as early as E12-13 (Carnegie Stages 5-6a, CS5-6a) [28, 29]. Recent studies based on in vitro-fertilization embryos identified nascent hPGCs around E12 [30, 31]. Single-cell transcriptomic studies of an E16-17 human embryo (CS7) characterize a small number of hPGCs in the posterior gastrulation region [32]. Microscopic observations of embryo samples from the Kyoto and Carnegie collections reveal that hPGCs localize near the yolk sac wall close to the allantois at E24 (CS12). Subsequently, these cells migrate through the hindgut to the developing genital ridges, where they begin oogenesis or spermatogenesis [7, 28]. To reconstruct hPGC development in vitro, a series of hPGCLC systems have been established [22, 23], based on the gradual discoveries of the key factors regulating hPGCLC specification. hPGCLCs are induced by BMP and WNT signals, which activate the expression of downstream PGC activity factors such as EOMES [33, 34], SOX17 [22], PRDM1 [22, 23], and TFAP2C [33, 35]. Next, we will focus on these hPGCLC induction systems that advance our understanding of the initiation, maintenance, and regulation of human germ cell lineage, followed by highlighting the elucidation of regulatory mechanisms governing the specification and development of hPGCLCs.

The development of hPSC-based *in vitro* PGCLC induction strategies

The reconstitution of PGCs in vitro is first achieved in mice, resulting in mouse PGCLCs (mPGCLCs) resembling the in vivo mPGCs [18]. These mPGCLCs contribute to the generation of viable and fertile offspring, the golden rule of functional germ cell fate [36–39]. The emergence of mPGCLCs has provided a conceptual framework and feasibility for the reconstruction of human germ cell development in vitro. Prior to this, hPGCLCs were generated at a low frequency by spontaneous differentiation of hPSCs in vitro, due to the different pluripotent states of human embryonic stem cells (hESCs)/human induced pluripotent stem cells (hiPSCs) compared with mouse embryonic stem cells (mESCs)/mouse induced pluripotent stem cells(miPSCs) [18, 36, 37, 40–43]. Since 2015, several groups have established different methods for robustly inducing hPGCLCs under defined conditions to explore the molecular mechanisms underlying hPGC specification [22, 23, 44, 45]. Based on these pioneering explorations, a series of in vitro hPGCLC induction systems have been developed to induce hPGCLCs more efficiently and with higher throughput, as well as to obtain hPGCLCs that are closer to the in vivo hPGCs [46–54]. We will highlight some of these key

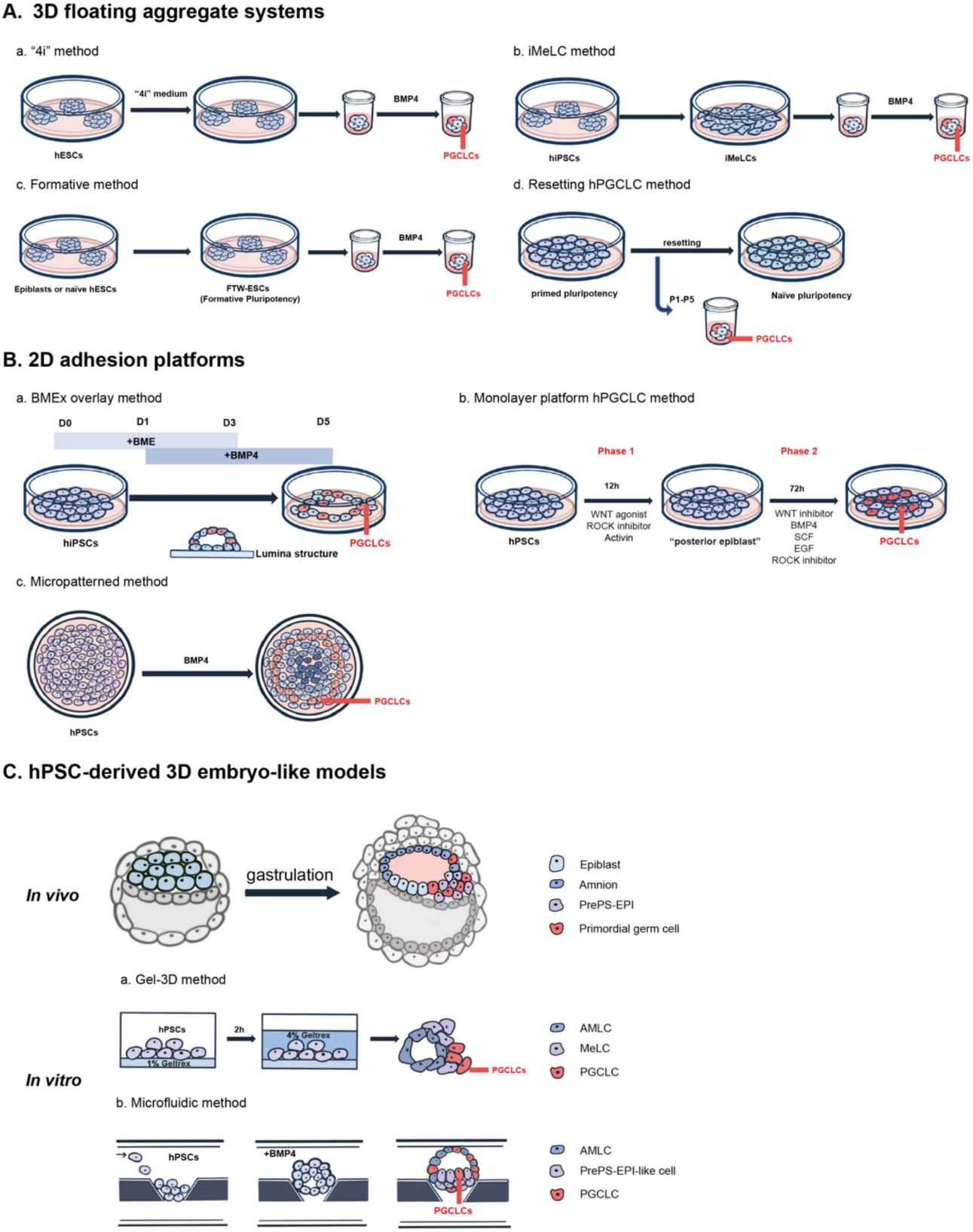

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Fig. 2 Summary of hPSC-derived hPGCLC induction methods

(**A**) Schematic illustration of hPGCLC induction in floating 3D aggregates systems, including the hPGCLCs induced from 4i hESCs, iMeLCs, formative and resetting hPSCs. hPGCLCs are shown in red. (**B**) Schematic illustration of hPGCLC induction in 2D adhesion systems, including the BMEx, monolayer, and micropatterned methods. (**C**) Schematic illustration of hPGCLC induction by hPSC-derived embryo-like models, including the Gel-3D and microfluidic methods

strategies that broaden and deepen our understanding of the specification and development of human germline.

Induction of hPGCLCs in 3-dimensional (3D) floating aggregates

PGCs are specified and differentiate in the 3D environment in developing embryos by interacting spatially with neighboring somatic cells [3, 6, 7]. Therefore, PGC fate induction is designed in a floating culture system where competent cells interact with each other in a spatially organized pattern. When hPSCs are grown in a 4-inhibitor (4i) media, they are competent for specification of hPGCLCs by adding growth factors such as BMP2 or BMP4. These in vitro induced hPGCLCs express key PGC genes, demonstrating the reliability and robustness of the 4i method. RNA sequencing (RNA-seq) reveals that hPGCLCs generated using the 4i method resemble the sorted in vivo hPGCs from week 7 gonads and the human seminoma-derived cell line TCam-2(Fig. 2A) [22]. Based on this system, SOX17 is discovered as the critical hPGC fate specifier [22]. This is in contrast to the findings in mice, where SOX17 is not involved in PGC specification. Furthermore, the functions of SOX17, PRDM1, and DMRT1 in the specification and development of hPGCLCs, as well as the migratory potentials of the hPGCLCs, have been elucidated based on this 4i method [22, 55, 56].

Another widely used method of inducing hPGCLCs in 3D aggregates starts by converting hPSCs into the incipient mesoderm-like cells (iMeLCs) using defined media containing ACTIVIN A (ACTA) and the WNT signaling agonist CHIR99021(Fig. 2A). To induce hPGCLCs, these iMeLCs are transferred to the hPGCLC media supplemented with BMP4 and other growth factors for 3D aggregation. It is notable that stem cell factor (SCF) has been found to be non-essential for the induction of hPGCLCs via the iMeLC method [34]. The hPGCLCs induced through the iMeLC method express key germ cell markers, with a gene expression profile closely resembling that of both in vivo gonadal PGCs and in vitro hPGCLCs obtained by the 4i method, thus serving as a convenient and reliable model to study hPGCLC specification [23]. Based on the iMeLC method, the roles of TFAP2C, SOX17, PRDM1, EOMES, and T have been thoroughly investigated. EOMES, activated by WNT signaling, activates the expression of SOX17 to drive the specification of hPGCLCs. In contrast, the key downstream transcription factor of WNT signaling in mice is T, and T activates the expression of Blimp1 and Prdm14 for the specification of mPGCs. In addition, the extended culture of hPGCLCs obtained from iMeLC method has been achieved from which the epigenetic reprogramming of germ cell fate has been elucidated, as well as the first attempts at genetic screens for identifying more hPGCLC regulators have been conducted [23, 31, 33–35, 54, 57–59].

hPGCLCs can also be induced directly from formative PSCs (fPSCs), a state of "naïve-primed intermediate" that possesses a unique capability to maintain germline competency (Fig. 2A) [47, 48, 60]. Given that fPSCs can be cultured stably for long periods in vitro, this one-step induction method is simpler compared with the two-step hPGCLC induction method using primed hPSCs. Furthermore, the differentiation from fPSCs to hPGCLCs reflects early embryonic development for hPGC fate induction. Therefore, fPSCs have emerged as a promising source for inducing hPGCLCs to decipher the functions of key factors that regulate the establishment and development of human germ cell lineage.

Besides primed and formative states, hPGCLCs can be induced from naïve hPSCs after they undergo a process called capacitation, which transitions them between different pluripotency states (Fig. 2A) [52]. The capacitation process converts hESCs from naïve to primed pluripotent states, while resetting reverses this process, creating a transient state that enhances hPGCLC induction. These resetting hESC-derived hPGCLCs (rhPGCLCs) possess distinct transcriptomic and epigenetic signatures, conferring them greater competence for germ cell development compared to other methods [52].

Overall, the design of the 3D floating aggregate methods is based on the previously established mPGCLC method. First, primed pluripotent hPSCs need to be induced to an intermediate state (with germline competency), and then the hPGCLC fate is acquired by cytokines (such as BMP4) in the aggregate state. The main difference between the above four methods lies in the way hPSCs acquire germline competency. Among them, the 4i method and the fPSC method generate intermediate cells that can be stably cultured in a long-term manner while maintaining the germline competency [22, 47, 48, 60]. The transcriptomes of 4i hESCs and fPSCs are similar, representing the intermediate pluripotent state of human formative epiblasts. In contrast, the cell state of germline competency obtained by the iMeLC method and the rhPGCLC method is a transient window and cannot be maintained stably. The resetting state is a

similar transitional pluripotency state that occurs during the transition from naive to primed pluripotency, and iMeLCs represent incipient mesoderm/primitive streak-like cells [23, 52]. Using hPSCs from different pluripotent states for induing hPGCLCs not only expands our tools for specifying germ cell fate in vitro, but also provides different contexts to explore the molecular nature of the competency for germ cell fate.

Induction of hPGCLCs in two-dimensional (2D) adhesion platforms

The above 3D aggregation systems for hPGCLC induction have revolutionized the field, leading to the fundamental discoveries for the specification and regulation of human germ cell fate. However, they suffer from low efficiency and scalability. To address these limitations, a novel method for inducing hPGCLC differentiation in scalable 2D cell culture has been developed using low BMP4 concentration and attenuated basement membrane extract (BMEx) (Fig. 2B) [51]. Notably, BMEx overlay increases the BMP response, thereby allowing the generation of hPGCLCs at a high rate (30–50% efficiency) within 5 days, despite requiring much lower BMP4 concentrations than standard protocols. This method is highly scalable and cost-effective, opening up the possibilities for high-throughput experiments such as clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-based genome-wide screens [61–66]. In addition, single-cell RNA-seq (scRNA-seq) analysis identifies the TFAP2A + hPGCLC progenitors, which is consistent with the previous findings based on the iMeLC method [31, 51]. 2D cultured hPGCLCs also possess the capacity to further differentiate into DDX4+/DAZL + germ cells when co-cultured with cells isolated from human fetal ovaries [51]. Mechanistically, BMEx treatment creates a luminal structure on the 2D culture plane, which may increase the contact between BMP receptors on cell surface and BMP4 signals in the media, resulting in a lower BMP4 concentration requirement and higher hPGCLC induction efficiency.

Another 2D monolayer method generates hPGCLCs from hPSCs within 3.5 days by initially activating and then inhibiting the WNT signaling pathway (Fig. 2B) [50]. WNT inhibition significantly improves the efficiency of hPGCLC specification, increases the expression of key germ cell markers, and represses mesodermal genes. hPGCLCs are purified based on specific surface markers, CXCR4+/PDGFRA-/GARP-, a combination of both positive and negative selection, and exhibit transcriptomic similarity to in vivo hPGCs. The advantage of the monolayer differentiation system is that it creates an environment for cells to more efficiently receive signals (such as BMP4) compared with 3D aggregates. Another key optimization is the precise control of the time windows of signaling molecule stimulation, including intermittent stimulation of BMP4, removal of LIF, and subsequent inhibition of WNT signaling. This simplified monolayer culture system allows for efficient and reproducible generation of hPGCLCs across various hESC and hiPSC lines.

A 2D induction system could also be achieved using micropatterned hPSC models, a quantitatively reproducible system that permits precise control over the size and shape of cell colonies, leading to high-efficiency hPGCLC induction (over 50%) with just BMP4 treatment (Fig. 2B) [49]. When hPSCs are pre-treated with ACTA, the efficiency increases to about 70%. Micropatterning ensures that hPSCs differentiate into self-organized cell fate patterns within small colonies, thereby simulating human in vitro gastrulation. The smaller colony structure allows most cells to be close enough to the edge to receive BMP4 signals, thereby improving the differentiation efficiency of hPGCLCs. Therefore, the micropatterned system facilitates stable and reproducible hPGCLC differentiation, providing a simple but powerful tool for precise single-cell studies and large-scale experiments [49]. Altogether, these 2D strategies offer efficient, scalable and cost-effective systems for generating hPGCLCs, supporting high-through experiments, such as genome-wide screening and genomics strategies for interrogating gene functions and regulatory networks.

Compared with 2D monolayer culture platforms, 3D aggregate culture systems create cell-cell interactions in a spatial manner for the specification of hPGC fate. The 3D aggregate structure potentially affects the degree of diffusion of extracellular signals deep within the aggregates, and the induction efficiency is relatively variable among different batches and different hPSC lines, which poses a challenge to systematically and quantitatively determine how and where hPGCLCs are specified in differentiation models. In addition, the low throughput of 3D differentiation also results in limitations for high-throughput downstream experimental applications. 2D differentiation sacrifices cell-cell interactions in spatial organization and may lose some key regulation in in vivo 3D environment, but achieves advantages in terms of controllability, scalability, and cost of hPGCLC differentiation. Nevertheless, more strategies should be designed to further investigate the key questions regarding the specification and development of hPGCLCs.

Induction of hPGCLCs by hPSC-derived 3D embryo-like models

Although the 3D aggregation and 2D adhesion strategies have substantially advanced our understanding of human germ cell development, they create disordered structures compared to the highly organized developing embryos. These methods lack the elegant temporal-spatial

orchestration of different cell types that naturally communicate and interact to build the precisely organized embryos. To address this limitation, new strategies incorporating microfluidics systems have been employed to generate 3D embryo-like structures, in which hPGCLCs are observed (Fig. 2C) [46]. In this system, hPSCs recapitulate several key events of the development of the epiblasts and amnion in the post-implantation human embryo in a highly controllable and reproducible manner [46]. The system comprises channels for injecting hPSCs, for loading Geltrex to form gel pockets required for the growth of embryonic-like sacs, and for delivering the morphogens needed for differentiation. By adding BMP4 to the culture media, posteriorized embryonic-like sacs (P-ELS) containing early-stage hPGCLCs are generated [46]. Importantly, scRNA-seq analysis confirms that these hPGCLCs express PGC markers, indicating their reliability [46]. These hPGCLCs derived from orderly differentiated 3D embryonic-like structures may closely resemble the in vivo hPGCs compared to those derived from the disordered 3D cell cluster methods. Therefore, the hPSC-based microfluidic model offers significant advantages for disease modeling, cell therapy, and high-throughput drug screening and toxicity assessment.

Another innovative approach has established a 3D biomimetic hPSC culture system, which uses a combination of a 3D extracellular matrix (ECM) overlay and an ECM gel bed to mimic the environment of peri-implantation human epiblasts (Fig. 2C) [53]. This Gel-3D culture system promotes the differentiation of hPSCs into amniotic ectoderm-like cells (AMLCs), which in turn induce hPGCLC specification through paracrine signaling. This biomimetic platform has been successfully applied to eight hPSC lines derived from patients diagnosed as non-obstructive azoospermia, demonstrating high efficiency and compatibility for hPGCLC induction, opening new avenues for high-throughput screening applications, especially for disease-related drug or small molecule screening.

The above two methods simulate the peri-implantation stage of human epiblasts, at which the formation of hPGCLCs is observed in the corresponding embryo-like models. Given that the in vivo primate PGCs are observed in both amniotic and gastrulating regions, hPSC-derived 3D embryo-like models provide a more in vivo-like developmental environment, which helps to analyze the molecular and genetic mechanisms for hPGC specification.

The induction of hPGCLCs and the applications for the reconstitution of gametogenesis *in vitro*

In general, the efficiency of nascent hPGCLCs induced by 4i or iMeLC methods is only around 20% [22, 23], indicating significant room for improvement in hPGCLC induction systems. An interesting question arises: why do only partial cells successfully differentiate into hPGCLCs under the same culture conditions? Does this suggest an inhibitory mechanism for hPGCLC induction? The emergence of 2D methods has reduced the difficulty of hPGCLC induction technology, accompanied with the improvement of the induction efficiency and the number of hPGCLCs [50, 51]. This has lowered the threshold for studying hPGCLCs and expanded the range of available technologies for hPGCLC-related research, which is crucial for elucidating the molecular mechanisms governing hPGCLC development. The microfluidics method provides a revolutionary new experimental approach and perspective for hPGCLC induction [46]. Particularly important is that this microfluidics system offers choices of different growth factors on apical or basal sides of hPSCs, resembling the in vivo situation compared to 3D and 2D systems in which cells are directly exposed to the growth factors in the culture media. Therefore, microfluidics-based embryo-like structures serve as critical models for investigating how hPGCLC induction is regulated by the external signals and the spatial organization of cells.

Another key feature of the above-mentioned methods is that hPGCLCs could be induced from primed, formative, and naive states of hPSCs. Primed hPSCs could be cultured in 4i media or induced into iMeLCs, both of which are competent for specification of hPGC fate [22, 23]. Naïve hPSCs could be capacitated to obtain hPGCLC competency [52]. In contrast, formative hPSCs can be directly differentiated into hPGCLCs, potentially elucidating the molecular basis of germline competency [47, 48, 60]. According to transcriptomic analysis, 4i cells, iMeLCs, and capacitating cells are probably at the formative state in the spectrum of continuum of pluripotency [22, 23, 31, 33, 35, 52], pointing to the hypothesis that hPGC fate is specified from formative state cells corresponding to the embryonic cells at peri-implantation stage around the time of in vivo hPGC specification. Additionally, the ability to differentiate formative state cells into hPGCLCs may also depend on several other factors, such as cell-to-cell contacts, stiffness of the extracellular matrix, and a unique epigenetic landscape. These factors may guide future improvements in the hPGCLC induction system, laying a foundation for in vitro reconstitution of the whole process of gametogenesis.

hPGCLCs induced from PSCs do not express DDX4 or DAZL, markers of late hPGCs, indicating their early developmental properties at the nascent stage before migration [22, 23]. Following hPGCLC establishment, efforts have been made to differentiate them to later stages to recapitulate gametogenesis in vitro, which holds significant potential for addressing infertility issues. One approach involves co-culturing hPGCLCs with hindgut organoids derived from hESCs [52]. This method,

which partially mimics the migration of hPGCs, successfully induces the expression of DAZL and DDX4 after 25 days. It is important to note that the hPGCLCs must be derived by capacitating the resetting precursors, specifically hESCs during the transition from naive to primed pluripotent state. This co-culture approach provides a novel method for recapitulating and investigating hPGC migration, a stage following hPGC specification (Fig. 1).

Another method used co-cultured hPGCLCs with ovarian somatic cells from mouse neonatal embryos to generate xenogeneic recombinant ovaries (xrOvaries) [67]. Within 21–77 days of culture, hPGCLCs in this system successfully differentiate into later stages, with significant expression of germ cell markers including DDX4, DAZL, and meiosis-related genes. By day 120, these cells show advanced differentiation, resembling human oogonia [67]. Similarly, hPGCLCs co-cultured with gonadal somatic cells from mouse embryonic testis can be further induced into pro-spermatogonia-like cells [68]. These studies represent the significant advancement towards human in vitro gametogenesis.

A recent strategy involves modulating BMP signaling to induce the differentiation of hPGCLCs into mitotic pro-spermatogonia or oogonia-like cells [54]. BMP2-driven differentiation involves attenuation of the MAPK (ERK) pathway, and both *de novo* and maintenance DNA methyltransferase activities, achieving extensive amplification of in vitro germ cells. Transcriptomic analysis indicates that BMP2-mediated signaling enhances the expression of germ cell markers such as DAZL and DDX4, reflecting advanced development stages [54]. This study offers a novel method for recapitulating human gamete development in vitro. Taken together, investigations based on all these hPGCLC induction systems provide critical insights into the regulatory mechanisms underlying hPGC specification and development.

The regulation of hPGCLC specification by collaborative signaling pathways

Cell lineage allocation during early embryogenesis is governed by key signaling pathways and developmentally regulated transcription factors [69]. Germ cell specification in mammals is initiated in pluripotent epiblasts through specific signals from surrounding somatic tissues [70, 71]. While the transcription factors regulating PGC specification may vary among species, the major signaling pathways, including the BMP family, WNT and TGF-β signaling, are evolutionarily conserved [7].

The significance of the BMP family signaling pathways to PGCs is primarily elucidated through intensive studies using mouse mutant embryos. Initially, it is demonstrated that Bmp4 produced in the extra-embryonic ectoderm is crucial for inducing mPGC precursors, while Bmp4 produced in the extra-embryonic mesoderm is vital for mPGC localization and survival [70–74]. Meanwhile, studies on Bmp8b-knock-out and Bmp2-knock-out embryos further confirm the importance of BMP signaling, as both exhibit significantly reduced mPGC numbers [21, 75, 76]. Notably, in vitro experiments on mPGCLC induction identify Bmp4 as the critical factor, as it alone is sufficient to induce mPGCLCs from the epiblasts [21]. In the hPGCLC-induction system, the role of the BMP family largely mirrors the findings in mPGCLCs, with both BMP4 and BMP2 described as sufficient to induce hPGCLC fate. No hPGCLCs are observed without the supplement of BMP4, suggesting the necessary role of BMP4 for hPGCLC induction [22, 23], while the function of BMP8b has not been examined in this context. In the study on the induction of hPGCLCs by micropatterned hPSCs, blocking BMP4 signaling leads to the loss of PRDM1 and reduced TFAP2C expression, which eventually leads to the decrease in hPGCLCs [49]. These studies highlight BMP signaling as the essential specifying factors for PGC fate.

Following the BMPs, WNT and TGFβ signaling pathways have been implicated in germ cell specification. In mice, Wnt3 homozygous mutant embryos fail to respond to Bmp4, suggesting that Wnt3 assists epiblasts in responding to Bmp4 and determining the fate of PGCs [21, 77]. Additionally, the capacity for hPGCLC induction is linked to primitive streak-related genes, primarily through NODAL/ACTA (TGFβ) and WNT signaling pathways [33, 34, 58]. Inhibition of TGFβ and WNT signaling, without affecting BMP4 signaling, completely prevents the induction of hPGCLCs [34]. These observations suggest that while BMP4 signaling primarily stimulates the hPGCLC induction program, pluripotent cells must also retain the ability to respond to TGFβ and WNT signaling for successfully capacitating competency for germ cell fate. In the micropatterned hPSC-induced hPGCLC system, blocking WNT signaling 12 h after BMP4 addition results in hPGCLC loss, while blocking WNT after 24 h has minimal effect, indicating that unlike BMP4, continuous WNT signaling may not be required [49]. Furthermore, replacing WNT with ACTA and stimulating downstream NODAL signaling of downstream of WNT partially rescues the hPGCLC decrease phenotype caused by WNT inhibition, suggesting that the role of WNT signaling in hPGCLC formation is achieved indirectly by activating NODAL in stem cells [49].

Comparative analyses across mammals highlight the conserved functions of WNT and BMP signaling pathways in PGC specification. In porcine embryos, WNT3A and BMP2/4 pathways are detected in the posterior epiblasts [78, 79], while in cynomolgus monkey embryos, WNT3A/BMP4 expression and SOX17+/TFAP2C + putative cyPGCs are observed in the amnion [27]. For human embryos, although in vivo investigation

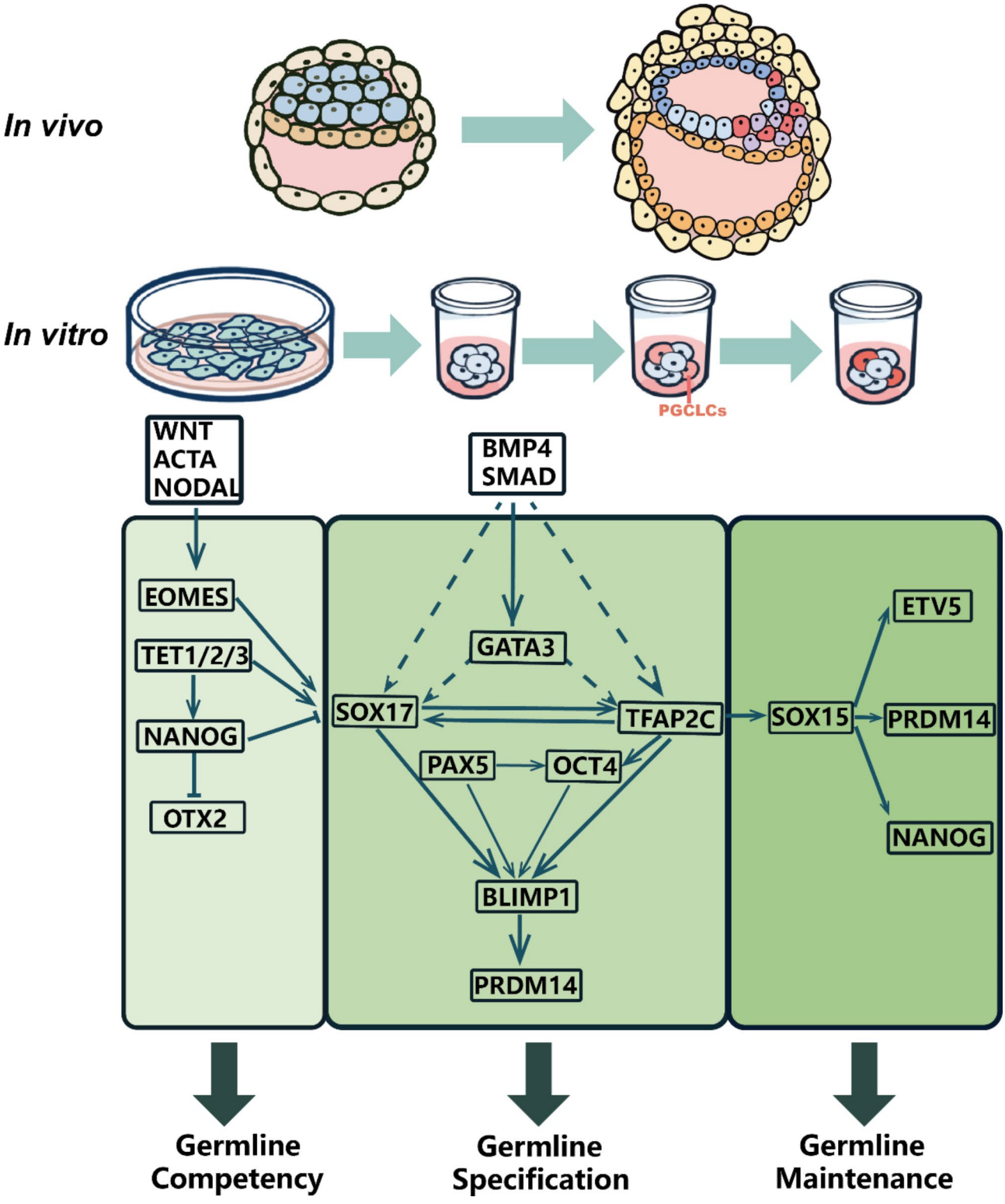

Fig. 3 (See legend on next page.)

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Fig. 3 Regulation network for hPGC specification and differentiation

ACTA/WNT/NODAL activates the expression of EOMES, which is critical for germline competency. TET1/2/3 maintains the expression of NANOG which inhibits the expression of OTX2 to ensure competency for hPGCLC induction. BMP4 and ACTA/WNT/NODAL signaling pathways promote hPGCLC specification by regulating TFAP2C and SOX17, the key regulators of hPGCLC specification. SOX17 is activated by EOMES and TET1/2/3 and inhibited by NANOG. On the other hand, TFAP2C and SOX17 function downstream of GATA3 and upstream of PRDM1. OCT4 functions downstream of PAX5 and TFAP2C, while PRDM1 functions downstream of PAX5 and TFAP2C, and upstream of PRDM14 to composite the regulator network for hPGCLC specification. Finally, TFAP2C activates SOX15 to upregulate ETV5, PRDM14, and NANOG to maintain the hPGCLC fate. Arrows and blunt arrows indicate positive and negative regulation, respectively. Dashed lines indicate possible regulation

of signals is not feasible, BMP4, WNT and TGF-β signals have been shown to be necessary in hPGCLC systems [22, 23]. Together, these signaling pathways capacitate precursor cells competent for germ cell lineage and safeguard the precisely stepwise differentiation of the germline for generating gametes for reproduction.

In summary, during early embryonic development, germ cell fate is regulated by key signaling pathways, including BMP, WNT, and TGF-β. BMP signaling plays a central role in PGCLC induction, with Bmp4 essential for the formation and survival of PGC precursors, while other BMP family members, such as Bmp2 and Bmp8b, also support PGC precursor numbers and viability. WNT signaling supports the response of epiblasts to BMP4 signals, promoting germ cell fate by enabling NODAL activation. The TGF-β pathway, primarily via the NODAL/ACTA axis, maintains cell responsiveness to differentiation signals, ensuring germ cell formation from pluripotent stem cells. These pathways are essential for germ cell lineage specification, particularly in the in vitro hPGCLC induction system, where inhibition of BMP4, WNT, or TGF-β signaling leads to a reduction in PGC(LC) numbers. Elucidation of molecular mechanisms of these signaling pathways, especially how they function with transcription factors to specify germ cell fate, is key to reconstituting functional germ cells in vitro for potential clinical applications and providing mechanistic insights into the pathogenesis of infertility-related diseases.

The orchestration of transcription factors governing the specification and development of hPGCLCs

Exogenous pathways, such as BMP, WNT, and TGFβ signaling, specify PGC fate by activating downstream effectors, primarily transcription factors. Although the requirements of these signaling for PGC specification are conserved between mice and humans, the gene regulatory networks that determine hPGC fate have diverged. Here, we focus on the transcription factors critical for human germ cell fate. The combination of different transcription factors functions at different stages of germline competency, specification, and maintenance (Fig. 3).

RNA-seq analyses of different hESC and hiPSC lines identify EOMES as a critical factor for hPGCLC competency [34, 58]. EOMES is expressed in iMeLCs, formative PSCs, and 4i cells, the competent cells for hPGCLC induction. Knock-out of EOMES in hESCs results in impaired induction of hPGCLCs. Regulated by WNT and TGF-β signaling, EOMES functions upstream of SOX17 for the specification of hPGCLCs [33, 34, 58]. Therefore, EOMES represents a positive regulator for hPGCLC competency. In contrast, OTX2 functions as a negative regulator that restricts the entry of germ cell lineage. In mice, Otx2-knock-out leads to an increased number of mPGCs and mPGCLCs [80]. In humans, OTX2 performs a similar repressive role, operating upstream of PGC transcription factors. WNT signaling and upregulated NANOG expression in response to NODAL signaling lead to transient OTX2 reduction in a subset of precursor cells, presumably conferring the competency for germline entry [80–82](Fig. 3). Identification of more positive and negative regulators for germline competency is essential for deciphering the regulatory network for PGC fate, constituting the basis for in vitro gametogenesis with clinical applications.

More transcription factors have been identified for the specification of germline (Fig. 3). Mouse PGC specification is characterized by the re-acquisition of Sox2 expression [83]. Surprisingly, SOX17, but not SOX2, is critical for hPGCLC specification [22]. High expression of SOX17 and almost no expression of SOX2 is detected in hPGCLCs. When SOX17 is knocked-out in hESCs, no hPGCLCs are detected, indicating that SOX17 is indispensable for hPGCLC induction. Further studies confirm the expression and functions of SOX17 in hPGCLCs, establishing SOX17 as one of the most critical regulators for hPGC fate specification [23, 31, 33]. TFAP2C represents another key regulator for hPGCLC fate specification. TFAP2C-knock-out results in complete loss of hPGCLCs, implying the essential functions of TFAP2C in hPGCLCs, which are not conserved in mice [31, 33, 35, 84]. In hESCs, TFAP2C is involved in the transition from primed to naïve states, consistently, TFAP2C ensures the naïve-like pluripotency in hPGCLCs. Moreover, further investigation reveals that TFAP2C functions upstream of SOX17 during the specification of hPGCLCs, highlighting the multifunctional and critical roles of TFAP2C in human germ cell fate [31, 35, 85]. PRDM1, as one of the most critical cell fate determinants for mPGCs [20], also plays distinct roles in hPGCLCs. PRDM1-knock-out leads to impaired hPGCLC development. At the transcriptomic level accessed by RNA-seq,

PRDM1-knock-out day 4 hPGCLCs resemble day 2 control hPGCLCs, suggesting that PRDM1 is dispensable for the specification but critical for the progressive development of hPGCLCs [22, 23, 33]. These studies emphasize the critical transcription factors SOX17, TFAP2C, and PRDM1 in the regulation of human germline specification and development (Fig. 3).

PAX5 plays an essential role in the intricate transcriptional network involving OCT4 and PRDM1 for the specification of hPGCLCs. In the generation of hPGCLCs from hESCs, PAX5, along with OCT4 and PRDM1, co-occupies genomic loci, thereby influencing the shift from pluripotency to germ cell lineage specification [86]. More specifically, PAX5 acts upstream of OCT4 and PRDM1, activating their promoters and ensuring the suppression of non-germline genes. Loss of PAX5 reduces the ability of hESCs to become hPGCLCs, while its overexpression enhances hPGCLC induction, highlighting its importance in the regulatory network [86]. Additionally, OCT4 represses ectodermal genes and, with PAX5, activates PRDM1 to suppress mesodermal and endodermal genes, effectively inducing and maintaining the germline program. Therefore, the PAX5-OCT4-PRDM1 network functions with TFAP2C and SOX17 to form the core regulatory hub for the specification of hPGCLCs from hPSCs, an axis that is divergent from that of mouse (Fig. 3).

GATA family members represent an additional layer of regulation linking BMP4 signaling to hPGCLC specification. GATA2 and GATA3 are immediate effectors of BMP4, critical for hPGCLC specification. Their expression is significantly upregulated in hPGCLCs after BMP4 stimulation [33, 87], and GATA2/GATA3 knock-out mutants exhibit dose-dependent impairments of hPGCLC specification. Both GATA2 and GATA3 can induce hPGCLC differentiation when combined with SOX17 and TFAP2C, even in the absence of BMP4 signaling, suggesting that the GATA-SOX17-TFAP2C system can drive hPGCLC specification independently. Compared with GATA2, GATA3 is a factor with a more prominent role in the differentiation process by interacting and functioning with SMAD, stimulating the expression of endogenous BMP4 [87]. These studies reveal GATA2 and GATA3 as important regulators for hPGCLC specification, which has not been deeply investigated in mice (Fig. 3).

PRDM14 is a core transcription factor in mPGC specification and plays a role in human PGCs as well. Over-expression of PRDM14, PRDM1, and TFAP2C is sufficient to induce PGC fate from mouse epiblast-like cells in the absence of growth factors including BMP4. More critically, even over-expression of PRDM14 alone is sufficient to induce PGC specification in mice, highlighting the central role of PRDM14 in the cell fate determination of mPGCs [38, 39, 88]. In humans, PRDM14 is expressed at low levels in hPGCLCs and gonadal hPGCs, and localized at cytoplasm in gonadal hPGCs in contrast to nucleus in hPGCLCs [22, 44, 45]. Acute depletion of PRDM14 using an inducible degradation system during hPGCLC induction results in significantly reduced specification efficiency and an aberrant transcriptome of hPGCLCs, suggesting its importance in both hPGCLC specification and maintenance [24] (Fig. 3).

Another member of the SOX family, SOX15, is not required for the initial specification of hPGCLCs but is crucial for their maintenance afterward. SOX15 exhibits specific expression patterns in hPGCs and hPGCLCs [22, 89]. In SOX15-knock-out cells, the percentage of hPGCLCs decreases over time, indicating that SOX15 may act primarily in the maintenance of hPGCLCs [89, 90]. SOX15 binds to the proximal regulatory regions of pluripotency-related genes, including ETV5, NANOG, and PRDM14. Moreover, SOX15-knock-out leads to downregulation of ETV5 in hPGCLCs, and ETV5-knock-out results in a reduced proportion of hPGCLCs, establishing the functional axis of SOX15-ETV5 for hPGCLC maintenance. Therefore, SOX15 functions through ETV5 to maintain the progressive development of hPGCLCs after specification (Fig. 3).

Altogether, the regulatory network for hPGCLCs is a complex interplay of signaling pathways and transcription factors (Fig. 3). The BMP4/SMAD signaling pathway initiates germ cell fate by inducing the expression of the key BMP effector GATA3, which in turn activates SOX17 and TFAP2C. Additionally, BMP4/SMAD can directly promote the expression of SOX17 and TFAP2C. Additional critical pathways involved in hPGC specification are the ACTA/NODAL and WNT signaling pathways. They activate the expression of EOMES, which in turn leads to the activation of SOX17 (Fig. 3). In addition, TET family proteins (TET1/TET2/TET3) enhance the expression of both SOX17 and NANOG [91, 92]. NANOG, in turn, inhibits the expression of OTX2 for supporting germline competency and SOX17 for preventing the entry of endodermal lineage (Fig. 3). The interaction between SOX17 and TFAP2C is critical for germ cell fate determination. TFAP2C promotes the activation of OCT4 and PRDM1. In addition, the PAX5-OCT4-PRDM1 network plays a crucial role in repressing genes from the three germ layers and promoting germ cell fate determination. Meanwhile, SOX17 promotes the expression of PRDM1, which further stimulates PRDM14 expression. SOX15, regulated by TFAP2C, supports germline maintenance by regulating downstream factors such as ETV5, PRDM14, and NANOG [90]. Therefore, these signaling pathways and transcription factors function with each other to precisely specify the hPGC fate

from competent precursors and promote the progressive differentiation of germ cells in a tempo-spatial manner to coordinate the germ-soma segregation and interactions.

Epigenetic reprogramming in the development of germ cell lineage

In addition to extracellular signals and transcription factors, epigenetic regulation also plays key roles in the development of germ cell lineage [7, 93]. During the development of human germ cell lineage, DNA methylation undergoes a significant reprogramming process. This dynamic reprogramming begins as PGCs are specified and migrate towards the genital ridge, during which they experience a drastic reduction in DNA methylation levels, dropping from approximately 70% in somatic cells to around 4.5% by week 8. The demethylation is primarily achieved through passive mechanisms driven by the repression of DNA methyltransferases, notably DNMT3A, DNMT3B, and UHRF1 [94–96]. In hPGCs, this demethylation process is accompanied by the activity of TET enzymes (TET1 and TET2), which catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [94–97]. The role of DNA demethylation mediated by TET proteins (TET1, TET2, TET3) in hPGCLC specification has recently been revealed. The TET-mediated balance between NANOG and SOX17 is critical for hPGC induction [91]. A recent study also reports the importance of TET1 catalytic activity for hPGCLC induction [92]. Despite this widespread demethylation, certain regions termed "escapees" retain methylation marks, including evolutionarily young retrotransposons like SVA elements and specific regulatory sequences, potentially through DNMT1-mediated mechanisms [94]. These complex and tightly regulated methylation dynamics are critical for the successful differentiation of PGCs and the maintenance of genomic integrity.

Histone modifications are another layer of epigenetic regulation that significantly influences the development of human germ cell lineage. During the PGC migration towards the genital ridges, key histone modifications undergo dynamic reorganization, which is essential for maintaining genome stability. A distinctive feature is the progressive depletion of H3K9me2, which is a repressive histone modification [91, 94]. In contrast, H3K27me3, another repressive histone modification, exhibits a unique temporal pattern in hPGCs, showing transient enrichment during migration followed by depletion upon arrival at the genital ridges [42, 94]. Unlike mPGCs, hPGCs maintain constant levels of H2A/H4R3me2s throughout development [7, 98]. These coordinated changes in histone modifications, in conjunction with DNA methylation, are essential for the proper erasure of somatic cell memory and the establishment of a germ cell-specific epigenetic landscape.

To be noted, the roles of transposon-related epigenetic regulation should not be neglected and require further study. TRIM28 is necessary for the differentiation of hESCs into hPGCLCs. It silences HERVH and SVA in primed hESCs, and regulates DNA methylation and chromatin status of LTR, HERVK, and LINE, thereby ensuring the germline competence of hESCs [99]. LTR5Hs, specific transposable elements in humans, act as enhancers during germline development. They increase chromatin accessibility, promote DNA demethylation, enrich H3K27ac modifications, and recruit key hPGC transcription factors, thus promoting hPGC specification [100]. It is reported that TET1 binds to LTR5Hs in hESCs and hPGCLC-specific transcription factor binding sites to promote the induction of hPGCLCs [92]. Above all, the functional discoveries of TRIM28, LTR5Hs, and TET proteins highlight the importance of TE-related epigenetic regulation in hPGC specification, underscoring the complexity and precision of human germ cell development.

The dual origin hypothesis of hPGC(LC)s

The above-mentioned in vitro hPSC-based models have been established for inducing hPGCLCs for intensive functional studies, representing valuable platforms for investigating the signaling pathways and transcription factors that govern cell fate transition and maintenance during hPGCLC development. However, they have provided limited insights into one of the most significant unanswered questions regarding hPGCs: What is their origin(s) in the human embryo?

In mice, PGCs originate from the proximal posterior epiblasts at around E6 [3, 8, 20]. Due to the inaccessibility of human embryos at around weeks 2–4, the understanding of the origin of human PGCs has long been inferred from studies in mice. The establishment of the hPGCLC model has revealed significant molecular differences in PGC development between humans and mice, raising the key question whether hPGCs share a similar induction origin compared to those of mice [7](Fig. 3). Importantly, the structures of mouse and human embryos at the PGC specification stage are entirely different, with a cup shape for mice and a bilaminar disc for humans [7, 101]. Therefore, non-human primate and pig embryos, which have structures more similar to those of humans [27, 78], have become more reliable models for exploring the origin of human PGCs.

A groundbreaking discovery about the origin of primate PGCs was made in cynomolgus monkeys, demonstrating that cyPGCs first emerge in the amnion around E11 [27]. SOX17+/TFAP2C+/PRDM1 + cells are identified as cyPGCs in the dorsal amnion as early as E11,

prior to the onset of gastrulation, challenging previous speculations about the origin of hPGCs [27]. Studies on porcine embryos reveal that porcine PGCs (pPGCs) are specified in the posterior end of the nascent primitive streak at E11.5-E12, the earliest time window when SOX17+/PRDM1 + pPGCs are clearly observed and originate from gastrulating cells (pre-mesendodermal cells) [79]. Importantly, this PGC specification pathway is confirmed in human and monkey in vitro systems. Thus, in humans, monkeys and porcine, PGC fate may derive from gastrulating cells. However, since the appearance of the amniotic membrane in porcine embryos occurs after the primitive streak stage, which is later than in humans and monkeys (before the primitive streak stage), the possibility remains that primate PGCs may originate from the amniotic membrane. These observations point to the hypothesis that hPGCs may originate from both amnion and gastrulating cells.

The possible dual origin of hPGCs has been further investigated using various in vitro models of hPGCLCs. scRNA-seq analyses of cells from hESCs to day 4 aggregates establish the human germline trajectory from hESCs to day 4 hPGCLCs. Lineage tracing within the germline trajectory identifies TFAP2A + cells in day 1 aggregates as immediate precursors for hPGCLCs, termed as day 1 progenitors. These progenitors exhibit both amnion-like and gastrulation-like transcriptome signatures, supporting the dual-origin hypothesis of hPGCLCs [31]. The microfluidic post-implantation amniotic blastocystoid (μPASE) system-based embryo-like 3D structures offer another potential model to trace the origin of hPGCLCs [46, 102]. Upon unilateral BMP4 stimulation, hESC clusters generate P-ELS with AMLCs, epiblast-like cells, and hPGCLCs. TFAP2C+/NANOG+/SOX17 + hPGCLCs are detected in a dispersed manner throughout the P-ELS 24 h after induction, including (i) the central amniotic ectoderm-like compartment, (ii) the junction of the epiblast-like and amniotic ectoderm-like compartment, and (iii) the epiblast-like compartment. Lineage tracing of the developmental trajectories in μPASE reveals that hPGCLC progenitors may have derived from the nascent AMLCs before fully committing to the hPGCLC fate [102]. However, due to the limitations of μPASE and the early developmental plasticity of cells in human embryos, other sources of hPGCs cannot be excluded.

Current methods to explore the origin of hPGCs heavily rely on the in vitro hPGCLC induction system based on hPSCs and studies of embryos from other species, particularly non-human primates. The earliest single-cell transcriptome data from in vivo human embryo are at the developmental stage of CS7 (E16-E19), when only a few PGCs are detected in primitive streak clusters [32]. Clearly, none of these findings directly answer the question of where hPGCs are specified. Overall, current research favors the model that both amnion and gastrulating epiblasts serve as the precursors for hPGC fate specification. Sophisticated simulation systems are needed to advance our understanding until the ethical 14-day limit on in vitro culture is resolved [103–105].

Summary and Outlook

The hPGCLC models derived from hPSCs are crucial tools for studying hPGCs and represent a significant starting point for establishing in vitro gametogenesis to address clinical issues such as infertility, low fertility, germ cell tumors, and birth defects [7, 11, 17, 106, 107]. Currently, direct understanding of hPGCs relies primarily on a limited number of human embryo resources and various in vitro hPGCLC models, with indirect insights extending from studies in mammals such as mice and non-human primate PGCs.

Several in vitro methods for hPGCLC induction have been developed, including 3D aggregation-based differentiation systems (disordered systems based on low-attachment environments and ordered systems utilizing in vitro embryo-like structures), as well as 2D adhesion-based differentiation systems (which involve micropattern cultures and additional modifications using BMEx) [22, 23, 34, 46, 49–53, 60]. It is noteworthy that the initial pluripotent state of hPSCs is crucial for hPGCLC induction, reflecting direct or indirect germline competency and influencing subsequent developmental potential of hPGCLCs (Fig. 2). However, compared to mice, the progressive differentiation of hPGCLCs remains inadequate, necessitating more robust systems for continuous differentiation to better functionally evaluate existing hPGCLC models. Nonetheless, these studies from hPGCLC models illustrate a germline regulatory network significantly distinct from that of mice. Extracellular signaling pathways, transcription factors, and epigenetic factors crucial for hPGCLC specification and cell fate maintenance are still being identified, and the functional regulatory relationships among them are still undergoing investigation (Fig. 3).

The hPSC-based hPGCLC models also provide valuable insights into the origin of hPGCs, a key unresolved question in human early embryogenesis. Detailed studies have been conducted in cynomolgus monkey and porcine embryos, along with various hPGCLC models, supporting the potential origins of hPGCs from amnion and gastrulating epiblasts [26, 27, 31, 79]. However, this dual-origin hypothesis remains inconclusive. To definitively determine the origin(s) of hPGCs, further studies, particularly those involving in vivo lineage tracing or similar techniques, are necessary.

The development of hPGCs is a complex process regulated by multiple mechanisms. The induction of

hPGCLCs from hPSCs is currently the most widely used model for studying human PGCs, facilitating the identification of key growth factors, transcription factors, and epigenetic factors involved in human germline development, as described above (Fig. 2). Key transcription factors for hPGCLC induction, such as SOX17, PRDM1, TFAP2C, EOMES, PRDM14, have been identified through in vitro induction experiments using mutant hPSC lines [22–24, 31, 34, 35]. However, analyzing the specific molecular mechanisms of these transcription factors is challenging, as it relies heavily on traditional RNA-seq analysis. Bulk RNA-seq data from sorted hPGCLCs using cell surface markers or PGC-specific reporters can be powerful and insightful, especially by comparing control and mutant hPGCLCs to characterize the differentially expressed genes for deeper analyses. However, caution should be taken when analyzing the bulk RNA-seq data for key regulators such as TFAP2C and SOX17, where no or very sparse "hPGCLCs" are induced in the mutants. These sorted mutant "hPGCLCs" could be non-hPGCLCs due to the surface markers/reporters used, or random cells due to the technical issues of flow cytometry. scRNA-seq analysis could be applied to overcome these limitations [108, 109]. Notably, scRNA-seq identifies heterogeneity in cell populations by capturing all cells and supports cell lineage tracing analysis, providing a natural advantage in dissecting the molecular mechanisms of mutants important for hPGCLCs. Based on single-cell transcriptomic analysis, TFAP2A + cells from day 1 aggregates are identified as hPGCLC progenitors through lineage tracing. More importantly, by aligning both control and TFAP2C mutant cells within the germline trajectory from hESCs to day 4 hPGCLCs and comparing the differentially expressed genes between control and TFAP2C mutant cells based on single-cell transcriptomic analysis, TFAP2C is discovered to specifically upregulate SOX17 in hPGCLC progenitor cells for ensuring germ cell fate specification, a finding difficult to be uncovered in previous studies [31]. scRNA-seq-based lineage tracing will be critical for deciphering the precise mechanisms of the identified transcription factors and other factors important for hPGCLCs. Furthermore, combining spatial genomics will provide more insights into the tightly regulated and tempo-spatially organized process of the specification and differentiation of germ cell lineage, promoting the in vitro reconstitution of gametogenesis for clinical applications and disease modeling.

Another layer of regulation that warrants deeper investigation is post-transcriptional regulation mediated by RNA-binding proteins. Current mechanistic understanding of early hPGC specification primarily relies on knockout experiments in hPGCLC differentiation models in vitro. The focus has been predominantly on extracellular signals (such as BMP and WNT), intracellular transcriptional regulation (such as TFAP2C, SOX17, and PRDM1), as well as epigenetic factors (such as TET1), with much less attention to post-transcriptional mechanisms. For instance, although NANOS3 is commonly regarded as a specific marker gene of early hPGCs [22, 23, 110], its precise molecular function remains poorly understood. RNA-binding proteins have been found to be essential for germ cell fate in other species. Knock-out of DND1 in mice leads to defects in PGCs, similarly, knock-down of dnd1 in zebrafish results in abnormal PGC migration and survival [111–115]. Furthermore, TIAR knockout leads to defects in mPGCs, and Adad1 knockout in zebrafish reduces the number of germline stem cells [115, 116]. These studies of RNA-binding proteins underscore the potential importance of post-transcriptional regulation mediated in PGC development. Further studies are needed to uncover the roles of RNA-binding proteins in the specification and differentiation of hPGCLCs from hPSCs. Altogether, with the hPSC-derived hPGCLC systems and model organisms, elucidation of the complex regulatory network including transcriptional, epigenetic, and post-transcriptional regulation of human germ cell lineage will provide insights into the mechanisms governing hPGC development, and strategies reconstituting gametogenesis in vitro for potential clinical applications.

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