Human pluripotent stem cell-derived DDX4 and KRT-8 positive cells participate in ovarian follicle-like structure formation

HIGHLIGHTS

- hPSC-derived DDX4 PGCLCs participate in ovarian follicle-like structure formation
- Human granulosa cells as a niche environment are participating in folliculogenesis
- Keratin 8 plays an essential role in primordial germ cell migration
Human pluripotent stem cell-derived DDX4 and KRT-8 positive cells participate in ovarian follicle-like structure formation

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SUMMARY
Understanding the mechanisms of human pluripotent stem cells (hPSCs) specification, development and differentiation to gametes are useful for elucidating the causes of infertility and potential treatment. This study aims to examine whether hPSCs can be induced to DDX4 extracellularly expressing primordial germ cell-like cells (DDX4ec PGCLCs) and further into ovarian follicle stage in a combined in vitro and in vivo model. The transcriptional signatures show that these DDX4ec PGCLCs are characteristic of PGCs and express ovarian folliculogenesis markers. We also verify that keratin (KRT)-8 is highly expressed in the DDX4ec PGCLCs and plays a crucial role in germ cell migration. By co-culturing DDX4ec PGCLCs with human granulosa cells (GCs), these cells are further induced into ovarian follicle-like structures in xenograft mice model. This approach can in the future design practical strategies for treating germ cell-associated issues of infertility.

INTRODUCTION
Germ cells are critical players in human reproduction. In modern infertility treatment, the quality and quantity of oocytes remain the major determinants of successful outcomes. However, the female reproductive potential is limited by the restricted number of eggs available in the ovaries and the quality of eggs that decline with age. For infertile patients with premature ovarian failure or due simply to ovarian aging, the reduced quality of eggs in the ovaries is often the primary reason leading to the subfertility. It is thus useful to explore efficient techniques to derive human gametes for the purpose of disease modeling to improve egg number and quality, or hopefully be used as a direct application to assisted reproduction. Human pluripotent stem cells (hPSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to develop into all cell types including germ cells in human. Although functional oocytes from mouse ESCs (mESCs) and iPSCs (miPSCs) have successfully been derived (Hayashi et al., 2011, 2012), mature human gametes have not yet been established from stem cells, except one recent report by Yamashiro et al., who reported the generation of human oogonia and immediate precursory states for oocytes from hiPSCs in culture (Yamashiro et al., 2020). Up to now, our knowledge of human mammalian primordial germ cell (PGC) specification and epigenetic reprogramming remains imprecise. Knowledge about mammalian PGC specification was initially established in mice (Saitou et al., 2002; von Meyenn et al., 2016), specified from post-implantation epiblast cells on embryonic day (E) 6.25. Subsequently, PGCs migrate through the hindgut to the developing genital ridges in response to bone morphogenetic protein 4 (BMP4) pathway signaling before sexual differentiation (Lawson et al., 1999; Saitou and Miyauchi, 2016). Evidence shows that the treatment with multiple cytokines and aggregated with male or female gonadal somatic cells leads to the differentiation of mESCs and miPSCs toward epiblast-like cells (EpiLCs), to PGC-like cells (PGCLCs), as well as the spermatogenesis and oogenesis (Hayashi et al., 2012; Hikabe et al., 2016; Kimura et al., 2014). However, significant differences exist in germ cell specification between mouse and human such as the developmental timeline, efficiency, signaling pathways, transcriptional, translational, and metabolic properties of developmentally similar cell types (Yamashiro et al., 2020). We thus need to explore the differences and identify the ways of deriving germ cells from human PSCs.

Previous studies have made a significant improvements toward the identification and characterization of PGCs in mice and human by the markers such as PRDM14 (Yamaji et al., 2008), cell surface makers KIT (Gkountela et al., 2015; Guo et al., 2015), CD38 (Irie et al., 2015), or combination with TNAP in mice...
In addition, human and mouse PGCs share several key genes that regulate germ cell fate, including BLIMP1 (PRDM1), SSEA1, OCT4, TFAP2C, DAZL, and DDX4 (Canovas et al., 2017). Expression of DDX4 (VASA) has been reported in several mammalian PGCs including human (Castrillon et al., 2000), rhesus macaques (Hermann et al., 2007), and mice (Rebourcet et al., 2016). DDX4/Ddx4 is a member of the DEAD-box family of protein and germline-specific RNA helicase which plays an important role in germ cell meiosis (Medrano et al., 2012) and is expressed in migratory PGCs in the region of gonadal ridge (Castrillon et al., 2000). Ddx4 has been demonstrated in mouse ESC-derived PGCLCs to retain the capacity of involvement in both spermatogenesis (Hayashi et al., 2011) and oogenesis (Ishikura et al., 2016). DDX4 has been reported to express a C-terminal domain (extracellularly; DDX4ec), with the N-terminal domain is located intracellularly (intracellularly; DDX4). Previous reports show that by DDX4 C-terminal antibody-based fluorescence-activated cell sorting (FACS), potential germline stem cells can be isolated from the human ovary (Zou et al., 2009) and show a functional ability to form oocyte-like structures (Clarkson et al., 2018; White et al., 2012). The transcriptome and proteome of human DDX4ec PGCLCs derived from hPSCs have not yet been systematically analyzed, with the function of these cells remaining largely unknown. The role and function of DDX4ec PGCLCs derived from differentiated human ESCs and iPSCs also remain especially un-established. Considering the critical role of DDX4 in the differentiation of human PSCs in germline lineage, it is important to analyze the role of DDX4ec PGCLCs in germline development in the field of human PSC differentiation to germ cells in more details. Directed induction of PSCs to germ cells in vitro and in vivo evidently needs the appropriate and timely use of specific growth factors and/or microenvironment.

Previous studies show that activin A and basic fibroblast growth factor (bFGF) play important roles in the differentiation of mouse PGCLCs (mPGCLCs) from ESCs-derived EpilCs, leading to a decrease in the expression of pluripotency markers and an increase in the expression of specific germ cell markers (Zhou et al., 2016). In addition, it has been demonstrated that activin A enhances the efficiency of human primordial follicle in oocyte development in vitro (Telfer et al., 2008). Retinoic acid (RA) signaling is essential during meiotic induction of PGCs. In a male mouse model, it was shown that the CYP26B1 regulates endogenous RA, which is induced by fetal-stage gonadal somatic cells to coordinate male germ cells into meiosis (MacLean et al., 2007; Zhou et al., 2016). In addition, several meiotic and self-renewal genes were also explicitly expressed in human female fetal germ cells in response to RA pathway signaling (Li et al., 2017; Zhou et al., 2016).

Previous studies also demonstrate that the three morphogens (activin A, BMPs and RA) regulate the expression of several germline genes and the initiation of meiosis in PGCs derived from ESCs (Koubova et al., 2014; Zhou et al., 2016). We made use of these morphogens for the initial induction of germ cells in this study. In addition, for further germ cell maturation and migration, a physiological niche microenvironment is usually needed. In terms of physiology, the movement of germ cells is important in determining germline developmental processes, regeneration, and cell migration. The expression of keratin (KRT) proteins such as KRT 8 and 18 can support the necessary shape changes and provide the stability needed for cell translocation (Seetharaman and Etienne-Manneville, 2020). Specifically for in vivo female germ cell growth, a stage of ovarian follicle formation is likely essential, in which the oocyte can be found to be surrounded by granulosa cells (GCs). Currently human GCs can be relatively easy to harvest during oocyte retrieval in an in vitro fertilization program, provided that IRB approval and patient informed consent are obtained. Previously we reported that human GCs can be successfully derived from hPSCs (Lan et al., 2013). These critical ovarian somatic cells thus can be used for the maturation of the DDX4ec PGCLCs derived in this study.

Here, we tentatively named these DDX4ec-sorted hPSC-derived germ cells as DDX4ec PGCLCs. We have conducted a comparative analysis of DDX4ec PGCLCs derived from hESCs and hiPSCs and investigated the developmental potential of these cells in a xenograft animal model.

**RESULTS**

**Derivation of human PGCs from pluripotent stem cells**

A schematic diagram of the differentiation strategy for human PGCLCs (hPGCLCs) formation is illustrated in Figure 1A. To generate hPGCLCs from hESCs and cord blood cell-derived iPSCs (CBiPSCs; a human iPSC line), we modified and improved the strategy described for mouse PGCLCs (mPGCLCs) (Oliveros-Etter et al., 2015) and hPGCLC specification in previous reports (von Meyenn et al., 2016; West et al., 2009).
A. Serum and feeder free, non-attachment culture

Day

0

3

5

7

+ActivinA +BMP4 +RA

+ActivinA +BMP4 +RA

bFGF, N2/B27 FBS growth factors

B. hPSCs EBs PGCLC aggregates

C. Log2 Gene signature. Spearman ρ = 0.896; p = 0.7881

D. 

E.  

F.  

G.  

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Characterization of DDX4ec PGC-like cells

First, to demonstrate the ability of purifying DDX4ec PGCLCs using a C-terminal extracellular epitope, we performed the double immunofluorescence analysis for DDX4 and E-cadherin in day 7 differentiated cells. Our results demonstrated that transmembrane protein E-cadherin confirmed the DDX4ec PGCLC localization (Figure S1B). To deeply understand the role of DDX4ec PGCLCs, we performed FACS to sort the DDX4ec PGCLCs from the differentiated stem cells (Figure 2A). DDX4ec PGCLCs can be isolated from approximately 10% day 7 differentiated cells after ABR treatment (Figure 2A). No significantly different percentages for DDX4ec PGCLCs were observed between the differentiated cells originating in hESCs and CBiPSCs (Figure S2). To further characterize the DDX4ec PGCLCs, we initially carried out an RNA-array to compare DDX4ec PGCLCs derived from hESCs and CBiPSCs (Figure 2A). We found that the transcriptional signatures of DDX4ec PGCLCs were differentially expressed between hESCs and CBiPSCs (Figure 2B). A heatmap of the 476 differentially expressed genes of pairwise comparisons and the GO analysis of the 3172 differentially expressed gene clusters revealed and identified the significant over-representation of 499/1413 signaling pathways (File S1). Our data showed that the expression of DDX4 and SSEA1 (an early PGC marker) significantly increased by using ABR treatment in PGCLC aggregates. Interestingly, we found that most of the DDX4-positive cells were not co-localized with SSEA1-positive cells (Figure 1G and see Figure S1A), suggesting the distinctive expressing pattern and timing of DDX4 in hPGCLCs.
**A**

hESCs and/or CB iPSCs differentiation

![Diagram showing hESCs and CB iPSCs differentiation](image)

**B**

DDX4ec PGCLCs

![Heatmap showing GO terms](image)

**C**

NESCs

DDX4ec PGCLCs vs Others

![Bar chart showing fold change](image)

**D**

Other Cells

DDX4ec PGCLCs

![Heatmaps comparing gene expression](image)
ZP1, ZP2, ZP3, DAZL, Stella, Blimp1, and SYCP3 were expressed at higher levels in DDX4ec PGCLCs (Figure 2D). No expression of BMP15 and NOBOX were observed in DDX4ec PGCLCs and ZP1 was not expressed in DDX4ec PGCLCs derived from CBiPSCs (Figure 2D).

DDX4 PGCLCs are participating ovarian follicle-like structure formation

Physiologically, at the third to fifth week of gestation, hPGCs start to migrate from the yolk sac wall through the hindgut to the developing genital ridge. In order to further explore whether the DDX4ec PGCLCs obtained in our culture were able to form ovarian follicle-like structures, subcutaneous xenografting was performed in NOD-SCID mice. After collecting DDX4ec PGCLCs by FACS, the cells were mixed with human GCs, encapsulated by matrigel as an extracellular matrix (ECM) and transplanted subcutaneously in NOD-SCID mice and observed for 6 weeks (Figures 3A and S3). The tissues were then removed from mice (Figure 3B) after 6 weeks and examined. We observed that ovarian follicle-like structures appeared in the transplants both in the cells derived from hESCs and hiPSCs (Figure 3C (hESCs) and Figures S4A and S4B (hiPSCs)). Balbiani body (orange arrow) and germinal vesicle-like staining in the middle of the follicle-like structures (red arrow) accompanied with the surrounding cells suggested that these follicle-like structures were likely at the stage of primary follicles (Figure 3C). No follicle-like structures were observed in the GCs-only (Figure 3D) transplants. To further confirm the follicle-like structures to contained early stage germ cells and surrounding somatic cells, we stained the cells with DDX4 and AMHR2 (a GC marker). Immunostainings showed that these follicle-like structures expressed DDX4 in the center (presumably the oocyte) and AMHR2 at the peripheral locations, likely the surrounding GCs (Figure S4C). We also demonstrated AMHR2 but not DDX4 expression in the control GC transplants (Figure S4D), and no follicle-like structures were observed in GC control (Figure S4D) and DDX4ec PGCLC control only (Figure S4E). Our data herein provide strong evidence that DDX4ec PGCLCs were capable of developing into early ovarian follicle-like structures, when cultivated with human GCs. Taken together, after in vitro treatment with an appropriate cocktail of growth factors (activin A, BMP4 and retinoic acid) combined with a suitable in vivo environment with GC co-culture, human PSCs carry the potential to develop toward germ cell lineage, up to the stage of early ovarian follicles.

Keratin 8 is predominantly expressed in DDX4ec PGCLCs

From the RNA array analysis, we surprisingly found that KRT family member Keratin 8 (KRT8) was highly expressed in DDX4ec PGCLCs compared with the other differentiated cells (File S2). To confirm the results, we stained the cells with DDX4 and KRT8 in 7 day PGCLC aggregates from hESCs and CBiPSCs. Here, we demonstrated that approximately over 50% of KRT8 positive cells were localized to the cell membranes of DDX4ec PGCLCs (Figure 4A). We then conducted a comprehensive proteome analysis of sorted DDX4ec PGCLCs from hESCs and CBiPSCs. The most predominant band in DDX4ec PGCLCs derived from hESCs and CBiPSCs compared with the other differentiated cells were selected and analyzed by mass spectrometry. We found that KRT8 was the top high-level protein species in the proteins (Figure 4B). We next confirmed that the mRNA expression levels of DDX4 and KRT8 in DDX4ec PGCLCs derived from hESCs and CBiPSCs were significantly higher than the un differentiated hESCs, the undifferentiated CBiPSCs and the other differentiated cells (Figure 4C). We further demonstrated that KRT8 was predominantly expressed in DDX4ec PGCLCs both from hESCs and CBiPSCs, and that KRT8 (lower level) and KRT8 (higher level) were also detectable in the other differentiated cells by using immunoblotting (Figure 4D). Our data suggest that KRT8 might be an important marker in PGC developmental progression. Since keratin family is involved in cytoskeletal organization and KRT8 is highly associated with cell migration, cell adhesion, and metastasis in some tumors (Fang et al., 2017), we assume that KRT8 might play an important role in germ cell migration toward the gonadal ridge.

Keratin 8 is essential in DDX4 migratory PGCs

In order to confirm our hypothesis, we examined the expression of Krt8 and Ddx4 in mouse PGCs from E8-E14 (E: embryonic day) using immunofluorescence. We identified Krt8 on the surfaces of almost all Ddx4⁺ cells in migratory PGCs from E7.5 to E10 (Figure 5A). However, from E11.5 and E13.5 onward in migratory
**A**

Human granulosa cells → DDX4<sup>PGC</sup> PGCLCs → Matrigel capsule → NOD-SCID mice subcutaneous xenograft → 6 weeks → follicle-like structure → No follicle-like structure

**B**

GCs only

**C**

GCs and DDX4<sup>PGC</sup> PGCLCs co-culture

**D**

GCs only
Figure 3. Human pluripotent stem cells (PSC) - derived human primordial germ cell-like cells (hPGCLCs) develop into ovarian follicle-like structures with a granulosa cell microenvironment in a NOD-SCID mouse model

(A) Schematic timelines and the procedures for the DDX4ec PGCLCs derived from hPSCs to the harvesting of transplants in the xenograft mouse model. (B) The transplants of human granulosa cells (GCs) with or without the presence of DDX4ec PGCLCs. (C and D) Ovarian follicle-like structures were obtained from the transplants of mixtures of GCs and DDX4ec PGCLCs and (D) GCs only. The red arrow shows germinial vesicle-like staining and the orange arrow shows that the balbiani body is present in the follicle-like structures. The other dotted line areas show other follicle-like structures.

PGCs, Krt8, and Ddx4 proteins became uncoupled, with 30% (E11.5) and less than 10% (E13.5) of Krt8+ cells co-expressed with Ddx4+ (Figure 5A). Therefore, by demonstrating Krt8 and Ddx4 expression in this mouse model, we propose a common PGC migratory stage in mice from E8 to E14, in which Krt8 might be playing an important role in the regulation of PGC migration. To further reinforce our hypothesis in human PGCs, we assessed the chemotactic activity of KRT8-knockdown (KRT8-KD) and control differentiated cells using trans-well migration assays. The KRT8 mRNA levels were low in undifferentiated hESCs and were increased on day 7 in the ABR-treated differentiated cells (Figure 5B). As expected, the KRT8 mRNA levels were significantly reduced in the siKRT8-KD cells (KRT8-KD by siRNA) when compared with the control-KD cells (Figure 5B). Subsequently, we observed that the migration of KRT8-KD DDX4+ PGCLCs was significantly reduced in comparison to the control cells in response to 10% FBS gradients across gelatin-coated membranes in the trans-well migration assay (Figure 5B). By examining the cells which successfully migrated through the membranes, we found that the number of migratory KRT8/DDX4+ cells (cells-expressing both KRT8 and DDX4) were reduced by approximately 50% in KRT8-KD DDX4+ PGCLCs, when compared with the control cells (Figure 5C). No KRT8/DDX4+ cells (cells-expressing DDX4 but not KRT8) were observed in the migratory cells, suggesting the critical role of KRT8 expression in DDX4+ cell migration (Figure 5C). In addition, the number of KRT8/DDX4 cells in the migratory cell fraction of KRT8-KD group was also reduced in comparison to the migration of control cells (Figure 5C). Our results therefore suggest that KRT8/Krt8 is required for hPGC and mouse PGC migration.

**DISCUSSION**

Using PSCs to generate functional mature germ cells has long been considered, with the concept confirmed in the reconstitution of oogenesis in mouse models (Hayashi et al., 2012; Hikabe et al., 2016; Yamashiro et al., 2020). Recent studies have also successfully established early human PGCs and human oogonia, an immediate embryonic precursor for human oocytes from PSCs, but mature gametes have not yet been consistently derived from hPSCs, except recent reports about the feasibility of generating human oocytes in culture (Yamashiro et al., 2018, 2020). These results suggest although human PGCs likely have a unique gene profile, the developmental mechanisms of human PGCs into germ cells may be substantially similar to the other species, particularly in epigenetic reprogramming. Therefore, using the age-matched endogenous ovary tissue that the human stem cell-derived hPGCLCs might gain developmental competence (Irie et al., 2015; Kobayashi et al., 2017; Tang et al., 2015). DDX4 encodes an ATP-dependent RNA helicase and it is known that DDX4 is a germline-specific molecule in various organisms. In mammals, DDX4 is found to be expressed specifically in PGCs and mature germ cells in most phyla studies, suggesting that DDX4 plays a crucial role in germline development and maintenance. DDX4 has been reported to have a C-terminal domain which is expressed extracellularly (Zou et al., 2009). To explore the ill-defined role of DDX4ec PGCLCs, we here report the use of strategies in terms of a cocktail of several morphogens to drive the development of DDX4ec PGCLCs from human PSCs. Subsequently, a further appropriate in vivo niche environment created by co-cultivating with human GCs enhanced the formation of early ovarian follicle-like structures. We also observed the potentially critical role of KRT8 in the migration of DDX4ec PGCLCs to the genital ridge.

Resetting of the epigenome in hPGCs is critical for germline lineage development. Our data show that the pluripotency genes were downregulated by activin A, BMP4 and retinoic acid treatment. Furthermore, DDX4ec PGCLCs derived from hESCs and hiPSCs exhibited differential transcriptome signatures, though both cell types still share similar germline differentiation potential. For example, the DDX4ec PGCLCs derived from hPSCs (both hESCs and hiPSCs) exhibited several germline markers including DPPA3, BLIMP1, and meiotic makers including REC8, SYCP1, and folliculogenesis markers including ZP1, 2, 3, GDF9 and FIGLA. The expression of DNMT1, DNMT3a and DNMT3b decreased in DDX4ec PGCLCs, indicating the chromatin reorganization and comprehensive DNA hypomethylation. For this latter issue, a further in-depth study is necessary for confirmation, after the efficiency of cell differentiation can be further
We thus have good reasons to consider that these DDX4ec PGCLCs have likely exited the pluripotent state and entered meiosis, a critical step in germline development. Taken together, our data suggest that DDX4ec PGCLCs obtained in this study are similar to early native PGCs but also express some mid-late PGC markers, implying that these cells retain the potential to develop into more advanced-stage oocytes such as oocytes in the ovarian primary or secondary follicles. We thus examined if DDX4ec PGCLCs could be utilized to induce human folliculogenesis in vitro. GCs are somatic cells surrounding the oocytes in the mammalian ovarian follicles. It is known that GCs interact intimately with oocytes and can secrete growth factors and sex steroids to create a niche environment for the development and maturation of oocytes by paracrine and autocrine effects (Wigglesworth et al., 2013; Zhang et al., 2010). GCs, therefore, are essential for folliculogenesis and are indispensable for ovarian follicle formation and growth at various stages. In this report, we successfully demonstrate the possibility of the growth of PSC-derived DDX4ec PGCLCs enhanced.

Figure 4. The proteomic identity of human DDX4ec primordial germ cell-like cells (PGCLCs)
(A) Immunofluorescent staining of DDX4 and KRT8 and the quantification of DDX4-expressing (DDX4+) and KRT8-expressing (KRT8+) cells at day 7 hPGCLCs.
(B) Mass spectrometry analysis of the protein extracts in amino acids (AA) of DDX4ec PGCLCs and the other differentiated cells derived from H9 hESCs and CBiPSCs.
(C and D) (C) mRNA levels of DDX4 and KRT8 and (D) immunoblotting analysis of KRT8 in undifferentiated stem cells, DDX4ec PGCLCs and the other differentiated cells derived from H9 hESCs and CBiPSCs. Mann-Whitney U test was used for statistical analysis. Bar graph represents mean ± SEM from at least three independent experiments. *p < 0.05, **p < 0.01. Mw: molecular weight.
toward the ovarian primary follicle stage, though efficiency remains low. This observation is important since the step-by-step improvement of each step of ovarian follicle growth and maturation from PSC-derived PGCLCs will hopefully help produce a final harvest of mature follicles or oocytes that provides an in vitro model of research for gamete biology.

Mammalian germ cells are specified at the border between embryonic and extraembryonic tissues (Richardson and Lehmann, 2010). Subsequently, germ cells must migrate through and along various somatic tissues to reach and aggregate the niche of the gonad. Previous studies show that mouse germ cells depart from the hindgut by the bFGF/Wnt signaling pathway and then undergo a trans-epithelial migration (Chawengsaksophak et al., 2012; Takeuchi et al., 2005). Dynamic adhesion to the ECM is observed during these processes in germ cells of some species. For instance, isolated migratory mouse germ cells during exit from
the hindgut display more E-cadherin expression than germ cells isolated at the end of migration (García-Castro et al., 1997). The keratin family is a major component of the intermediate filament which connects to ECM and regulates cell adhesion and migration. Interestingly in this study, the data show that human PSC-derived DDX4ec PGCLCs express a significant increase in KRT family members, particularly KRT8, an observation further supported by the finding that migratory mouse PGCs also express high levels of Kr8 during the migration period when compared with the germ cells in the gonad. We further confirmed the importance of KRT8 expression in human PGC migration by demonstrating that KRT8 deficiency led to a profound impairment in human PGC migration. Taken together, these human and mouse studies strongly support the notion that keratin family member, especially KRT8, very likely play a critical role in mammalian germ cell migration to the genital ridge.

In conclusion, we have identified a critical role of KRT8 in the chemotactic migration of human PGCLCs derived from PSCs, which may direct our further study into the currently un-established mechanisms of human germ cell migration. Our data also suggest that when an appropriate niche environment is provided, DDX4ec PGCLCs can exit a pluripotency state and progress into mid-stage germ cells, showing evidence of meiosis and the potential of ovarian folliculogenesis. We previously showed that human GCs can be successfully derived from hESCs and hiPSCs (Lan et al., 2013). Thus by using the human PGCLCs derived in this study and the previously reported stem cell-derived GCs (Lan et al., 2013), both originating from autologous hiPSCs, we can explore the mechanisms of germ cell development in a fully autologous system and hopefully design applicable strategies for some important causes of infertility such as primary ovarian insufficiency or aged-related reduced oocyte quality and quantity. Finally, it is hoped that our findings provide new insights into unique human germ line development through an artificial cell model by way of in vitro and in vivo human PSCs differentiation.

Limitations of the study
The primary follicle-like structure in our study is developed from human stem cells derived-DDX4ec PGCLCscapsuled with human GCs and transplanted subcutaneously in NOD-SCID mice. The primary follicle-like structure here is an early stage and the exogenous GCs might present in any place including developmental follicle-like structure. It is challenging to get similar or parallel human primary follicle samples as a positive control demonstrating our results because it will be from the human fetal ovary. Future research is needed to identify the possible parallel human primary follicles.

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, DannyCw Yu (dcwyu@email.nchu.edu.tw).

Materials availability
The materials in this study will be available on the requests with a completed materials transfer agreement.

Data and code availability
This study did not generate code and the RNA array and mass spectrometric data can be downloaded from the supplemental file.

METHODS
All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2020.102003.

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AUTHOR CONTRIBUTIONS
DannyCW Yu: Conception, design, data analysis, interpretation and manuscript writing.

Fang-Chun Wu: Conception, design and data analysis.

Chia-Eng Wu: Data analysis and assembly of data.

Lu-Ping Chow: Data analysis and interpretation.

Hong-Nerng Ho: Design, financial support and administrative support.

Hsin-Fu Chen: Conception, design, interpretation, manuscript writing and final approval of manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Human pluripotent stem cell-derived
DDX4 and KRT-8 positive cells participate
in ovarian follicle-like structure formation

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Transparent Methods

Human Cells, PSC Culture and Differentiation

Human H9 ESCs (hESCs; karyotype 46, XX; WiCell, WI, USA) (Thomson et al., 1998) and NTU1 hESCs (karyotype 46, XX, derived in this lab) (Chen et al., 2007) were used in the present study. Culture protocols of hPSCs were modified and described from our previous reports (Chen et al., 2009a; Chen et al., 2009b; Chen et al., 2007; Robertson et al., 2007). The CBiPSCs (CB: cord blood) were generated using human cord blood-derived CD34+ progenitors with seven episomally expressed factors (Cat# A18945, Life Technologies) (Burridge et al., 2011). The hPSCs (both hESCs and hiPSCs) were continuously maintained on mitomycin-C treated mouse embryonic fibroblasts using ReproCELL serum-free medium (ReproCELL, Tokyo, Japan) and passaged weekly manually using 30-gauge insulin needles as described from our previous protocols (Chen et al., 2009b; Chen et al., 2007). Differentiation of PGCs was performed according to the protocols (Irie et al., 2015; Vincent et al., 2013). Briefly, embryoid bodies (EBs) were generated by putting approximately 300 PSCs in each drop and maintained in bFGF/N2B27 medium (West et al., 2009). On day 5 and 7, EBs were transferred to DMEM-based medium supplemented with 20 ng/ml activin A, 100 ng/ml BMP4 and 0.1 μM RA (the ABR combination). Human granulosa cells (GCs) were obtained from ovarian follicular aspirates during oocyte retrieval in in vitro fertilization programs conducted in National Taiwan University Hospital. The use of human cells had been approved by the Institutional Review Board of Academia Sinica and the Ethical Committee of National Taiwan University Hospital. Informed consents were obtained from all subjects.

Animals and Xenograft

Different days of HaM/ICR mouse embryos were purchased from BioLASCO, Taipei, Taiwan and College of Medicine, National Taiwan University. Briefly, slides of the embryos were formalin-fixed, paraffin-embedded and sectioned. Immunodeficient NOD/SCID mice (BioLASCO, Taipei, Taiwan) were used in the xenograft study. 1 x 10^5 FACS-sorted DDX4+ PGCLCs were mixed with 1 x 10^6 human GCs and embedded in Matrigel (an extracellular matrix; ECM). The cell capsules were transplanted into the backs of mice via subcutaneous injections. After 6 weeks, mice were sacrificed by carbon dioxide and the newly formed tissues were washed with DPBS and fixed in 10% buffered formalin before being paraffin-embedded. All procedures had been approved by the Institutional Review Board of the Ethical Committee for Animal Study of National Taiwan University Hospital.

RNA extraction, cDNA Preparation and Quantitative RT-PCR (qPCR)
Total RNA was harvested by Trizol® reagent (Invitrogen) following the manufacturer’s instruction. Reverse transcription was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, #L1642). The following qPCR procedure was initiated by 3 minutes at 95 degree, and followed by 40 cycles of 15 seconds at 95 degree, 30 seconds at 55 degree, and 30 seconds at 72 degree. The expression levels of the target genes were calibrated by the internal control GAPDH and the collected data were analyzed by ABI 7500 Fast Real-Time PCR system. Quantitative PCR (qPCR) reaction was performed on 1x EvaGreen reagent (Biotium, Fremont, CA, USA, catalog number 31014); 1 ul of diluted cDNA and 100 uM selected primers (IDT, San Jose, CA, USA, Table S1) were used at 2ul in 20ul of qPCR reaction. Quantification of all the samples by the software was calculated by the C_T and relative fold changes were calculated using the 2^(-ΔΔCT) (Yu et al., 2016).

**Immunocytochemistry, immunohistochemistry and Fluorescence Activated Cell Sorting**

The anti-human SSEA1 (MAB2155, R&D system), DDX4 (ab13840, Abcam), KRT8 (sc-52324, Santa Cruz) and E-cadherin (AF748, R&D system) antibodies were used at the dilution of 1:200. The secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG, donkey anti-goat IgG, Alexa Fluor 488 goat anti-mouse IgM and donkey anti-rabbit IgG (Abcam and Molecular Probes, Eugene, OR, USA). The presence of expression of SSEA1 and DDX4 in the cells and mouse tissues were measured with the NIS-elements BR2.30 software (Nikon, Tokyo, Japan) and Carl Zeiss LSM 780, and the numbers of immune-reactive cells in these areas were quantified in a blinded fashion. DDX4^ec^ cells were isolated by FACS Aria II benchtop flow cytometer running DiVa 6 software. The unlabeled and secondary antibody only–labelled dissociated differentiated hPSC samples are used each time as controls to gate against background autofluorescence and to rule out non-specific binding of the secondary antibody. DDX4 antibody was used at 1:200 dilutions.

**Mass Spectrometric Analysis**

The protein samples from DDX4^ec^ cells and DDX4^ec^-negative cells were determined by SDS page electrophoresis and specific bands were extracted and analyzed by mass spectrometric analysis. Briefly, each sample was reduced with 5mM tris-carboxyethyl phosphine hydrochloride (TCEP) for 1h at 37°C, alkylated with 10 mM methylethanethiosulfonate (MMTS) for 20 min at room temperature (RT). Each dried fraction was reconstituted in 100 ul of 0.1% formic acid and 2% acetonitrile and then analyzed using a Q-Star Elite mass spectrometer (Applied Biosystems Company; MDS-Sciex), coupled to a prominence HPLC system (Shimadzu). The mass
spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 300 – 2,000 m/z. Peptides with +2 to +4 charge states were selected for MS/MS and the time of summation of MS/MS events was set to 2s.

**Immunoblotting**

Cells were washed with cold DPBS twice before being lysed in RIPA buffer (BioProducts, Ashland, MA, USA) supplemented with phosphatase inhibitor cocktail set II (brand, Calbiochem, San Diego, CA). Immunoblotting analysis was performed as described from our previous report (Yu et al., 2016). All primary antibody incubations were performed at 4°C overnight at the following dilutions: SSEA1 1:1000; DDX4 1:1000; KRT8 1:1000, and mouse monoclonal anti-actin (Sigma), 1:10,000.

**Transcriptional Profiling of DDX4 cells by RNA Array**

0.2 ug of total RNA was amplified by a Low Input Quick-Amp Labeling kit (Agilent Technologies, USA) and labeled with Cy3 (CyDye, Agilent Technologies, USA) during the in vitro transcription process. 0.6 ug of Cy3-labeled cRNA was fragmented to an average size of about 50-100 nucleotides by incubation with fragmentation buffer at 60°C for 30 minutes. The labeled cRNA was hybridized to Agilent SurePrint Microarray (Agilent Technologies, USA) at 65°C for 17 h. After washing and drying, microarrays are scanned with an Agilent microarray scanner (Agilent Technologies, USA) at 535 nm for Cy3. Raw signal data were normalized by quantile normalization for differential expressed genes discovering. For the functional assay, we used cluster-profile for enrichment test for gene ontology (GO) and pathway (KEGG).

**RNA Interference**

On-target KRT8 plus SMARTpool (SR302611) and control siRNA were purchased from OriGene (Rockville, MD, USA). The siRNA of KRT8 was 3 unique 27mer siRNA duplexes targeting Locus ID 3856. The siRNAs were transfected into the cells in 12-well plate using DharmaFECT1 transfection reagent according to manufacturer's instructions. 6h after transfection, the media was replaced by fresh media. After further 24h, the cells were collected for further analysis.

**Migration Assays**

Chemotaxis/directed migration assay was performed using polycarbonate filter wells (transwell, 8-lm pores; Coaster, Corning, NY) coated with 1% gelatin. Transwell migration of DDX4<sup>ec</sup> PGCLCs was stimulated by 10% FBS to the culture medium in the lower well. After 24h, the upper surface of the insert was swabbed to remove non-migrating cells. The cells that had migrated to the lower surface were fixed and
recognized by HE stains (Dade Behring, Deerfield, IL). Cells migration was quantified by counting the number of cells in three random fields per insert.

**Statistical Analysis**
All results are presented as mean +/- standard error of the mean from a minimum of three independent experiments. Mann-Whitney U test was used to determine statistical significance and $p<0.05$ was considered significant.

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Figure S1. DDX4, SSEA1 and E-Cadherin expression in day 7 differentiated cells, Related to Figure 1.

(A) Double immunocytofluorescence for DDX4 (red) and SSEA1 (green) in hPGCLCs shows uniform expression of DDX4. (B) Transmembrane protein E-cadherin (red) is colocalized with DDX4 (red) PGCLCs.
Figure S2. The DDX4\textsuperscript{ext} PGCLCs were derived from HPSCs and analyzed by flow cytometry, Related to Figure 2.

(A) The negative control showed no DDX4 positive cells. The percentage of DDX4\textsuperscript{ext} PGCLCs derived from (B) H9 (hESCs) (C) CBiPSCs showed no significant difference.
Figure S3. DDX$^{\text{ext}}$ PGCLCs and human granulosa cells transplanted into mouse after 6 weeks, Related to Figure 3.

The red area was DDX$^{\text{ext}}$ PGCLCs mixed with human GCs and the black area was human GCs only.
Figure S4. Human Follicle-like structures, Related to Figure 4, were developed from DDX4ec PGCLCs derived from (A) H9 (hESCs), (B) CBiPSC (hiPSCs) in a xenograft model. Human Follicle-like cells were stained with DDX4 and AMHR2 in (C) DDX4ec PGCLCs co-culture with GCs transplant and (D) GCs transplant only and (E) DDX4ec PGCLCs only in a xenograft model. The arrow and dotted lines showed new formed follicle-like cells. Scare bars represent in the figures.
Supplementary Table:

Table S1. Primer list, Related to Figure 2.

| Primer   | Forward (5’→3’) | Reverse (5’→3’) |
|----------|-----------------|-----------------|
| DAZL     | TCGAACTGTTGTCAGG | TAGGATTCATCGTTGG | YBX2 |
|          | GTCCAAAAGGCTA    | GTGGTGTTGGGCT   |
|          |                  |                  |
| YBX2     | ACCCTACCCAGTACCCTGCT |                  |
|          |                  |                  |
| SYCP3    | GCAAGAAAGCAACC A | AACTCCAACCTCTCCAGCA |
|          | GAGGAG            | GA |
|          |                  |                  |
| NOBOX    | ATAAAACGCCAGAGATTGCCCAGA |                  |
|          |                  |                  |
| NOBOX    | AAGTCTGGTCAGAAGTCAGCAGCA |                  |
|          |                  |                  |
| NOBOX    | LHX8 (5’→3’) |                  |
|          | CAAGCACAATTGGCTCAGGA |                  |
|          |                  |                  |
| GDF9     | GGCACGTAGGCAGAATAAGC |                  |
|          |                  |                  |
| OCT4     | TAGGTTGAAGGAGGCTGTCAT |                  |
|          |                  |                  |
| VASA     | VASA (5’→3’) |                  |
|          | TCACCTCTACAACAACACT GTTCCGCT |                  |
|          |                  |                  |
| OCT4     | GACAGGGGGAGGGAGGAGCTAGG |                  |
|          |                  |                  |
| OCT4     | CTTCCCTCAACCAGTGCCCAAAAC |                  |
|          |                  |                  |
| OCT4     | STELLA (5’→3’) |                  |
|          | GCGGAGTTTCGTACGCATGA |                  |
|          |                  |                  |
|   | CCATCCATTAGACACGCAGAAA |
|---|------------------------|
| R | BLIMP1 (5'→3')          |
| F | TGGAGAAACGGCCTTTCAAAT  |
| R | CCTGGCATTCATGTGGCTTT   |
|   | ZP1 (5'→3')             |
| F | TGCTCCATCTCTGCTACCCTG   |
| R | GTCTTGTGCCACATCCACAC   |
|   | ZP2 (5'→3')             |
| F | CTCAGGCTGGTCAATGCAG     |
| R | TGGAAAGGCAGATTACCAAC    |
|   | ZP3 (5'→3')             |
| F | GACCGGGGCAGATACACT      |
| R | CATCTGGGTCTGCTAGCTA     |