Oocyte Arrested at Metaphase II Stage Were Derived From Human Pluripotent Stem Cells in Vitro

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Research

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Abstract

Background

Generation and maturation of human oocyte *in vitro* could facilitate studies of folliculogenesis and oogenesis. We have previously shown that human aminotic fluid stem cells giving rise to oocyte-like cells (OLCs), However, it was difficult to observe whether these OLCs enter meiotic stage.

Methods

Human induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs) were cultured by follicle fluid, cytokines and small molecule to induced oocyte-like cells (OLCs) formation through a three-step induction procedure. Surface marker expression and differentiation potential of germ cells were analyzed *in vitro* by flow cytometry, gene expression, immunocytochemistry, western blotting and RNA Sequencing.

Results

To induce hiPSCs differentiation into OLCs, cells were firstly cultured in a primordial germ cell medium for 10 days. The cells showed the morphology similar to primordial germ cells (PGCs), highly expressing germ cell markers and primordial follicle development associated genes. The induced PGCs were then cultured in the primordial follicle-like cell medium for 5 days to form the induced follicle-like structures (iFLs), which retained both primordial oocytes-like cells and granulosa-like cells. In the third step, the detached iFLs were harvested and transferred to the OLC-medium for additional 10 days. The cumulus-oocyte-complexes (COC) structures and OLCs in different sizes (50-150 μm diameter) with zona pellucida were observed. The in vitro matured OLCs presented the polar body and arrested at metaphase II (MII) stage. Some OLCs were self-activated and spontaneously developed into multiple-cell structures similar to preimplantation embryos, indicating that OLCs were parthenogenetically activated though in vitro fertilization potential of OLCs are yet proved.

Conclusions

In vitro maturation of OLCs derived from hiPSCs provides a new means to study human germ cell formation and oogenesis.

Background

Generation and culture of germ cells in the absence of the morphogenetic events of gastrulation is a crucial issue to understand the entire process of gametogenesis *in vitro*. Many studies have demonstrated that human and mouse pluripotent stem cells can produce oocyte-like cells (OLCs) [1–4] to reconstitute the process of oogenesis *in vitro*. Commonly, the suspension culture condition was used to induce the differentiation of pluripotent stem cells into primordial germ cells (PGCs) [1], which were continuously grown in media contained cytokines, such as BMP4, SCF, EGF, and LIF, and developed to

form germ cells-containing aggregates and follicle-like structures. Meanwhile, the OLCs were also derived from somatic stem cells and induced in the conditional medium supplemented with porcine follicular fluid and ovarian steroid hormones [5, 6] under monolayer culture condition. A successful reconstitution of mature oocyte was induced from mouse ESCs, which derived into PGCs combined with somatic cells from embryonic day 12.5 (E12.5) female gonad [7]. Recently, oogonia-like cells induced from human pluripotent stem cells were reconstituted with mouse embryonic ovarian somatic cells and formed xenogeneic ovary, which hold germ cells with epigenetic reprogramming and entered into meiotic prophase [4]. However, the differences of germ cell development among mammalian species are evident [8, 9]. Lately, a report showed that human ESCs were induced by the overexpression of DAZL and BOULE with recombinant GDF9 and BMP15 (also known as GDF9B) to enter into meiosis and further to form follicle-like structures (FLs) *in vitro*, in which the mixed cells of OLCs and granulosa-like cells were self-organized and resembled the primary follicles [3].

Our previous report demonstrated that hAFSCs were able to induce OLC formation in a two-step procedure [5]. Here, we further demonstrated that MII OLCs were derived from human iPSCs and ESCs in adherent monolayer culture through a three-step approach. The MII OLCs derived from hPSCs provides a new means to study human folliculogenesis and oogenesis.

Materials And Methods

Cell culture

The human pluripotent stem cells (hPSCs) used in this study are human induced pluripotent stem cells (hiPSCs, female XX, TAC153) and human embryonic stem cells (hESCs, female XX, H9), which were purchased from SiDanSai Biotechnology Co. and ATCC respectively. The hPSCs were grown on Matrigel (Becton Dickinson) in mTeSR™1 (Stem Cell Technology) till 80% confluence. And a three-step induction procedure was then conducted to generate OLCs.

Step 1, Formation of induced PGC cells (iPGCs, day 0 to day 10). The hPSCs were cultured in the medium of PGC-m for 10 days to form iPGCs. The medium of PGC-m consists of alpha minimum essential medium (alpha-MEM; Gibco) supplemented with 5% knockout serum replacement (KSR; Gibco), 5% bovine follicular fluid (bFF), 1% L-glutamine (Invitrogen), 1% nonessential amino acids (NEAA; Gibco), 0.1 mM b-mercaptoethanol (b-ME, Gibco), 1% penicillin/streptomycin, 50 ng/mL BMP4 (PeproTech), 200 ng/mL human LIF (Sino Biological), 100 ng/mL SCF (PeproTech), 50 ng/mL EGF (stemRD), and 10 μM ROCK inhibitor (Y-27632, Selleck). The hPSCs at 3×10^5 /3.5 cm plate pre-coated with Matrigel were cultured in PGC-m at 37°C, 5% CO_2. The medium was changed daily. At day 2 post induction, the hPSCs were became the flattened epithelioid morphology with distinct cell-to-cell boundaries. During differentiation, SOX17-positive cell clusters (25 to 50 μm in diameter) were gradually formed. At day 8–10 post induction, cell aggregates and the induced primordial germ cells (iPGCs) were formed, which were grown on the top of the monolayer cells.

Step 2. Formation of induced follicle-like structures (iFLs, day 11 to day 15). The iPGCs were then cultured in the medium for culturing follicle-like cells (FLC-m) for 5 days to form iFLs. The medium of FLC-m consists of alpha-MEM supplemented with 5% KSR, 5% bFF, 1% L-glutamine, 1% NEAA, 0.1 mM b-ME, and 1% penicillin/streptomycin. At day 11 post induction, cell aggregates were then incubated in FLC-m for additional 5 days. The medium was changed every day with the half of the liquid volume. At day 15, the induced follicle-like structures (iFLs) in 50 to 200 μm diameter were usually visible under the microscope.

Step 3. Formation of oocyte-like cells (OLCs, day 16 to day 25). For OLC *in vitro* maturation, iFLs were picked up, transferred into OLC-m, and cultured for another 10 days. In brief, iFLs were transferred into a 30 μL droplet, covered with mineral oil, and incubated in OLC-m for 10 to 15 days, changing half of the medium every 2 days. OLCs varying in size from 50 to 150 μm in diameter could be then observed. The medium of OLC-m consists of TCM 199 (Gibco) supplemented with 3 mg/mL bovine serum albumin (BSA, Sigma), 5 U/mL follicle-stimulating hormone (FSH, Sigma), 10 U/mL human chorionic gonadotropin (hCG, Sigma), 10 IU/mL pregnant mare serum gonadotropin (PMSG, Sigma), 0.23 mM pyruvic acid, 10 ng/mL epidermal growth factor (EGF, Sigma), and 1% insulin-transferrin-selenium (ITS, Gibco).

All media used in this study are listed in Table S1. To prepare bFF, the bovine ovaries from mature cows were collected from a licensed local slaughterhouse, Sanqiao Slaughterhouse in Xi`An, China, and transported to the laboratory within 6 h in sterile saline at 37°C. The bFF was aspirated from follicles using a 10-mL syringe with an 18-gauge needle. The cumulus-oocyte complexes (COC) and granular cells were removed from bFF by centrifugation at 3000 g for 20 min at 4°C. The bFF was passed through 0.22-μm sterile filter, and was then aliquot in 5 mL/tube and stored at -80°C for the future use.

iPGCs Purification

The iPGCs were purified from 7-day differentiated cell aggregates derived from hiPSCs by immunomagenetic isolation of DDX4 antibody. The iPGC cells were washed 3 times in PBS, placed into PBS containing 0.05% trypsin and then incubated at 37°C, 5% CO_2 for 5 min. Trypsin was neutralized by adding culture medium with 10% FBS. The dispersed cells were carefully collected and centrifuged at 1000 g for 5 min, the pellet was resuspended and washed 2 times with PBS. The cells were resuspended and prepared to conjunct with DDX4 antibody, which was pre-coated with goat anti-rabbit IgG microbeads. $DDX4^+$ iPGCs were separated by magnetic separation according to manufacturer's instruction.

Plasmid Transfection

The pBMP15-EGFP vector carrying with human BMP15 promoter sequence was constructed as described previously [10]. To conduct the transient transfection, the differentiated cells at day 5 and 10 were washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco) once and then transfected with 4 μg

pBMP15-EGFP plasmid by Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's introduction. Images were documented by the fluorescence microscope (Nikon).

Flow Cytometry

The iPSCs and 10-day differentiation iPGCs were treated with Accutase (Invitrogen), then washed added culture medium to assist in washing off cells, and pipette gently up and down. After washing once with DPBS containing FBS and 0.1% BSA, the cell suspension was filtered by using a cell strainer (BD Biosciences) to remove cell clumps, and was subjected to centrifugation. Cells were incubated with the anti-c-KIT antibody (#562094, BD Biosciences) on ice for 15 min. The nonbinding isotype-matched FITC-conjugated mouse IgG was utilized in control experiments. The collected cells were suspended in FACS buffer (0.1% BSA in DPBS) and analyzed by FACSCalibur Flow Cytometer (BD Biosciences, USA).

Gene Expression Analysis

Total RNAs were extracted by RNeasy Mini Kit (Qiagen) following the manufacturer's introduction. One microgram of RNA was reverse transcribed into cDNA by RevertAid™ kit (Thermo Fisher Scientific), and then used to perform PCR in 50 μL reaction volumes for 35 cycles. The GAPDH expression was measured as the internal control. Quantitative RT-PCR (qRT-PCR) was performed using the SYBR Premix Ex TaqTM kit (TaKaRa) on a programmed thermal cycler (ABI), following the manufacturer's protocol. The gene expression level was first normalized to the GAPDH and then normalized to the expression of a control sample in each set of experiments. The positive control sample was human ovary tissue. Primers used in this study are listed in Table S2.

Immunocytochemistry and Western Blotting

The procedure for the protein analysis was performed as described previously [10]. In brief, to perform the immunocytochemistry staining, the differentiated cells at day 0, 5, 10, 15, 25 and 35 were fixed in 4% paraformaldehyde in DPBS for 20 min at room temperature. The fixed cells were washed twice, incubated with DPBS containing 0.2% Triton X-100 for 10 min at room temperature. Cells were blocked with blotting buffer (1% BSA, 0.1% Tween 20 in DPBS) for 2 h, and then incubated with the primary antibody at 4°C overnight. Antibodies against OCT4 (1:300, SC-5279, Santa Cruz), SOX2 (1:400, #3579, Cell Signaling Technology), PRDM1 (1:300, SC-47732, Santa Cruz), SOX17 (1:300, SC-130295, Santa Cruz), STELLA (1:500, ab74531, Abcam), DDX4 (1:200, #8761, Cell Signaling), FRAGILIS (1:300, ab15592, Abcam), NOBOX (1:400, ab41521, Abcam), FOXL2 (1:400, NB100-1277, Novus Biologicals), SCP3 (1:500, ab181746, Abcam), ZP2 (1:300, 32894, Santa Cruz), c-KIT (1:500, ab5505, Abcam), and CX37 (1:300, ab83788, Abcam) were used. The same batch of the differentiated cells was used as control, which was incubated with isotype-matched IgG. After washing three times, cells were incubated with secondary antibody for 1 h at room temperature. Then, cells were washed as described above, and cellular nuclei were stained with DAPI dye at a concentration of 1 μg/mL for 5 min, and then washed again as described above, and visualized by the fluorescence microscope or confocal microscope. The procedure for western blot analysis was previously described [10]. The differentiated cells at day 0, 5, 10, 15, and 25 were collected after washed with cold DPBS and treated with 200 μL RIPA buffer (50 mM Tris, 150 mM NaCl,

1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, pH 8) with protease inhibitors (1 mM PMSF). Human ovary was used as control. The supernatant was measured for protein concentration and heated at 100°C for 5 min, and then loaded on 10% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane (LC2002, Invitrogen) by semi-dry electrophoretic transfer (Bio-Rad) for 45 min at 15V. The membrane was blocked with 5% non-fat milk for 1 h at room temperature, and then incubated with the primary antibodies, including anti-OCT4 (1:500, SC-5279, Santa Cruz), anti-BMP15 (1:1000, ab108413, Abcam), anti-SCP3 (1:1000, ab181746, Abcam), anti-ZP2 (1:500, 32894, Santa Cruz), anti-ZP3 (1:300, SC-398359, Santa Cruz), and anti-GAPDH (1:2000, KM9002, Sungene Biotech), for 2 h. After washing three times with TBS-T buffer (20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20), the membrane was incubated with an HRP-conjugated secondary antibody (1:3000, A0258, Beyotime) at 37°C for 1 h. After washing in TBST (Tris-buffered saline, 0.1% Tween 20) for three times at room temperature, the membrane was incubated in the enhanced chemiluminescent substrate (#32106, Pierce) for 1 min and detected with a Chemiluminescent Imaging System (ZY058176, Tanon-4200).

Light Microscopy

Follicle-like structures were picked and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer. Following several washes with 0.1 M phosphate buffer, FLs were post-fixed in 1% osmium tetroxide and washed with 0.1 M phosphate buffer, Then FLs were dehydrated in increasing concentrations of ethyl alcohol (30, 50, 70, 80, 90, and 2 × 100%) and infiltrated with epoxy resin overnight and polymerizing overnight at 60°C. For light microscopic examination of follicular structure, 1 μm thick epoxy sections were stained with 1% (w/v) aqueous methylene blue and examined using an Olympus BX50 microscope.

RNA Sequencing

hiPSCs were seeded onto six-well-plate coated with Matrigel, each well of iPGCs was lysed in 1mL TRIzol reagent (Invitrogen, 15596018) after 5 days differentiation in three independent experiments, and hiPSCs were used as control. A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 250 ~ 300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent

Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated, the multiplexed library was sequenced on an Illumina Hiseq 2000 platform according to the manufacturer's recommendations (Illumina) at Novogene Bioinformatics Institute, Beijing, China.

Statistical Analysis

All data were presented as mean ± SD. GraphPad Prism software was used to perform statistical analyses of Student's *T*-test where indicated in the figure legends. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and highly significant, respectively. At least three independent experiments were conducted to draw conclusions.

Results

Derivation of iPGCs from Human PSCs

Since hESCs and hiPSCs bear a primed pluripotency (Fig. S1A-B), we explored the potential of hiPSCs and hESCs to differentiate into iPGCs following the procedure showed in Fig. 1A. The morphology of hPSCs cultured in PGC medium (PGC-m) for 10 days became the flattened epithelial cell type with distinct cell-to-cell boundaries, subsequently, a small number of spherical cells and even some follicular-like cell aggregates were mostly formed at the bottom of the culture dish, and few of them floated in the culture supernatant (Fig. 1B, S2A-B). The expression of pluripotent genes *OCT4*, *SOX2*, and *PRDM14* were significantly reduced during the differentiation from day 0 to day 10 (Fig. 1C, S3A). To estimate the synthesis of reproductive hormones during the differentiation, the expression of genes related to estrogen biosynthesis was determined and showed that the expressions of two crucial genes involved in estrogen biosynthesis, *CYP19A1* and *CYP17A1*, and a gene for the follicle-stimulating hormone receptor (*FSHR*) were significantly increased, suggesting that the granulosa-like cells within the differentiated cells produced hormones to stimulate the development of female reproductive physiology. (Fig. 1C, S3A). Additionally, the expression level of c-KIT was increased from 0.45% in hiPSCs and 0.95% in hESCs to 50.6% and 42% in iPGCs after 10 days of differentiation, respectively (Fig. 1D, S3B). The interaction of c-KIT from PGC and SCF from somatic cells is able to modulate PGC adhesion to somatic cells and further contributes to oogenesis [11].

To further confirm iPGCs, Immunofluorescence analysis showed that cell clusters derived from both hiPSCs and hESCs expressed the key PGC transcription factors OCT4, PRDM1, SOX17, and STELLA at day 10 of differentiation (Fig. 2A, S3C). The expression of germline specific genes *PRDM1*, *TFAP2C*, and *SOX17* were significantly elevated versus the early stage of differentiation (day 5), which presented the observation similar to the report in mouse PGCs [12]. The early mesoderm marker *TBXT* (also known as *T* or *Brachyury*) was significantly activated, indicating that cells were turned into the incipient stage of mesoderm-like cells (iMeLCs) and activated to primordial germ cell fate that was reported previously [9,

13, 14] (Fig. 2B). In the late stage of iPGCs (day 10), some cell clusters composed of iPGCs were semi–suspended above the monolayer cells in the bottom of culture dish, those iPGCs could be purified by DDX4 antibody after immunomagenetic isolation. The $DDX4^+$ iPGCs showed the round-oval shape and large nucleus with 10–20 μm diameter (Fig. 2C). We also observed the expression of FRAGILIS, DDX4, and NOBOX after 7–10 days of induction in iFLs (Fig. 2D), which are the essential factors for folliculogenesis and regulation of oocyte development. These findings demonstrated that human iPSCs could be differentiated through iMeLCs into the late stage of PGCs and even early stage of ovarian follicle.

Transcriptome Analysis of iPGCs

To illustrate whether the iPGCs expressed the PGC specific markers, we performed the whole-genome transcriptome sequencing to analysis of the iPSCs (TAC153) and iPGCs (D5). Unsupervised hierarchical clustering of genes showed that each group clustered to its duplicate (Fig. 3A). Principal component analysis of the transcriptome datasets together with the Ovarian Kaleidoscope database (OKdb; http://ovary.stanford.edu), which provides information regarding the biological function, expression pattern, and regulation of genes that are expressed in the ovary, showed that many primordial follicle growth and development associated genes, such as *YAP1*, *SRC*, *BMP4*, *TGFBR1*, and *FOXO3* etc., were extensively upregulated in iPGCs. Alternatively, the mesodermal and stem cell-related genes, such as *KDR*, *FGF2*, *PPP6C*, *STK11*, *LIF*, and *KITLG* etc., were downregulated (Fig. 3B). The heat map data show that there are many differentially expressed genes between the hiPSCs and iPGCs, in which iPGCs have the most consistent downregulation of pluripotent genes, *OCT4, SOX2*, *NANOG*, and *TERT*. Many genes that are involved in differentiation and reproductive processes are extensively upregulated in iPGCs (Fig. 3C), and the differentiated cells had a certain correlation with the cells in the human follicle when compared the whole transcriptome to adult ovarian six main cell clusters [15] (Fig S2C). These results indicated that the hiPSCs exited from the pluripotent state when cultured in PGC-m for 5 days and differentiated into germ cells and started to initiate the folliculogenesis.

Formation and Growth of Induced Follicle-like Structures (iFLs)

When continuing the induction from day 10 to day 15, the iFLs were formed gradually and floated in the culture medium, presenting the morphological structure similar to the follicle with granulosa cells and COC (Fig. 4A, S2A-B). At this stage, many iFLs with granulosa-like cells were released from cell aggregates, and iFLs in different sizes were observed (Fig. S2A-B). Some iFLs were attached at the surface of culture plate and some were floated in the culture medium (Fig. 4A, and video S). The iFL that was transfected by pBMP15-EGFP plasmid showed the green fluorescence (Fig. 4B), indicating that the oocyte-specific BMP15, which shares high homology with GDF9 and plays crucial role in both oocyte maturation and fertilization [3, 10], was activated in the iFL. Western blot analysis further proved that BMP15 expression was activated in iPGCs and iFLs stages, meanwhile, the OCT4 expression was significantly reduced (Fig. 4C).

To determine the existence of granulosa cells around the iFL, we discovered the expression of granulosa cell marker *FOXL2* in 15-day and 25-day iFL samples, but it was absent in control (Fig. 4D). We also performed the qRT-PCR assay to measure the expression of oocyte-specific markers. Results showed that expression of *DAZL*, *FIGLA, and IFITM3* were significantly increased (Fig. 4E, S4A). We then used the BMP15 positive iFLs to perform the immunostaining with anti-FOXL2 and anti-DDX4 antibodies, in which DDX4 is a unique protein in germ cell lineages [16]. These results revealed that a BMP15/DDX4 positive OLC was surrounded with FOXL2 positive cells (Fig. 4F). We also checked the estrogen biosynthesis related genes *CYP19A1*, *CYP17A1*, and *FSHR* with high level expression in iFLs compared to the control from human ovary (Fig. 4G). These results indicated that follicle-like structures derived from iPGCs expressed multiple female germline cell markers and retained the immature OLC.

OLCs Derived from Follicles and in vitro Maturation

In 15 to 20 days of induction, the follicles with large nuclei could be observed. The detached iFLs were harvested and cultured in OLC-m for 10 days. OLCs ranging from 50 to 150 μm coating zona pellucida (ZP) were detached from iFLs and surrounded by granulosa-like cells (Fig. 5A). Though the thin ZP was fragile and extremely difficult to be manipulated with a micropipette, an OLC was able to be picked up by a holding pipette (Fig. 5B). ZP2, ZP3, and BMP15 expressions in OLCs were confirmed by western blotting (Fig. 5C, S3E). We then detected the expression of SCP3 a meiosis marker as well as initiation of ZP glycoprotein 2 synthesis in OLCs, and confirmed that SCP3 as well as ZP2 was co-localized in OLCs, but not in the surrounded granulosa-like cells (Fig. 5D). And the SCP3 protein and DDX4 were also detectable in BMP15 positive iFLs derived from hESCs (Fig. S3D). Additionally, to further determine the connection between granulosa cells and OLCs, the CX37 in OLCs was low expression and showed the dot form distribution in the surface of cell membrane compare to human oocytes (Fig. 5E). Which mediates the formation of gap junction communication between oocyte and granulosa cells and is mainly expressed at the membrane of oocyte [17].

In 25-day post-induction, germinal vesicle (GV) stage OLCs with a morphologically-normal human oocytes appearance were stripped off cumulus cells (Fig. 6A). The DAPI staining indicated a distinct nucleus in OLC (Fig. 6B, S3F) and the first polar body was observed in the individual OLC (Fig. 6C). Further, the meiosis specific marker, SCP3 showed highly expression in OLCs (Fig. 6D). These results indicated that OLCs reached and arrested at metaphase II (MII) stage.

Production of Parthenogenetic embryo-like structures

To continue culturing OLCs up to 35 days in OLC-m, some OLCs developed spontaneously into cleavage stage-like embryos structures similar to preimplantation. The nuclear staining showed that two- and multiple nuclei were detected in embryo-like structures (Fig. 7A, S3G), indicating that OLCs were parthenogenetically activated, although these embryos was fragile with a thin ZP membrane (Fig. 7B). To elucidate the embryo-like structures, we performed IF analysis and showed that DDX4 and c-KIT were expressed in the parthenogenetically activated embryos (Fig. 7C, S3G), suggesting that DDX4 and c-KIT were involved in the progression of embryogenesis during differentiation, and parthenogenetic embryo-

like structures had the development potential. The effectively modulates the parthenogenetic activation of OLCs and optimizes the culture condition for maintaining MII OLCs may help to reveal OLCs development ability *in vitro*.

Discussion

We have shown that a novel approach of three-step induction of hPSCs into iPGCs and OLCs in a monolayer culture. The previous reports showed that the induction of iPGCs was initiated following EBs formation, ESCs enter the spontaneous differentiation and further form iFLs and OLCs by adding cytokines and exogenous proteins [18, 19]. Here, we found that hPSCs in the monolayer culture were directly stimulated by BMP4, SCF, LIF, and EGF, and encouraged iPGCs formation, and then stimulated by bovine follicle fluid to form aggregates and iFLs. Under treatments of ITS and gonadotropins, the percentage of postmeiotic OLCs extruded from iFLs was increased and arrested at MII stage. Although OLCs could be observed through the formation of aggregates after incubated with retinoic acid (RA) in the medium [20], when follicle fluid was replaced with RA, the efficiency of OLC production was very low [5]. Compared with previous methods that OLCs derived from mouse ESCs in the monolayer culture without cytokines [21] and OLCs derived from porcine stem cells in treatments with porcine follicular fluid, cytokines, and gonadotropin for 30–50 days [22], we found that OLCs generated in our method could be matured in a shorter term and higher efficiency without transgenes.

SOX17 is a key critical marker in human PGC, but not in mouse PGC [9, 23], human iPGCs robustly induced from ESCs, in particular through iMeLCs, expressed the high level of *SOX17*, *PRDM1*, *TFAP2C*, and *TBXT*, and the SOX17 and PRDM1 positive cells were gradually formed clusters. Those were further confirmed in RNA-Seq data, which indicated this differentiation approach used for hPSC could confer competence for germline fate. However, late germ cell markers, including *DAZL*, *VASA*, and *MAEL* were not detected in iPGCs after 5 days induction. Another interesting finding is that the gene *GATA6* and *KITLG* showed ovarian somatic cell contained distinct sub-populations [24].

We further tested if the differentiated cells could through formation of aggregates and generation of iFLs, the iFLs retained OLCs were in the middle of an aggregate and surrounded by granular-like cells. By further investigation of the existence and function of granulosa cells, we confirmed the expressions of *DDX4*, *FRAGILIS*, *NOBOX*, *FOXL2*, *CX37*, and other genes related with female reproductive formation, indicating that mixed cells in the aggregates had the ability to self-organize into an ordered biological entity [25]. Additionally, overexpression of DAZL and BOULE with cytokines GDF9 and BMP15 could induce to form human ovarian iFLs [3, 17]. To evaluate OLC production, the expression of ZP2 and ZP3 were significantly increased during generation of OLCs in later stage of induction. ZPs are membrane glycoproteins that surround the oocyte and mediate species-specific sperm binding. ZP2 acts as a secondary sperm receptor, and ZP3 is essential for sperm binding and zona matrix formation [26]. And we also detected *P450*, *CYP17*, and *FSHR* expressions during the induction, which are essential for estrogen production and OLC development. These observations showed that a specification microenvironment for germ cells development was created by using the three-step induction procedure.

We found that bFF in media of PGC-m and FLC-m significantly promoted the germ cells generation. follicular fluid contains numerous cytokines and hormones, including GDF9 [27], bFGF [28], BMP15 [29], insulin-like growth factor [30], and gonadotropins [27]. These factors are secreted from oocyte or somatic cells, and could effectively promote the formation of iFLs and maturation of oocytes [6, 10].

Differentiation of mouse ESCs into fully potent mature oocytes was reported recently [7], which required aggregation of gonadal somatic cells from mouse fetal ovaries. However, the similar strategy is difficult to implement for hESCs. Our findings indicated that hPSCs-derived OLCs were morphologically similar to human oocytes, although the OLC development was not synchronized, as shown by different sizes of OLCs (50–150 μm in diameter). It might be caused by proliferation and maturation of granulosa cell layer, which was limited in the late stage of differentiation and OLC development. In order to improve the efficiency of OLCs induction, we found that the master plate, in which the first run OLCs had been picked up, could be maintained in OLC-m and continued growth for another two weeks. During this period, OLCs could frequently be released from iFLs, and the quality of OLCs was improved due to having the thickness of ZP. Some OLCs with normal ZP were operated by micropipette though many OLCs with thin and fragile ZP were extremely difficult to be handled by micromanipulator. Additionally, it is possible to improve the efficiency of differentiation by using advanced 3D culture systems [31].

Conclusions

We conclude that OLCs could reach the MII stage of post-induction, but the efficiency of MII OLCs derivation was very low. The fertilization potential of OLCs comparing with the normal eggs from *in vivo* is still an important issue that remains to be answered. Overall, our three-step induction procedure invents human OLCs derived from hPSCs, which can be used to explore human folliculogenesis and oogenesis *in vitro*.

Abbreviations

OLCs: Oocyte-like cells

hiPSCs: Human induced pluripotent stem cells

hESCs: Human embryonic stem cells

PGCs: Primordial germ cells

iPGCs: Induced primordial germ cells

hPSCs: Human pluripotent stem cells

PGC-m: Primordial germ cell medium

FLC-m: Follicle like cell medium

KSR: Knockout serum replacement

bFF: Bovine follicular fluid

NEAA: Nonessential amino acids

b-ME: b-Mercaptoethanol

BSA: Bovine serum albumin

FSH: Follicle-stimulating hormone

hCG: Human chorionic gonadotropin

PMSG: Pregnant mare serum gonadotropin

EGF: Epidermal growth factor

ITS: Insulin-transferrin-selenium

cDNA: Complementary DNA

qRT-PCR: Quantitative reverse transcription PCR

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

qPCR: Quantitative polymerase chain reaction

DPBS: Dulbecco's phosphate-buffered saline

iFLs: Induced follicle-like structures

COC: Cumulus-oocyte-complexes

MII: Metaphase II

PGCLCs: Primordial germ cell-like cells

FLs: Follicle-like structures

FSHR: Follicle-stimulating hormone receptor

iMeLCs: Mesoderm-like cells

ZP: Zona pellucida

GV: Germinal vesicle

RA: Retinoic acid

Declarations

Availability of data and materials

Data supporting the current study are available from the corresponding author upon reasonable request.

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Author's contributions

Xiao-Li Yu and Ning Wang contributed equally to this work. They designed experiments, performed experiments, analyzed data, and wrote the manuscript. Ying-Xin Zhang, Xiang Wang, Yi-Kai Qiu, Hong-Yan Wang, Xiu-Ying Pei, Ya-Yui Ren, Ping Chen, and Li Wang carried out the experiments and analysis of data. Guo-Ping Wang and Chun-Fang Ha provided human biological samples. Xiao-Li Yu and Hua-Yan Wang, conception and design, financial support, data analysis and interpretation, and final approval of manuscript.

Ethics declarations

Ethics approval and consent to participate

The protocols of handling human and animal specimens were approved by the Ethical Review Board in General Hospital of Ningxia Medical University and the Ethics Committee of Ningxia Medical University (approval number 2015056).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

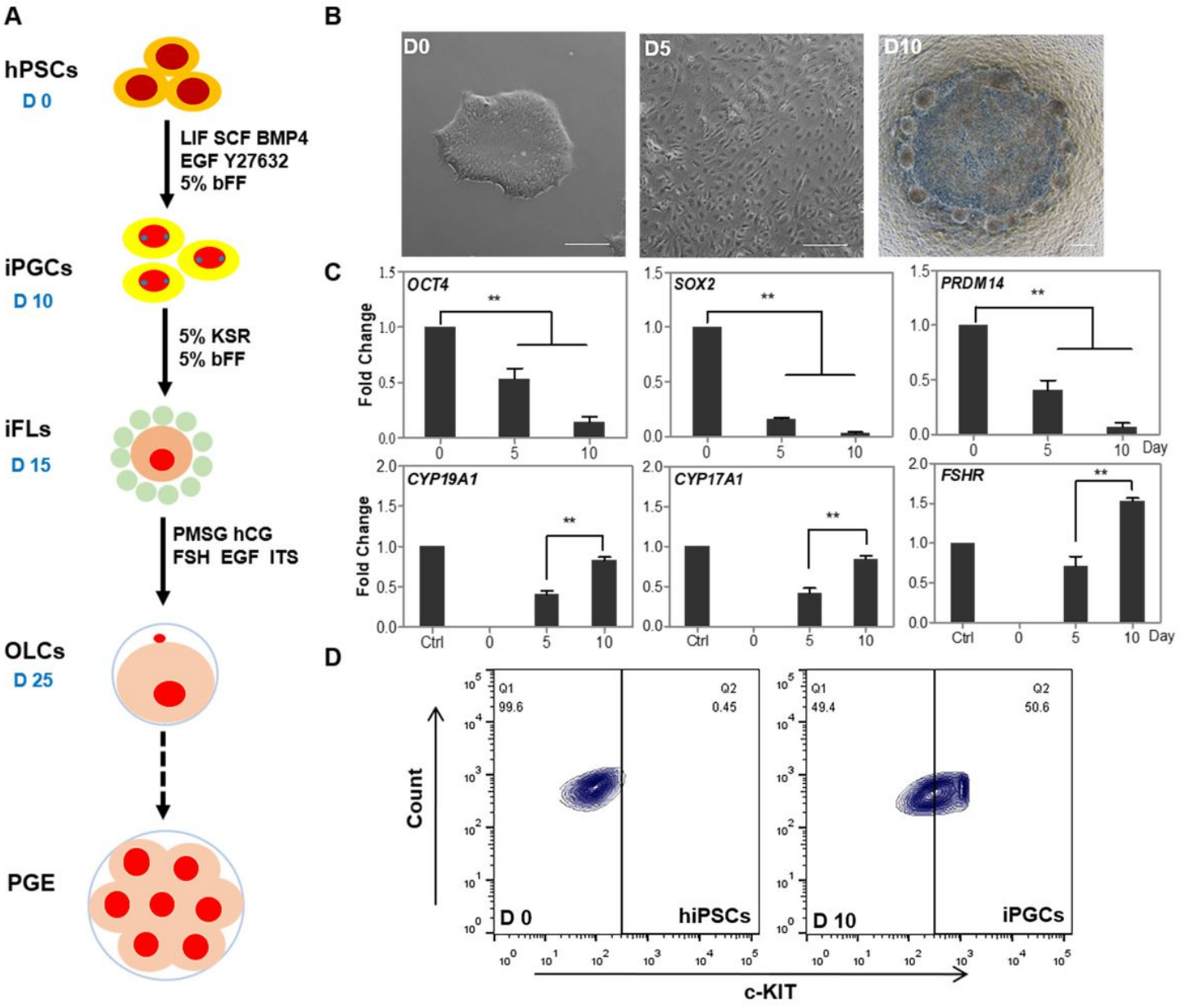

Figure 1

Differentiation of hiPSCs into early stage of iPGCs. (A) Schematic diagram of OLCs derived from hPSCs. (B) Morphology of early stage of iPGCs derived from hiPSCs at day 10 (D10). Scale bar, 200 μm. (C) qRT-PCR analysis of pluripotent genes (OCT4, SOX2, and PRDM14) and hormone related genes (CYP19A1, CYP17A1, and FSHR) in iPGCs. Ctrl, human ovary tissue was used as positive control. (D) Flow cytometry analysis of c-KIT expression in hiPSCs and early stage of iPGCs at day 10. Data indicate mean ± SD. **P <0.01, n=3.

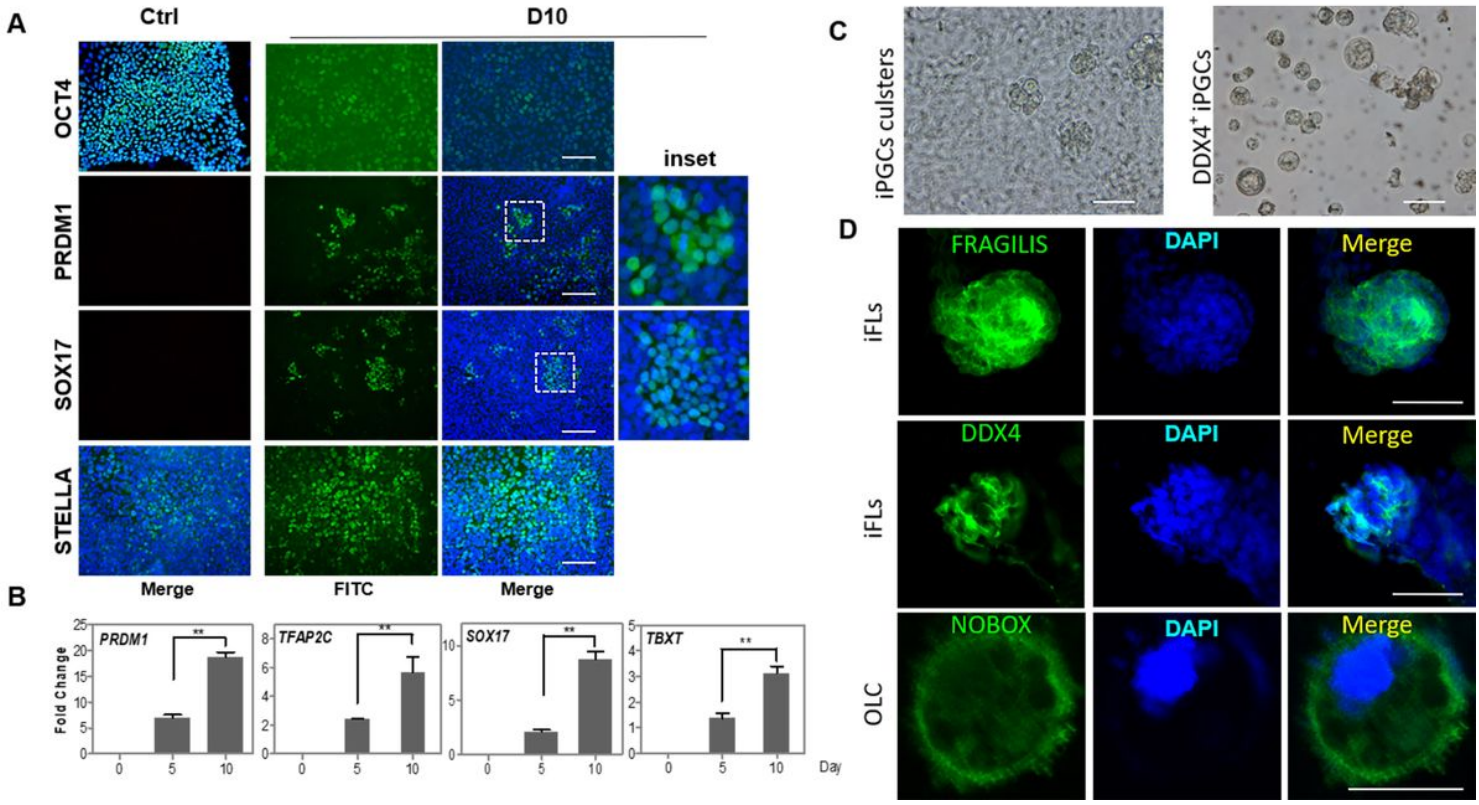

Figure 2

Characterization of iPGCs. (A) Immunocytochemistry of OCT4, PRDM1, SOX17, and STELLA expressions in iPGCs at day 10 (D10) of post induction. Ctrl, hiPSCs were used as control. Scale bar, 50 μm. (B) qRT-PCR analysis of PGC-related genes in iPGCs. (C) iPGCs clusters were semi-suspended above the monolayer cell at day 10 of post induction. The iPGCs were successfully purified by DDX4 antibody using immunomagenetic isolation. Scale bar, 50 μm. (D) Immunocytochemistry of FRAGILIS, DDX4, and NOBOX in iFLs (Scale bar, 100 μm) and OLC (Scale bar, 50 μm) at day 8 of post induction.

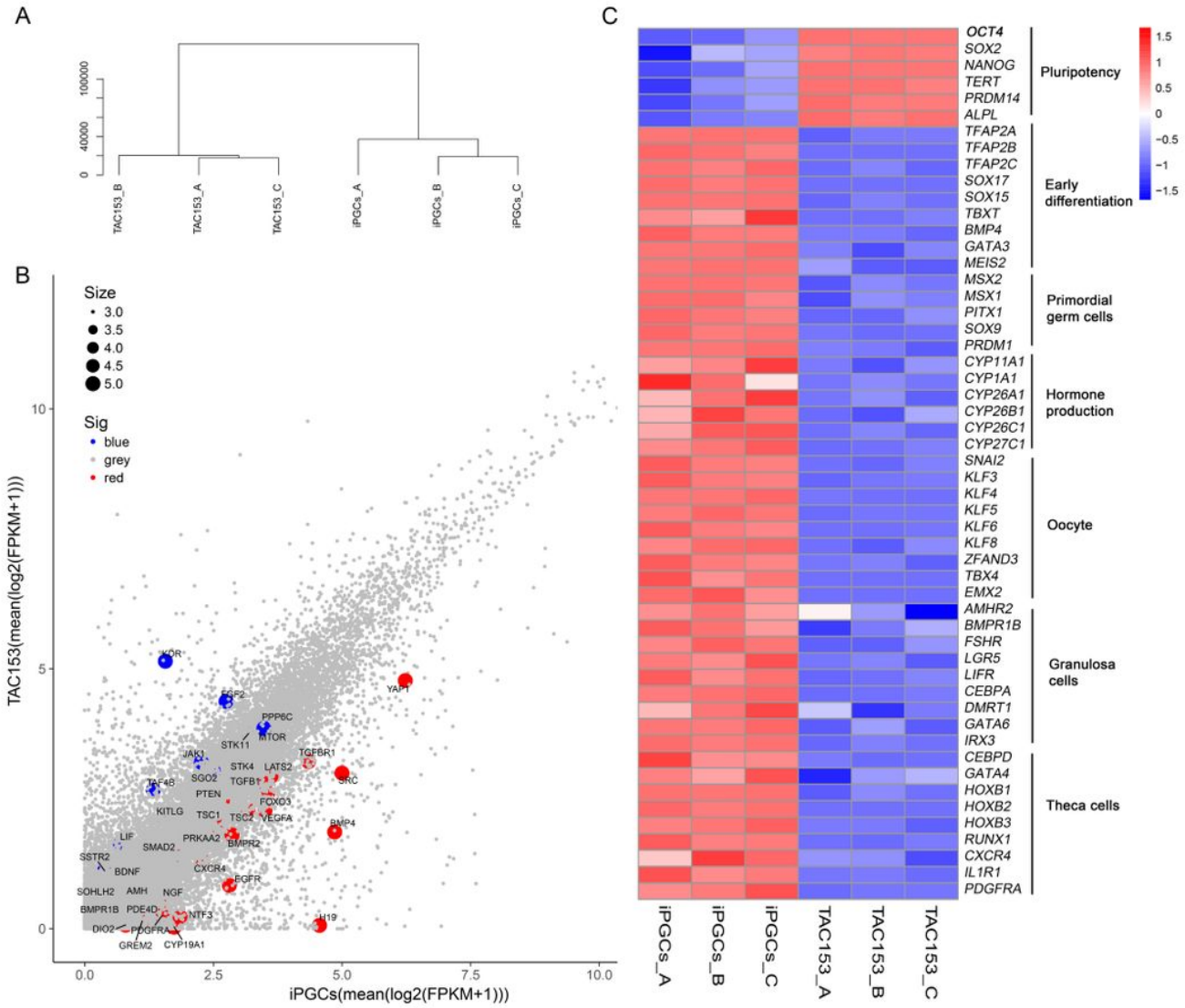

Figure 3

Transcriptome sequencing of iPGCs. (A) Unsupervised hierarchical clustering of gene expression in hiPSCs (TAC153) and iPGCs (D5). Independent biological duplicates of each group were subjected to whole-genome RNA sequencing. (B) Scatter plot analysis of transcripts between iPSCs and iPGCs (D5). Red dots show that genes enriched in reproductive cells (oocytes and granulosa cells) are highly expressed in iPGCs. Blue dots show genes that are enriched in iPSCs. (C) Heat map of gene expression of iPSCs and iPGCs (D5) shows the pluripotent stem cell and germ cell-specific differentially expressed

transcripts. Red and blue indicate higher and lower levels of expression respectively. Hierarchical clustering was performed on log2 signal intensity data. These values were resized to Row Z-Score scale for any single genes (from - 1.5, the lowest expression to + 1.5, the highest expression).

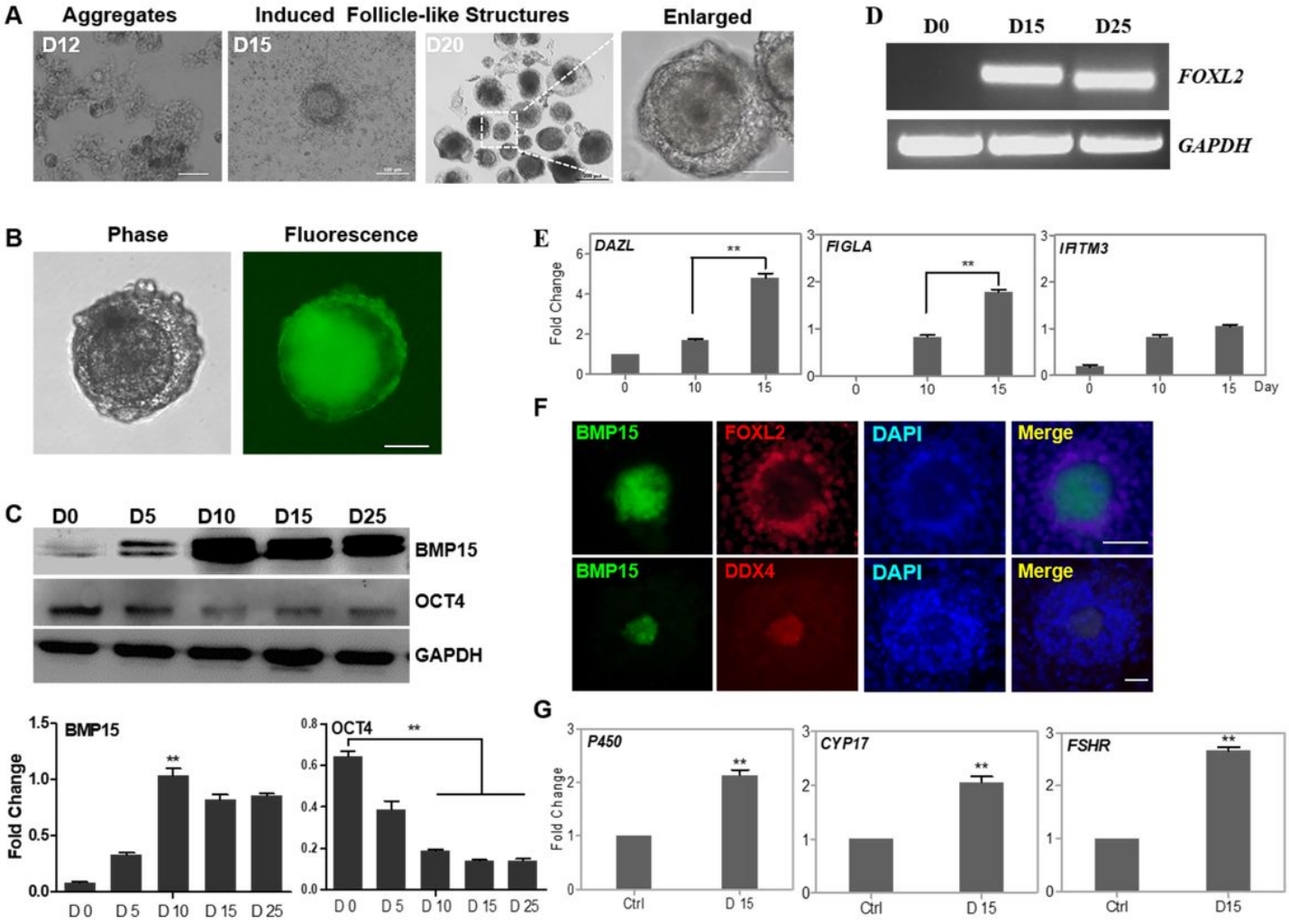

Figure 4

Characterization of iFLs. (A) Cell aggregates and iFLs at 12 to 20 days post-induction. (B) Expression of BMP15-EGFP fusion protein in iFLs that were transiently transfected by the pBMP15-EGFP vector. (C) Western blotting (upper) and densitometry analysis (lower) of BMP15 and OCT4 expressions in iFLs. GAPDH was an internal control. (D) RT-PCR analysis of FOXL2 expression in iFLs. GAPDH was an internal control. (E) qRT-PCR analysis of oocyte-specific markers. (F) Immunocytochemistry analysis of FOXL2 and DDX4 expressions in iFLs that were transfected by pBMP15-EGFP vector. (G) qRT-PCR analysis of hormone related genes in iFLs at 15 days post-induction. Ctrl, human ovarian tissue as positive control. Scale bar, 50 μm for A and F; 100 μm for B. Data indicate mean ± SD. **P <0.01, n=3.

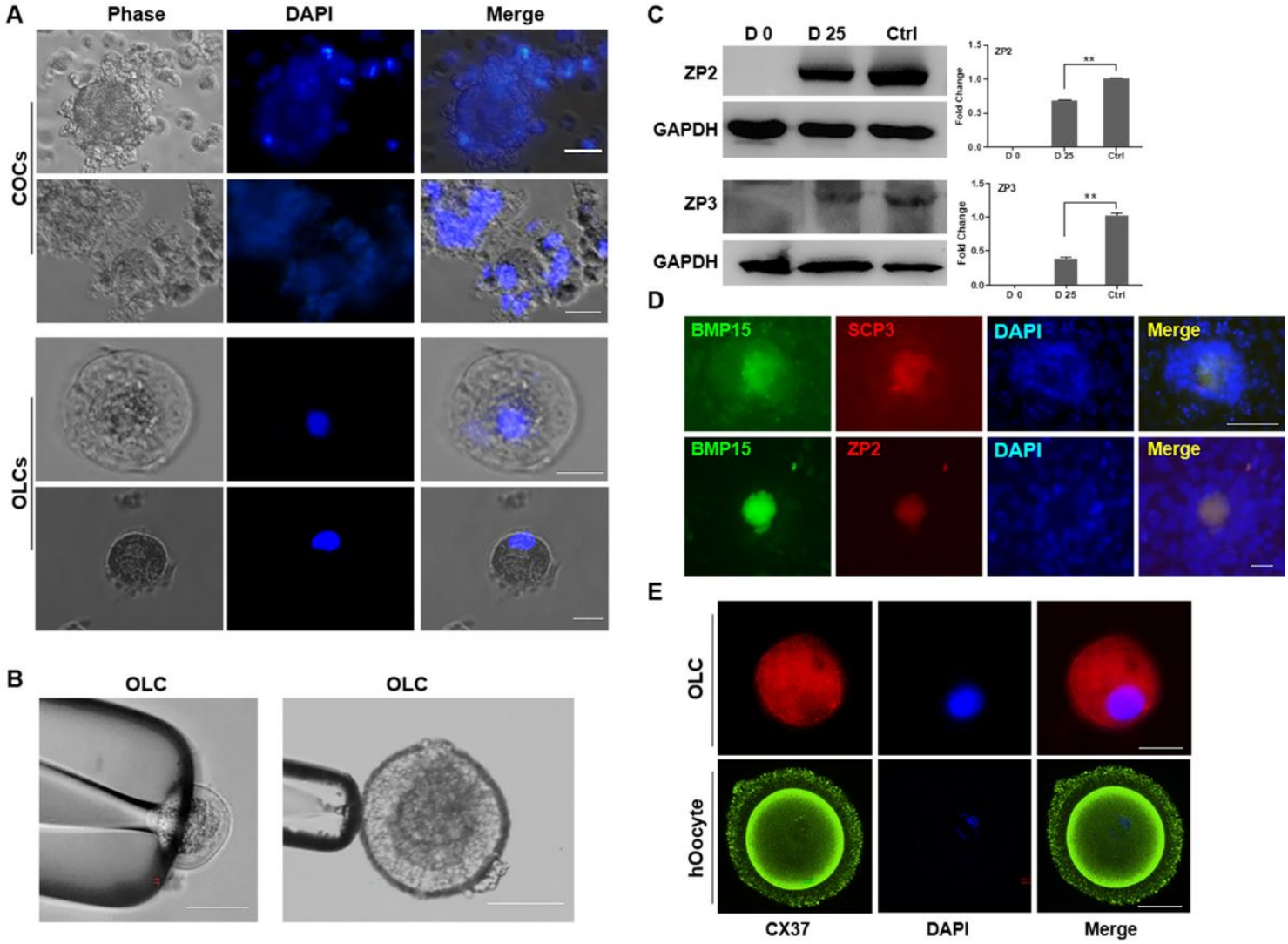

Figure 5

OLCs derived from iFLs. (A) COCs and OLCs derived from iFLs. Nuclei were stained by DAPI. (B) OLCs were held by the micromanipulator glass holding pipette. Scale bar, 50 μm (left) and 100 μm (right). (C) Western blotting (left) and densitometry analysis (right) of ZP2 and ZP3 expression in OLCs. GAPDH was an internal control. (D) Immunocytochemistry analysis of SCP3 and ZP2 in OLCs that were transiently transfected by pBMP15-EGFP vector. (E) Immunocytochemistry analysis of CX37 expression in OLCs. Human oocyte (hOocyte) was used as positive control. Scale bar, 50 μm for A and D; 100 μm for E.

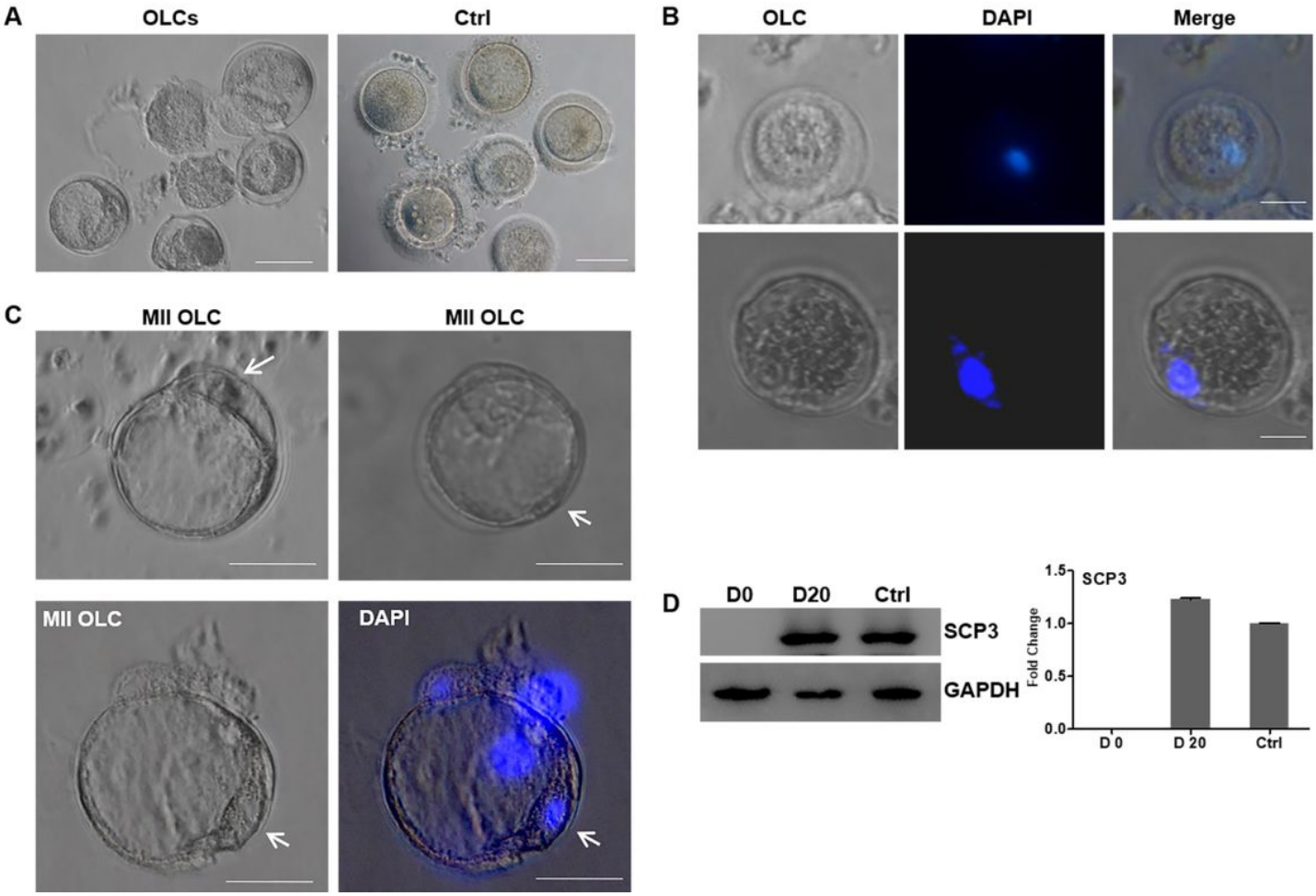

Figure 6

OLCs maturation in vitro. (A) The in vitro matured OLCs. Ctrl, human oocytes as positive control. (B) OLCs were stained by DAPI. (C) OLCs cultured in OLC-m were arrested at MII stage. Arrows indicate the first polar body. Nuclei were stained by DAPI. (D) Western blotting (left) and densitometry analysis (right) of SCP3 expression in OLCs. Ctrl, human ovary tissue as the positive control. GAPDH was as internal control. Scale bar, 50 μm for B; 100 μm for A and C.

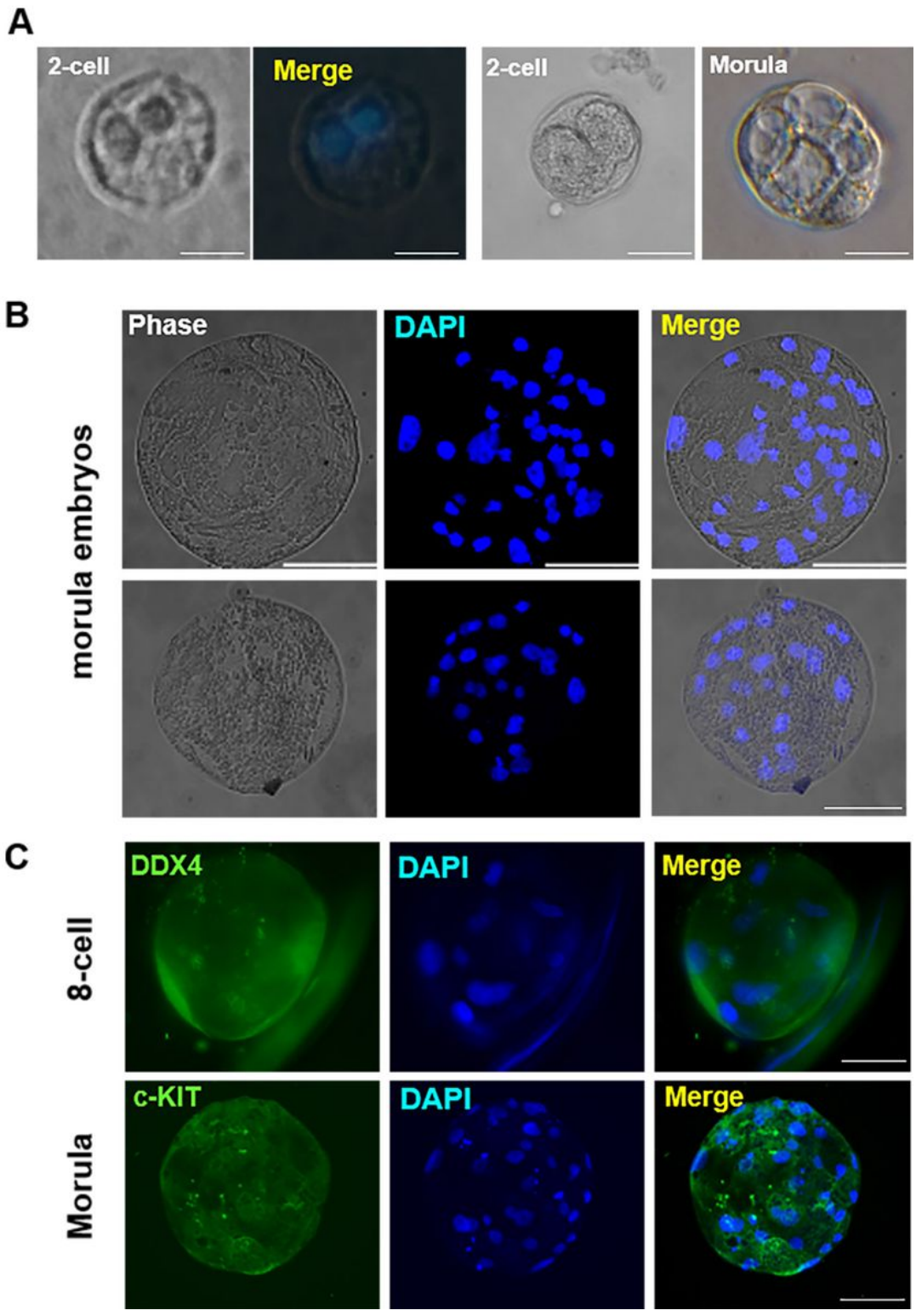

Figure 7

Parthenogenetic development of embryo-like structures in vitro. Over 30 days induction, OLCs arrested at MII stage in OLC-m were able to further develop due to the spontaneous parthenogenetic activation. (A) The 2-cell and early stage of morula embryos. (B) The late stage morula embryos. (C) Immunocytochemistry analysis of DDX4 and c-KIT in embryo-like structures that were developed into 8-cell (DDX4) and morula (c-KIT) stages. Nuclei were stained by DAPI. Scale bar, 50 μm.

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