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Characterization of Female Germ-Like Cells Derived From Mouse Embryonic Stem Cells Through Expression of GFP Under the Control of *Figla* Promoter

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ABSTRACT

Previous studies have demonstrated that germ cells can be derived from mouse embryonic stem cells (ESCs). However, there is still no efficient system, which can visualize the stage of germ cell specification in vitro, and further to identify and enrich germ cells derived from ESCs. Figla (factor in the germline, alpha) gene encodes a germ cell specific transcription factor that coordinates the expression of the oocyte-specific zona pellucida (Zp) genes and is essential for folliculogenesis in mouse. Here, we first constructed a pFigla-EGFP recombinant plasmid that expressed enhanced green fluorescent protein (EGFP) under the control of Figla promoter, and generated and characterized an ESC line stably carrying this pFigla-EGFP reporter construct. Then the ESCs were induced to differentiate into female germ-like cells by culturing adherent embryoid bodies (EBs) in retinoic acid (RA) induction medium or transplanting ESCs under the kidney capsule with ovarian cells. A population of differentiated ESCs expressed GFP, and these cells were analyzed by RT-PCR and immunofluorescence. The GFP positive cells showed the expression of germ cell markers Vasa, meiotic specific gene Stra8, Scp3, oocyte markers Gdf9, Zp3 and Figla, indicating that this method could be used for the purification and selection of female germ cells. Our study establishes a new selective system of female germ-like cell derivation and offers an approach for further research on the development and the differentiation of germ cells derived from stem cells. J. Cell. Biochem. 113: 1111–1121, 2012.

KEY WORDS: EMBRYONIC STEM CELLS; GERM CELLS; RETINOIC ACID (RA); FIGLA; MOUSE

Germ cells are highly specialized cell populations that are indispensable for the continuation and evolution of the species. Human germ cell development has been difficult to study because the important early events occur after implantation in womb [Nagano, 2007]. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst, which could differentiate into almost all cell types in the body, and thus potentially provide unlimited cells for organ and cell replacement therapies [Thomson et al., 1998]. Recently, reports have shown that murine and human ESCs, induced pluripotent stem cells (iPSCs) can differentiate into primordial germ cells (PGCs) and subsequently develop into early gametes (oocytes and sperms) that further fertilize to form blastocysts [Hübner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Lacham-Kaplan et al., 2006; Hua and Sidhu, 2008, 2011; Panula et al., 2011]. Derivation of oocytes from stem cells will be a potential resource for therapeutic cloning. Thus, we may circumvent the ethical issues pertaining to the use of discarded embryos. However, derivation of female germ cells (oocytes) was rare and preliminary update [Hua and Sidhu, 2008, 2011]. The core problem is that we do not know the detailed information on the female germ cell differentiation process from ESCs [Zwaka and Thomson, 2005; Zheng and Dean, 2007; Roig et al., 2011].

Additional Supporting Information may be found in the online version of this article.

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An oocyte-specific gene, factor in the germline alpha (Figla or FIGα), a basic helix-loop-helix (bHLH) transcription factor, plays a crucial role in the formation of primordial follicles and the expression of multiple oocyte-specific genes, including those that initiate folliculogenesis and those that encode the zona pellucida (Zp) required for fertilization and early embryonic survival [Soyal et al., 2000; Huntriss et al., 2002; Joshi et al., 2007]. Female mice lacking Figla are unable to form primordial follicles which results in massive depletion of oocytes and sterility. The expression of Figla was limited to oocytes and required for ovarian follicle formation within the ovary [Liang et al., 1997]. Figla transcription factor regulates expression of the zona pellucida (Zp) proteins Zp1, Zp2, and Zp3 through an E-box motif (CANNTG). Figla knockout mice cannot form primordial follicles and lose oocytes rapidly after birth, whereas male gonads are unaffected [Liang et al., 1997; Choi and Rajkovic, 2006]. In human, premature ovarian failure (POF) harbors mutations in Figla [Pangas and Rajkovic, 2006; Suzumori et al., 2007]. Figla plays critical roles in female germline and follicle development [Zheng and Dean, 2007].

In this study, we established an ESC line stably carrying a pFigla-EGFP reporter construct and investigated their differentiation potentiality by RA addition or kidney capsule transplantation with ovarian cells. Our study establishes an efficient selective system of female germ-like cell derivation and offers an approach for further studying on the development and the differentiation of germ cells.

MATERIALS AND METHODS

GENERATION OF THE pFIGLA-EGFP CONSTRUCT

The promoter region for the Figla gene was analyzed using the Promoter On-line Analysis Database (Promoter 2.0, Promoter ScanII, BDGP). A 925 bp promoter fragments of the *Figla* gene was amplified using primers incorporating restriction sites for *Sal*I (forward primer: 5′-CGGTCGACCCCCATCTAGCCTCCACACG-3′) and *Bam*HI (reverse primer: 5′-GCGGATCCGTCAAGAC-CACGGGCAGCAG-3′). The resulting products of polymerase chain reaction (PCR) from were purified using the Qiagen gel purification kit (Qiagen) according to manufacturer's instructions and cloned into the pMD-18T vector (TaKaRa, Biotech. Co. Ltd), which was confirmed by PCR using the same primer sets detailed above. Then the purified Figla promoter fragment was constructed into pEGFP-1 vector which was digested by *Sal*I/*Bam*HI. The constructed vector pFigla-EGFP plasmid was identified by digestion with *Sal*I/*Bam*HI restriction enzymes and sequencing.

CULTURE OF MOUSE ESCs AND TRANSFECTIONS WITH pFIGLA-EGFP

Mouse ESCs (J1) were cultured on mitomycin C (Sigma, St. Louis, MO)-treated mouse embryonic fibroblasts (MEFs) in 0.1% gelatin-coated tissue culture plates in H-DMEM (Invitrogen, Carlsbad, CA), supplemented with 15% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 100 mg/ml penicillin/streptomycin and 1,000 U/ml leukemia inhibitory factor (LIF; Millipore).

Subcultured mESCs were inoculated into a 6-well plate and transfected with recombinant plasmid pFigla-pEGFP using TurboFect^{TM} Transfection Reagent (Fermentas). The DNA was diluted in water according to the manufacturer's instructions. One milliliters of serum-free Opti-MEM (Gibco) was added to sterile tubes and mixed with 6 μg DNA. Then, 12 μl TurboFect^{TM} was added and the tube was incubated for 15–20 min at room temperature. Tissue culture plates containing mESCs at a confluence of 60% were washed with fresh medium, and the Opti-MEM/TurboFect^{TM}/DNA mix was carefully added drop-wise and the plates incubated for 4–5 h. After 48 h of transfection, the cells were harvested and subcultured, then selected the stable transformants based on the expression of the bacterial neomycin phosphotransferase gene (Neo). Colonies resistant to G418 (200 μg/ml) were selected after 2 weeks.

Additionally, the functionality of constructs was tested by F9, PT67, MEF and mouse primary ovarian cells through transfection. These cells were cultured in H-DMEM (Invitrogen), supplemented with 10% or 15% FBS (Hyclone), 2 mM L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 100 mg/ml penicillin/streptomycin.

DIFFERENTIATION OF pFIGLA-EGFP EMBRYONIC STEM CELL LINES

ESCs carrying pFigla-EGFP were suspended and plated in a culture plate (Nunc) pretreated with 0.2% gelatin, which allows rapid MEF adherence to prepare cell suspension MEF-free. After 15 min, non-adherent cells were collected and plated on the Petri dishes with 3×10^5 cells per 3.5 cm dish. After 3 days of culture, the cells aggregated and formed "embryoid bodies" (EBs). The resulting EBs which diameters approximated 100 μm were transferred in a 48-well culture plate (10–15 EBs per well) supplemented with 0.1 μM retinoic acid (RA; Sigma) [Hu et al., 2010b] differentiation medium, which was changed every 2 days to avoid its degradation. Seven days later, the EBs and suspension cells were harvested and analyzed.

OVARIAN TISSUE AGGREGATION AND KIDNEY CAPSULE TRANSPLANTATION

Adult mouse ovaries were collected and minced after the removal of adherent tissues. The specimens were digested with 1 mg/ml collagenase at 37°C for 15 min with shaking at intervals. After centrifuged at 1,500 rpm for 5 min, the specimens were incubated in a dissociation solution that consisted of a mixture of 0.2% (v/w) trypsin (Invitrogen) and 1.4 mg/ml DNase (Invitrogen) for 10 min at 37°C. The ovarian cell suspension from 1 female mouse ovary per graft was then mixed with 1×10^7 ESCs. Cell suspensions were pelleted into grafts and transplanted under the left kidney capsules of busulfan-treated female Kunming mice [Nicholas et al., 2009].

AP STAINING

To detect alkaline phosphatase (AP) activity, the cells cultured under the normal conditions were fixed with 4% paraformaldehyde (PFA) for 10–15 min at room temperature. Fixed cells were washed three times with PBS and stained with naphthol AS-MX phosphate (200 μg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM Tris buffer, pH 8.2–8.4, for 10–30 min at room temperature, and washed with PBS to terminate staining [Zhang et al., 2011].

TERATOMA FORMATION

ESCs carrying pFigla-EGFP were cultured in ESC medium consecutively for at least 24 days and dispersed into single cells by incubation in 0.05% trypsin for 2 min at 37°C. Figla-EGFP mESCs (2×10^6) were transplanted into the kidney capsules of busulfan-treated female Kunming mice [Nicholas et al., 2009]. Tumor formation was identified by manually palpating the injected area since 20 days after injection. After 35 days of growth, tumor tissue was removed, fixed in 4% PFA, and processed for paraffin sectioning.

RT-PCR

Total RNA was extracted with Trizol reagent (TaKaRa, Biotech. Co. Ltd) from ESCs or treated EBs. Single strand cDNAs were prepared from 0.5 μg RNA using a reverse transcription Kit (TaKaRa, Biotech. Co. Ltd) and specific gene expressions were analyzed. The RT-PCR primers used are described in Table I, which are markers of germ cells or oocytes [Hübner et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Dyce et al., 2006; Nayernia et al., 2006ab; Hua and Sidhu, 2008, 2011; Hua et al., 2009]. PCR conditions were: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, the annealing temperature used was in accordance to the primer sequence for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min. The PCR primers and the length of the amplified products are shown in Table I. The PCR products were analyzed in 2% agarose (Invitrogen) gel electrophoresis, stained with ethidium bromide (Invitrogen), and visualized under UV illumination.

TABLE I. The Primer Sequences and PCR Reaction Conditions

Gene	Primer (5′ → 3′)	T_m/°C	Size/bp
β-actin	Forward: GCGGCATCCACGAAACTAC Reverse: TGATCTCCTTCTGCATCCTGTC	58	138
Figla	Forward: CTCTGCTGCCCGTGGTCTT Reverse: CTGCTCTGTGGTAGAAACGGC	58	304
Oct4	Forward: CGCCCGCATACGAGTTCT Reverse: GCACCAGGGTCTCCGATTT	58	487
C-myc	Forward: CTGGTGGGCGAGATCATCA Reverse: CACTGCCATGAATGATGTTCC	54	304
Sox2	Forward: GCCCAGGAGAACCCCAAGAT Reverse: GGGTGCCCTGCTGCGAGTA	54	520
Nanog	Forward: GATTCTTCTACCAGTCCCAAAC Reverse: ATGCGTTCACCAGATAGCC	54	376
Klf4	Forward: CCAGGAGAACCCCAAGATGC Reverse: GGGTGCCCTGCTGCGAGTA	58	518
B-III-tubulin	Forward: CTTTTGGCCAGATCTTTAGACC Reverse: CTCGTTGTCAATGCAATAGGTC	58	377
Brachury	Forward: AAGGTGGCTGTTGGGTAGGGAGT Reverse: ATTGGGCGAGTCTGGGTGGATGT	58	451
Pdx1	Forward: AACGCAGGAACCACGATGAGAGG Reverse: AAGGGGTCGCCCGAGTAAGAATG	67	483
Stra8	Forward: AGCAGCTTAGAGGAGGTCAAGA Reverse: TACTCGGAACCTCACTTTTGTC	58	111
Scp3	Forward: CTAGAATTGTTCAGAGCCAGAG Reverse: GTTCAAGTTCTTTCTTCAAAG	58	247
Zp3	Forward: GAGCTTTTCGGCATTTCAAG Reverse: AGCTTATCGGGGATCTGGTT	58	150
Gdf9	Forward: TAGTCCACCCACACACCTGA Reverse: CCAGAAGCCTGAGAACCAAG	62	197
GFP	Forward: GACGGGAACTACAAGACACG Reverse: CGAAAGGGCAGATTGTGTGG	54	349
Vasa	Forward: AAGCAGAGGGTTTTC CAAGC Reverse: GCCTGATGCTTCTGAATCG	57	61

QRT-PCR

The QRT-PCR reactions were set up in 25 μl reaction mixtures containing 12.5 μl 1× SYBR@ PremixExTaqTM (TaKaRa, Biotech. Co. Ltd), 0.5 μl sense primer, 0.5 μl antisense primer, 11 μl distilled water, and 0.5 μl template. The reaction conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 58°C for 30 s. All expression levels were normalized to β-actin in each well. Expression was quantified as the ratio of the mRNA levels obtained from undifferentiated mESCs [Cao et al., 2011].

IMMUNOFLUORESCENCE ANALYSIS

Samples of cells were fixed in 4% PFA, treated with 0.1% Triton X-100 for 10 min at room temperature. After blocking with 10% FBS for 30 min, the cells were incubated with primary antibodies against Vasa (1:200, Abcam), Figla (1:100, Santa Cruz Biotechnology, Inc., CA), Stra8 (1:200, Abcam), Scp3 (1:300, Santa Cruz Biotechnology, Inc., CA) and Zp3 (1:100, Santa Cruz Biotechnology, Inc., CA), β-III-tubulin (1:500, Santa Cruz Biotechnology, Inc., CA), Nestin (1:200, Chemicon), AFP (1:500, Chemicon), Pdx1 (1:500, Chemicon), Glut2 (1: 200, Chemicon), α-actin (1:500, Sigma) and CT3 (1:1,000, DSHB) respectively for overnight at 4°C. After washing three times in PBS, appropriate secondary antibodies were incubated for 1 h at room temperature in the dark. Untreated ESCs and ovarian cells were used as the negative control and the positive control, respectively. Images were captured with Leica fluorescent microscope.

FLUORESCENCE-ACTIVATED CELL SORTING OF FIGLA-GFP POSITIVE CELLS

Cells were harvested using 0.05% (v/w) trypsin with physical disaggregation of the monolayer by periodic pipetting over 5 min to give a single cell suspension. Cells were collected by centrifugation (1,500 rpm for 3 min), washed with phosphate-buffered saline (PBS), and then resuspended in PBS for FACS. Sorting was performed using a Becton Dickinson FACS aria (BD Biosciences, San Diego).

ESTRADIOL MEASUREMENTS

The medium was pooled from culture dishes and stored at −80°C for hormone analysis. Estradiol (E2) was assayed using a commercial radioimmunoassay (RIA) kits [Wen et al., 2010], which were carried out in Yangling Demonstration Zone Hospital, based on the manufacture's protocol.

RESULTS

IDENTIFICATION OF FIGLA PROMOTER REGION

RT-PCR analysis confirmed the primary ovarian cells, the tissues of ovary and testis were positive for Figla, while ES, F9, PT67, MEF cells and the tissues of skin, heart, liver, spleen, lung, kidney, and brain were negative (Fig. 1A). Furthermore, immunofluorescent analysis demonstrated that Figla is restrictedly expressed in Leydig's cell of adult mouse testis, but not expressed in spermatogonial cells (Fig. 1B). However, Figla is expressed in ovarian follicular cells of 2-week and 2-month mouse (Fig. 1C,D). This indicated that the Figla gene is specifically expressed in ovarian cells and is detectable only in the tissues of the ovary and testis of the adult mouse. The promoter of Figla was cloned and ligated into the pEGFP-1 plasmid,

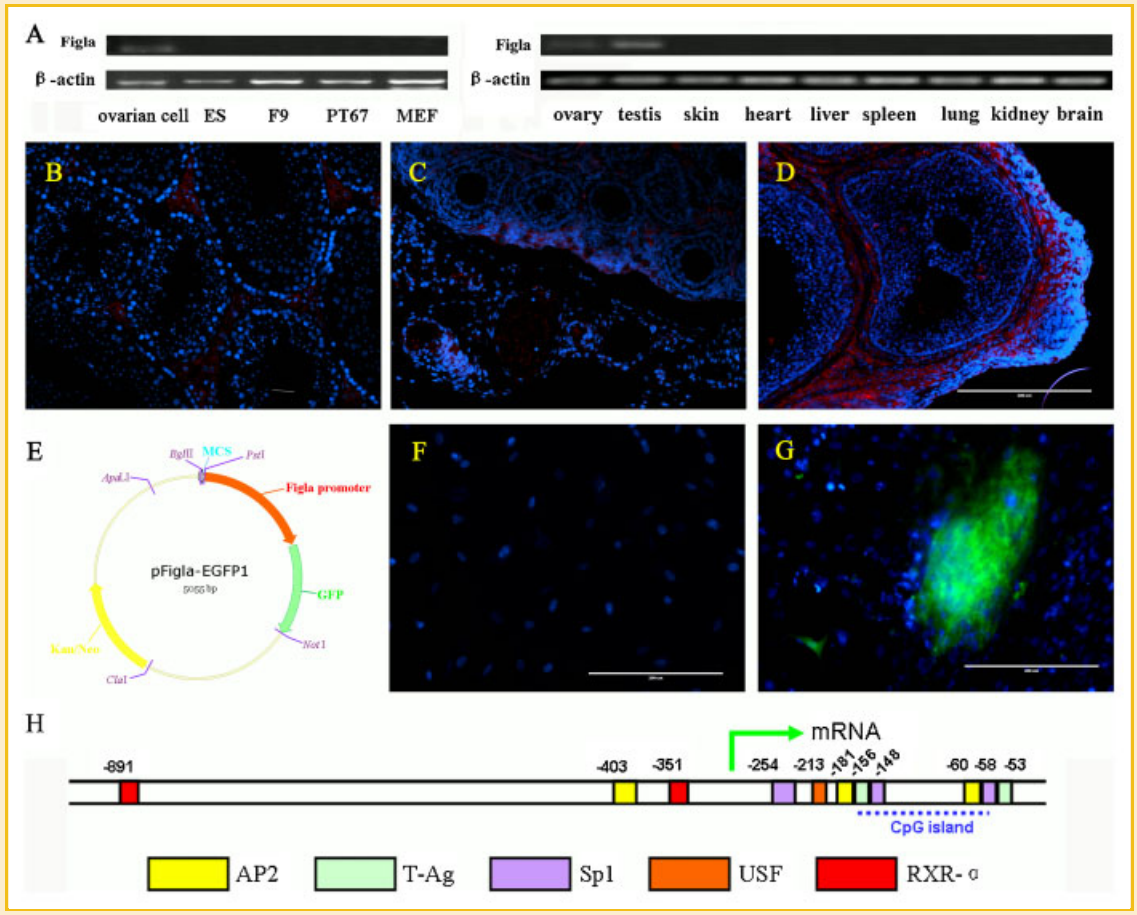

Fig. 1. Expression of Figla and the pFigla-EGFP recombinant plasmid. A: RT-PCR analysis showed the expression of Figla in the primary ovarian cells, tissues of ovary, testis, but not in undifferentiated ESCs, F9, PT67, MEF, skin, heart, liver, spleen, lung, kidney, and brain. Immunofluorescence analysis showed that Figla was expressed (red) in Leydig cells of testes (B), 2W (C) and 2M (D) ovaries, the nucleus was stained with Hoechst33342 (blue). E: The map of pFigla-EGFP recombinant plasmid. The expression of GFP in ovarian germ cells after transfected with pFigla-EGFP1 plasmid (G), while MEF was negative (F). H: The structural sketch map for Figla promoter. Scale bar = 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and constructed pFigla-EGFP recombinant plasmid (Fig. 1E). When MEF and mouse primary ovarian cells were transfected with plasmid pFigla-EGFP, the expression of enhanced fluorescent green protein (EGFP) was only detected in ovarian germ cells (Fig. 1G), while negative results were shown in MEF after transfection (Fig. 1F). This suggested that this pFigla-EGFP plasmid can be used to detect and purify certain stages of ovarian germ-like cells derived from differentiated mESCs.

The core promoter sequence of Figla gene was identified by analysis of the coding sequence and 2 kb upstream from the transcription start site. Firstly, the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) was used to predict the transcriptional start site of the core promoter and the result indicated that the transcription initiation site was located at −300 bp upstream of translation initiation sites (TIS). Secondly, the transcription factor binding sites were predicted by PROMOTER SCAN (http://www-bimas.cit.nih.gov/molbio/proscan/) and we found there contain binding sites for transcription factors AP-2, T-Ag, Sp1, USF, and RXR-α in the upstream of transcription start site (Fig. 1H). Furthermore, a single CpG island is highlighted by FirstEF online (http://rulai.cshl.org/tools/FirstEF/) whose position lies within the predicted promoter region as well. From the results above, we are confident that the assigned core promoter region contains a significant majority of the regulatory sequences of the Figla gene. This sequence was submitted to Genebank (HQ662521).

POTENTIALITY OF PROLIFERATION AND DIFFERENTIATION OF FIGLA-ESCs

ESCs carrying pFigla-EGFP (Figla-ESCs) at 6 (Fig. 2A) and up to 26 (Fig. 2B,C) passages maintained ESC characteristics, formed typical ESC colonies similar to wild mESCs expressing pluripotent markers including Oct4, Sox2, Klf4, Nanog, and C-myc analyzed by RT-PCR (Fig. 2D). The Figla-ESCs were also positive for AP staining (Fig. 2E). After 3 days of suspension culture, these cells spontaneously differentiated into typical embryonic bodies (EBs) (Fig. 2F), which expressed specific markers of three germ layers including β-III-tubulin (ectoderm), Brachury (mesoderm), and Pdx1 (endoderm) analyzed by RT-PCR (Fig. 2H).

The day 3 EBs attached to the plastic plates and the peripheral cells of the EBs formed monolayers that continued to spread some

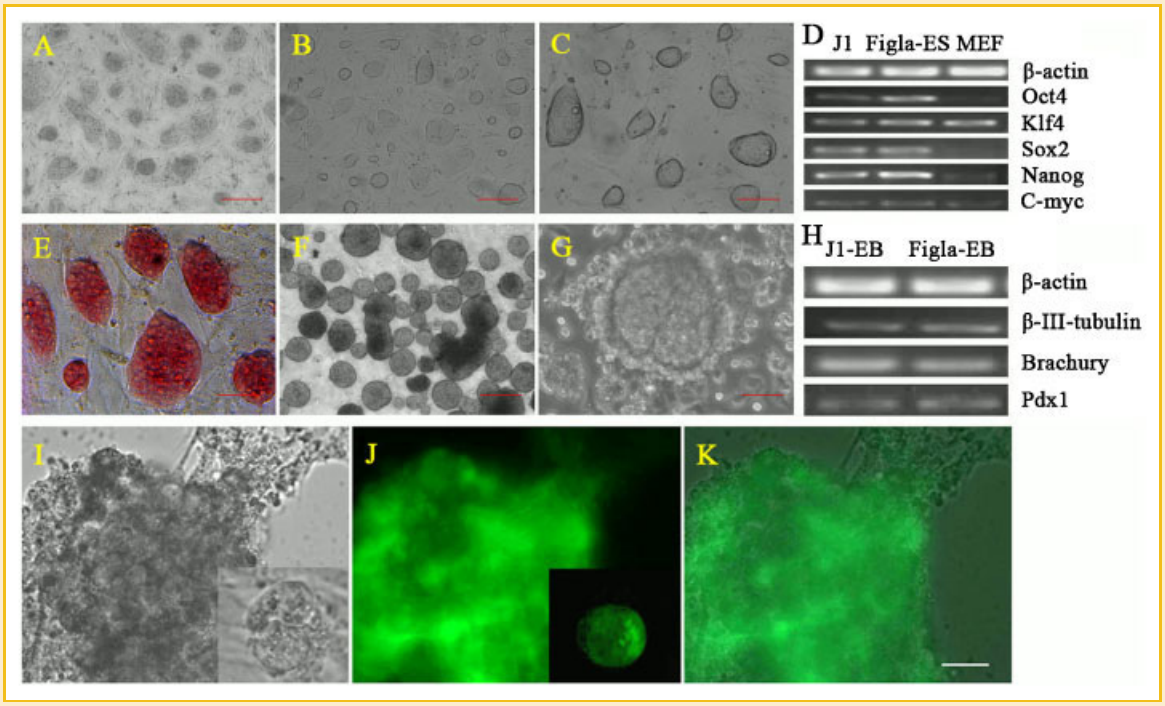

Fig. 2. The morphology of Figla-ESCs and their differentiation potentiality. ESCs carrying pFigla-EGFP formed typical ESCs colonies up to 6 (A) and 26 (B,C) passages on MEF feeder. D: ESCs expressed pluripotent markers analyzed by RT-PCR such as Oct4, Sox2, Klf4, C-myc, and Nanog. E: The Figla-ESCs were positive for AP staining. F: Figla-ESCs spontaneously differentiated into typical EBs. G: The day 3 EBs attached to the plastic plates and the peripheral cells of the EBs formed monolayers that continued to spread some large cells and small clusters around the center of the EB. H: EBs expressed specific markers of three germ layers including β-III-tubulin, Brachury, and Pdx1. I–K: A population of differentiated ESCs expressed GFP when allowed these EBs adherent and supplied with RA (I, phase contrast; J, fluorescent microscope; K, merged I and J). A, B, F: Scale bar = 200 μm; C: Scale bar = 100 μm; E, G, I–K: Scale bar = 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

large cells and small clusters around the center of the EB (Fig. 2G). When allowed the EBs attached and supplied with RA, a population of differentiated cells expressed GFP (Fig. 2J,K). By day 7, some large PGC-like cells were observed to spread out from the EBs. The cells in the center of clusters degenerated and the peripheral cells had varied morphological shapes with some remained floating (Fig. 2G,I–K).

To test whether the differentiated EBs derived from Figla-ESCs produced estradiol, the culture medium was collected between day 3 and day 23 after initial differentiation. The concentration of estradiol produced by the differentiating cells was increased compared to ESCs, and approached to the primary ovarian cells. The estradiol concentration in the different stages media was changed periodically (Supplemental Fig. 1). Immunofluorescent staining showed that the attached EBs expressed Nestin (ectoderm), β-III-tubulin (ectoderm), Pdx1 (endoderm), Glut2 (endoderm), cardiac α-actin (mesoderm), and Figla (Fig. 3).

FIGLA-GFP WAS A MARKER OF GERM CELL DIFFERENTIATION

To further detect the GFP positive cells, immunofluorescent and RT-PCR analysis were used. Immunofluorescent analysis showed that GFP positive cells were simultaneously expressed germ cell marker Vasa and meiotic-specific gene Scp3 after 7 days of differentiation (Fig. 4A). RT-PCR analysis showed that the expression of Gdf9, Figla, and GFP in RA-treated cells was up-regulated compared with untreated ESCs (Fig. 4B). Differentiated cells were positive for GFP which indicated the expression of Figla, while GFP expression in undifferentiated Figla-ESCs was negative. Figla-GFP positive cells were analyzed and enriched by FACS and the percentage of Figla-GFP positive cells increased to 5.2% of the total cell population on 6th day after RA induction (Fig. 4C). Quantitative RT-PCR analysis highlighted a significantly higher expression of several germ cell and meiotic specific markers (Vasa, Figla, Stra8, Scp3, and Zp3) in Figla-GFP positive cells compared with the undifferentiated ESCs (Fig. 4E). This demonstrated that pFigla-GFP could be a selective system of female germ cell derivation from ESCs.

DIFFERENTIATION OF FIGLA-ESCs FOLLOWING THE Co-TRANSPLANTATION OF MOUSE OVARIAN CELLS AND FIGLA-ESCs

We transplanted the co-aggregates of both ovarian cells and Figla-ESCs under the kidney capsule of recipient mice for four weeks. Two out of ten grafts formed oothecoma (Fig. 5A), which contained endoderm (gut)-, mesoderm (muscle)-, and ectoderm (neural epithelium)-like structures (Fig. 5B–F), and these structures expressed β-III-tubulin (Fig. 5G), Nestin (Fig. 5H), AFP (Fig. 5I), Pdx1 (Fig. 5J), cardiac α-actin (Fig. 5K), and CT3 (Fig. 5L) through immunofluorescent staining. Additionally, there were rare GFP positive cells in a fraction of the formed oothecoma (Supplemental Fig. 2).

We observed ESC-derived GFP positive cells in the grafts and these cells simultaneously expressed germ cell and meiotic markers including Vasa, Stra8, and Scp3. Meanwhile, some cells of

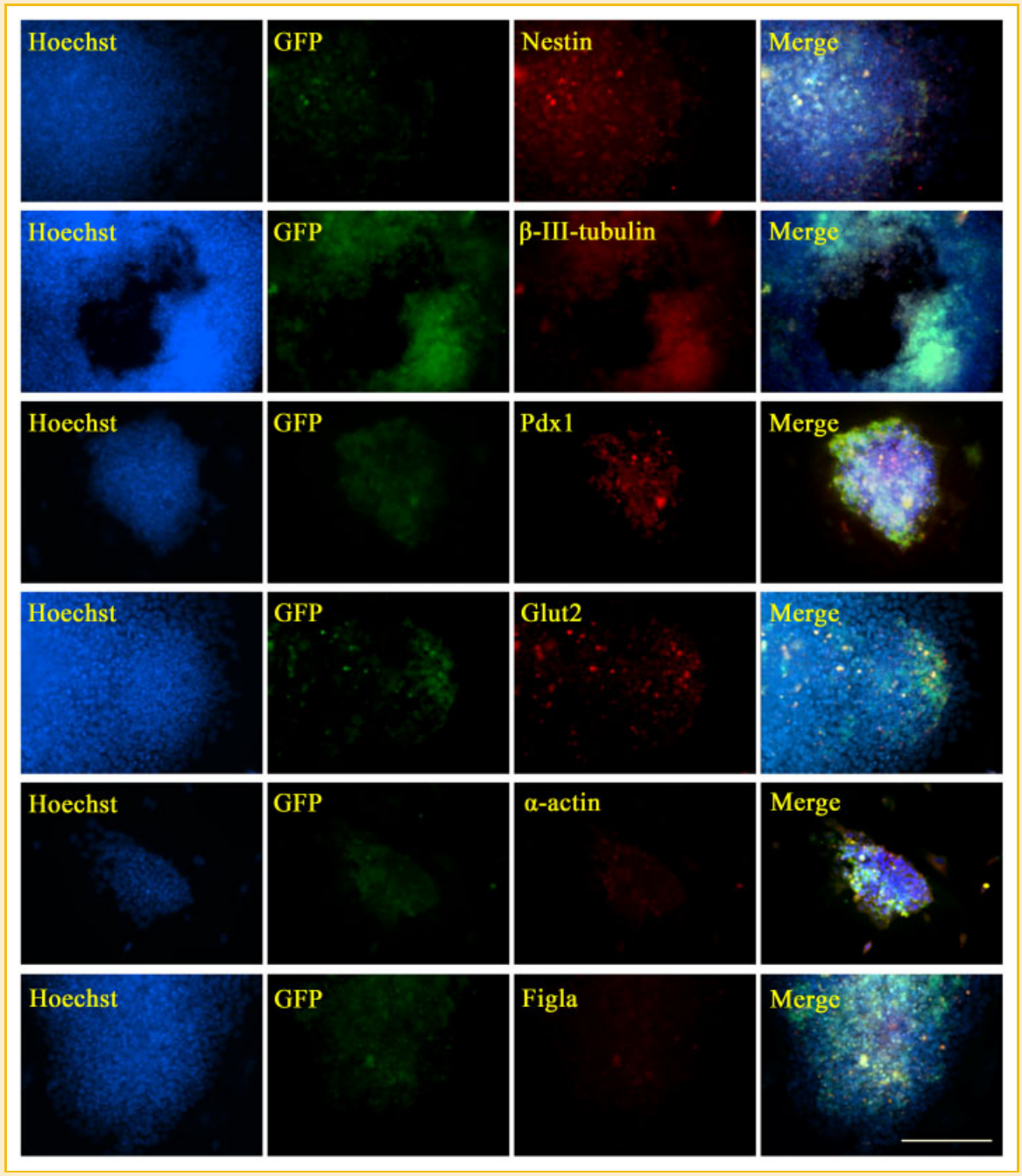

Fig. 3. Figla-ESCs differentiated into three germ layer cell types. Immunofluorescent staining showed that the EBs derived from Figla-ESCs expressed β-III-tubulin, Nestin, Glut2, Pdx1, α-actin, and Figla. Scale bar = 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

the co-aggregates differentiated into large cells, which were positive for germ cell and oocyte markers Figla and Zp3 (Fig. 6).

DISCUSSION

The limited knowledge of the development and regulation of mammalian oocyte formation, the different steps of folliculogenesis and the required conditions for oocyte to undergo proper growth, differentiation and maturation are major causes of the failure in obtaining viable offspring from in vitro cultured early oocytes from domestic animals and humans because it is a long and complex process in mammalian ovary [Hartshorne, 1997; van den Hurk and Zhao, 2005].

Murine embryonic stem cells (mESCs) can differentiate into PGCs and subsequently develop into early gametes (oocytes and sperms) [Hübner et al., 2003; Clark et al., 2004; Lacham-Kaplan et al., 2006; Kee et al., 2009]. In our study, we demonstrated that mESCs could

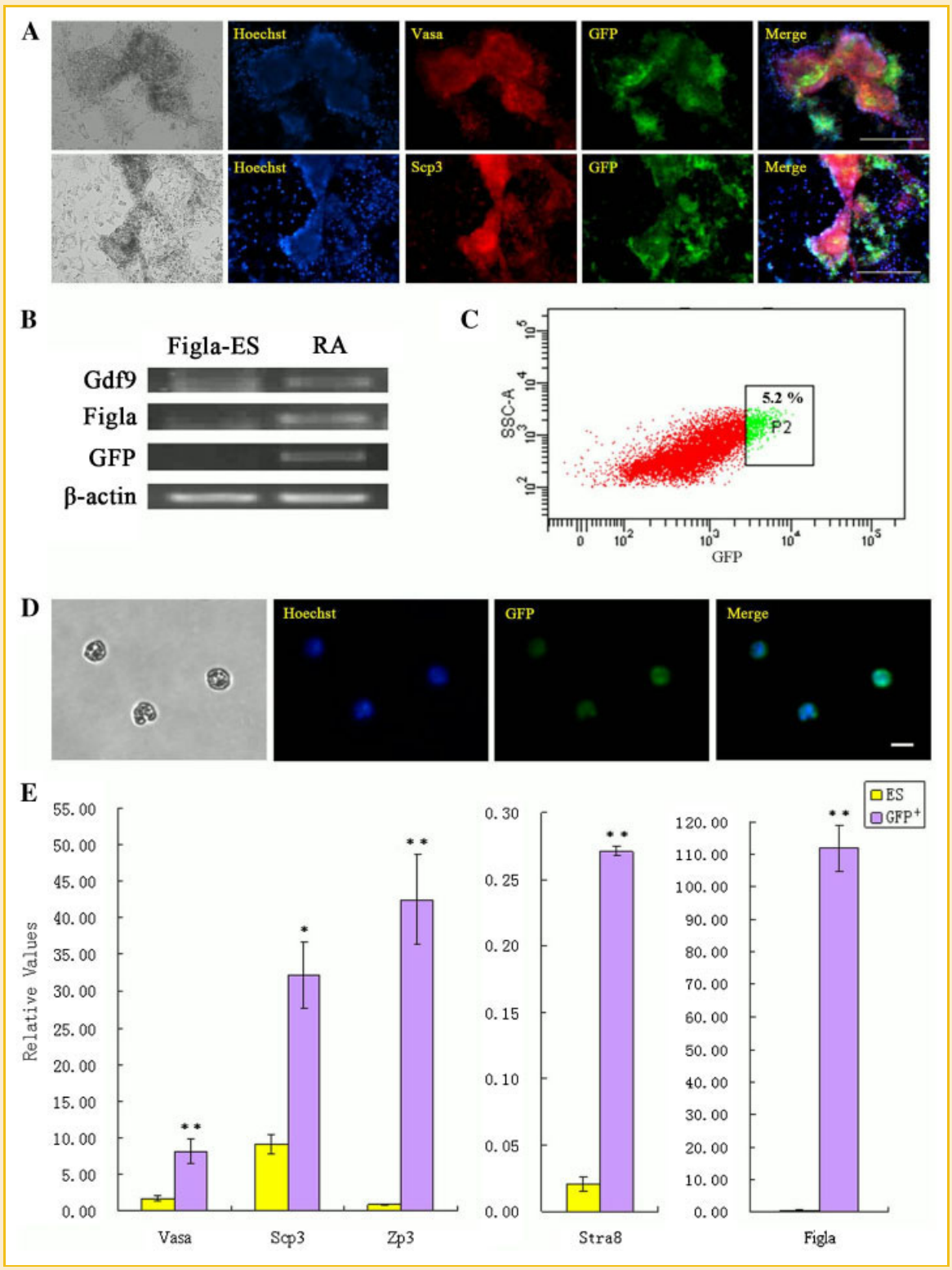

Fig. 4. Figla-ESCs differentiated into germ-like cells. A: Immunofluorescent analysis showed that GFP positive cells were simultaneously expressed germ cell marker-Vasa and meiotic specific gene-Scp3 after 7 days differentiation. B: RT-PCR analysis showed that differentiated cells were positive for GFP which indicated the expression of Figla, while undifferentiated Figla-ESCs were negative. The expression of oocyte markers-Gdf9 and Figla in RA-treated cells was up-regulated compared with undifferentiated Figla-ESCs. Figla-GFP positive cells were enriched by FACS (C,D) and the percentage of Figla-GFP positive cells increased to 5.2% of the total cell population on 6th day after RA induction (C). E: QRT-PCR analysis highlighted a significantly higher expression of several germ cell and meiotic specific markers in Figla-GFP positive cells compared with the undifferentiated ESCs ($P < 0.05$). A: Scale bar = 200 μm. D: Scale bar = 10 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

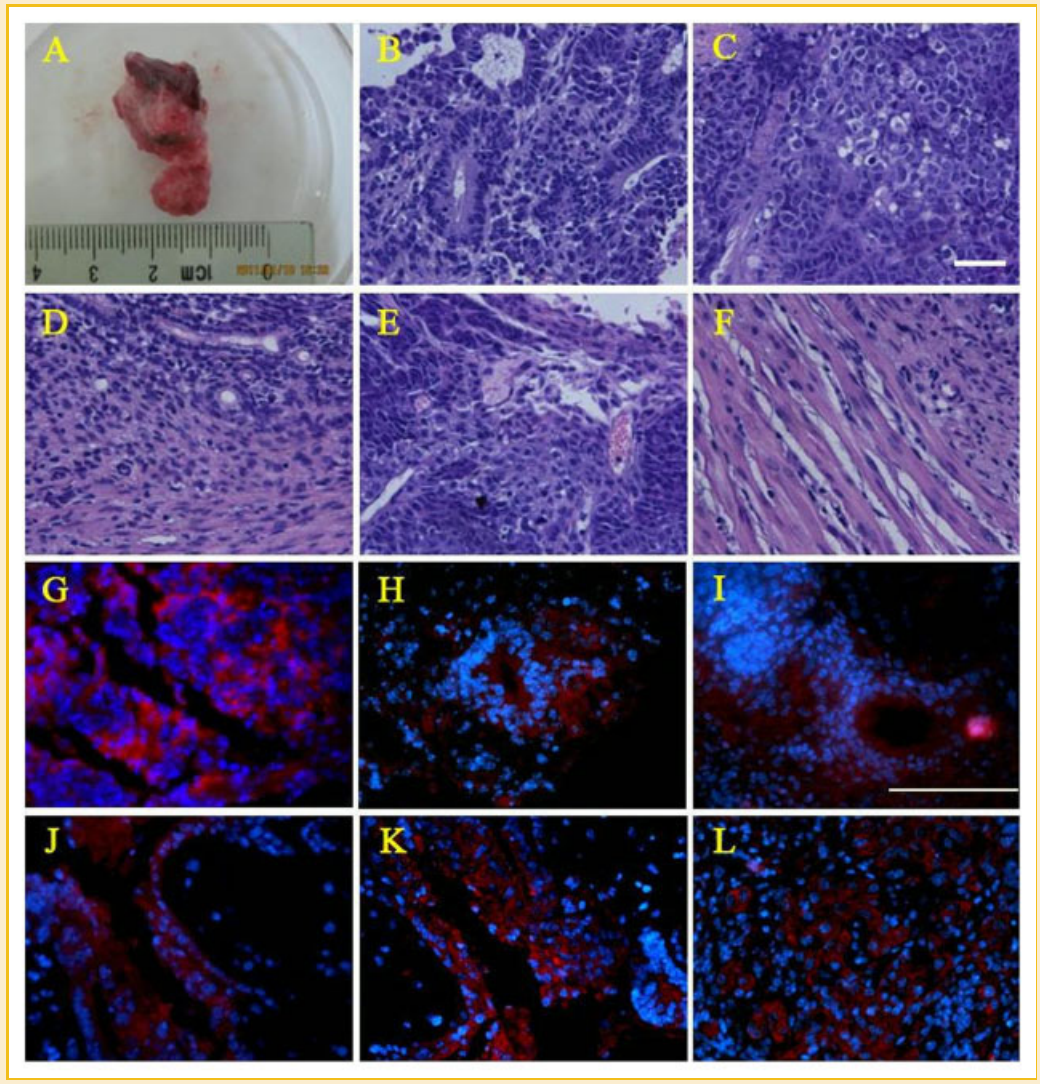

Fig. 5. Figla-ESCs formed teratoma. A: Teratoma derived from Figla-ESCs formed at 4 weeks after kidney capsule transplantation. The teratoma contained gut-, muscle-, cartilage-, and neural epithelium-like structures (B–F), and these structures expressed β-III-tubulin (G), Nestin (H), AFP (I), Pdx1 (J), α-actin (K), and CT3 (L) through immunofluorescent staining, the nucleus was stained with Hoechst33342 (blue). Scale bar = 100 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

differentiate into female germ-like cells, even oocyte-like cells evaluated by the morphology, immunofluorescent staining, RT-PCR analysis, and transplantation assay with production of estradiol. Ovarian estradiol production is the hallmark of pre-ovulatory follicular development [Hillier et al., 1994]. If the cell aggregates observed are truly follicle-like structures, they should contain granulosa and theca cells, in addition to oocytes.

Therefore, ESCs may provide a model for further research on the development and the differentiation of germ cells derived from stem cells. However, pre-meiotic germ cell markers are expressed in ESCs [Clark et al., 2004]. ESCs and germ cells may share aspects of a common molecular program and therefore express similar genes. The expression of germ cell markers may indicate spontaneous differentiation of part or all of the population of ESCs into germ cells [Clark et al., 2004].

A dynamic and visible marker may be valuable for the germ cell differentiation from stem cells. Germline specific transcription factor is essential for oocyte to survive and form primordial follicles, which implicated in postnatal oocyte-specific gene expression and played a key regulatory role in the expression of multiple oocyte-specific genes, including those initiate folliculogenesis and those encode the zona pellucida (Zp1, Zp2, and Zp3) required for fertilization and early embryonic survival. Factor in the Germline, alpha (FIGα), one of the first germ cell specific regulators to be implicated in primordial follicle formation [Jagarlamudi and Rajkovic, 2011], which is involved in the coordinate expression of the Zp genes, is a bHLH transcription factor first detected in oocytes at E13.5 that persists in adults [Huntriss et al., 2002]. Knockout analysis has shown that Figla is essential for oocytes to survive and form primordial follicles in the mouse. Results suggest

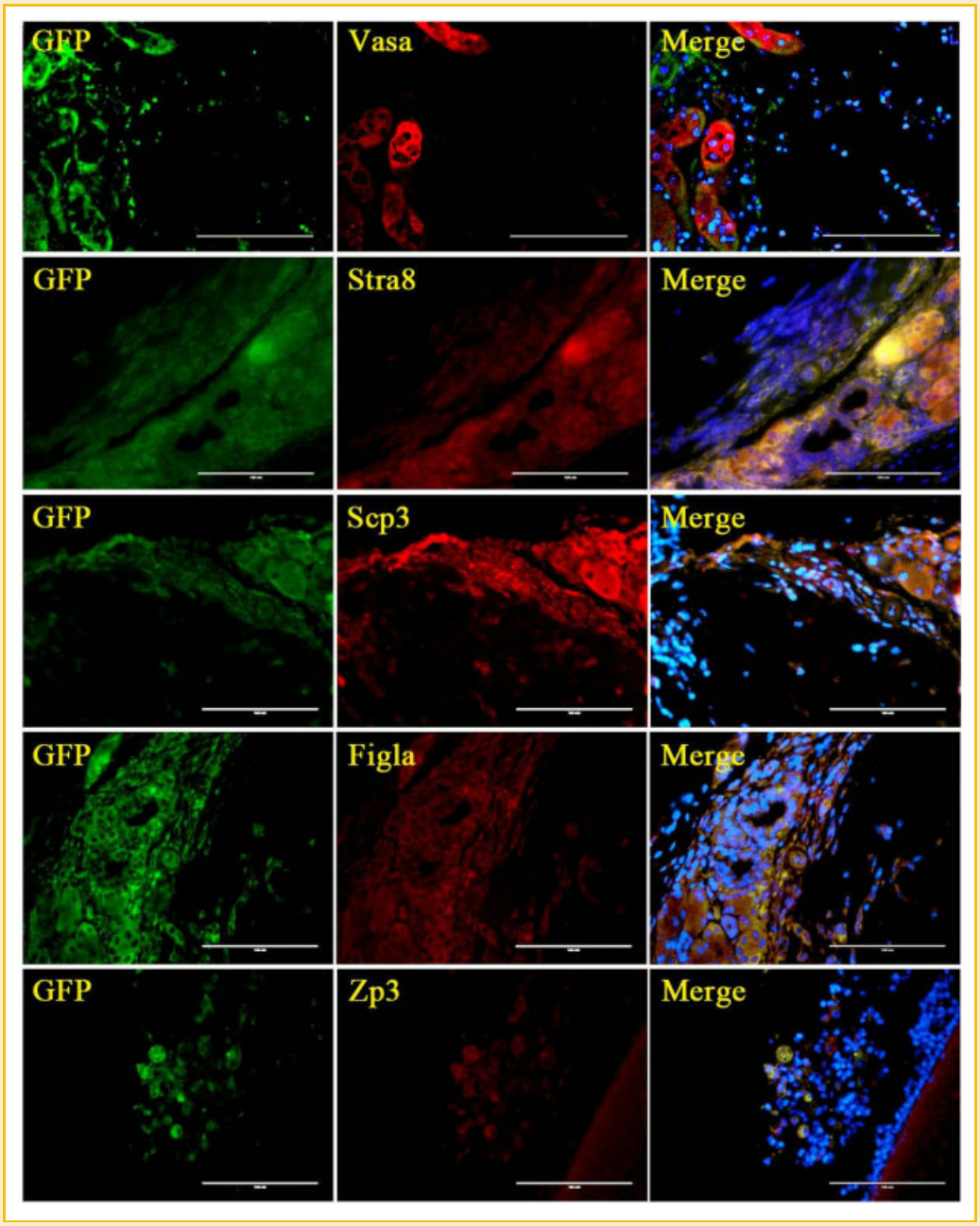

Fig. 6. Figla-ESCs differentiated into female germ-like cells in an ovarian niche. ESC-derived GFP positive cells were observed in transplanted grafts and these cells simultaneously expressed germ cell markers. Meanwhile, some cells of the co-aggregates differentiated into large round cells, which were positive for germ cell and oocyte markers. The nucleus was stained with Hoechst33342 (blue). Scale bar = 100 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

that Figla is involved in oocyte survival as primordial follicles form in the human as in the rodent ovary [Bayne et al., 2004]. Electrophoretic mobility shift assays with in vitro expressed human FIGLA proteins showed that, as in the mouse, Figla can heterodimerize with E12 protein and bind to the E-box of the human Zp2 promoter [Bayne et al., 2004].

FIGLA, also known as POF6, BHLHC8 or FIGALPHA, is a 219 amino acid nuclear protein expressed in fetal ovary and germ cells [Bayne et al., 2004]. Acting as a germline specific transcription factor and a key player of ovarian folliculogenesis, FIGLA regulates the expression of multiple oocyte-specific genes that are required for fertilization and early embryonic survival. In adult females, the persistence of FIGLA binds to the E-box (5′-CANNTG-3′) of Zps (Zp1, Zp2, Zp3) promoters, and regulates additional pathways that are essential for normal ovarian development [Bayne et al., 2004; Pan et al., 2011]. Ovarian folliculogenesis is an intricate process involving interactions between germ and somatic cells in mammalians. Mutations in the gene encoding FIGLA maybe the

cause of POF, a genetically heterogenous disorder that leads to hypergonadotropic ovarian failure and infertility [Zhao et al., 2008]. POF is characterized by amenorrhea, hypoestro-genism, and elevated serum gonadotropin concentrations [van Dooren et al., 2009]. Figla inhibits the expression of male germ cell specific genes during oogenesis [Rajkovic and Matzuk, 2002; Hu et al., 2010b].

Mouse transgenesis has been particularly useful in defining germ-cell specific genes and their roles in folliculogenesis (e.g., DAZLA, FIGLA, NOBOX, SOHLH1, YBX2, CPEB1, GDF9), fertilization (e.g., Zp1, Zp2, Zp3), and pre-implantation embryonic development [Joshi et al., 2007]. Figla and Nobox may be important for ovarian determination by suppressing male specific genes [Jagarlamudi and Rajkovic, 2011]. The expression of Figla was up-regulated during primordial follicle formation [Fowler et al., 2009]. Vasa is a specific marker of germ cell migration that enters into the gonads and meiosis [Anne-Marie et al., 2002]. Growth differentiation factor 9 (Gdf9), zona pellucida genes (Zp1, Zp2, and Zp3) are preferentially expressed in oocytes which play important roles during folliculogenesis [Dong et al., 1996; Lyrakou et al., 2002]. Some cells in RA treated EBs derived from Figla-ESCs expressed markers specific to different stages of female germ cell development (e.g., Vasa, Gdf9, Zp3). Furthermore, the meiotic marker genes, Stra8 and Scp3 were identified, increased and the female germ markers including Gdf9, Zp3 and Figla were increased clearly analyzed by QRT-PCR in RA treated Figla-GFP positive mESCs. Scp3 is a specific marker of meiosis in male and female germ cells [Acevedo and Smith, 2005]. Simultaneously, the GFP was expressed after Figla-ESCs were spontaneously differentiated and induced. We observed some oocyte-like cells derived from Figla-mESCs that could develop into blastocyst-like structures, also expressing GFP. Additionally, these Figla-ESCs maintained the typical characteristics of pluripotent cells, which expressed pluripotent markers, and had the potentiality to differentiate into three germ layer cell types [Thomson et al., 1998; Bucay et al., 2009; Park et al., 2009]. Our results demonstrated that Figla-ESCs may provide a dynamic and visible marker and an in vitro model to study the regulation of female germ cells derived from stem cells. In vivo, these cells may differentiate into female germ-like cells, even oocyte-like cells analyzed by graft transplantation [Dyce et al., 2011].

Studies suggested that the in vitro production of germ cells is possible from ESCs and somatic cells [Hua and Sidhu, 2011]. Overexpression of Vasa, Dazl or other germ cell specific genes, induction with RA, or addition with follicular fluid, hormones or extracts derived from germ cells, or co-cultured with Sertoli cells may induce the stem cells to differentiate into germ cells [Dyce et al., 2006, 2011; Bucay et al., 2009; Kee et al., 2009; Park et al., 2009; Hua and Sidhu, 2011]. However, the efficiencies of germ cell differentiation derived from stem cells were still low. Importantly, our results showed that Figla-ESCs may provide an efficient method to select and purify the female germ cells derived from ESCs, and maybe combining growth factors can enhance the efficiencies.

In conclusion, we established a mouse ESC line stably carrying this pFigla-EGFP reporter construct. These cells provide a new selective system of female germ cell derivation and offer an approach to study the development and the differentiation of germ cells derived from stem cells.

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