Am J Transl Res 2022;14(12):8828-8842
www.ajtr.org /ISSN:1943-8141/AJTR0145920

Original Article

Co-culture induces expression of female primordial germ cell-specific genes in human Wharton's jelly-derived mesenchymal stem cells

Bihui Bao[1*], Yuyan Li[2*], Limei Chen[1], Yuan Huang[3], Qifeng Tang[4], Zhiqing Liang[2]

[1]Department of Obstetrics and Gynecology, Qingbaijiang Women's and Children's Hospital (Maternal and Child Health Hospital), West China Second University Hospital, Sichuan University, Chengdu 610300, Sichuan, China; [2]Department of Obstetrics and Gynecology, The First Affiliated Hospital (Southwest Hospital), Army Medical University, Chongqing 400038, China; [3]Department of Gastroenterology, Tianjin Armed Police Corps Hospital, Tianjin 300163, China; [4]Department of Anesthesiology, Shanghai Hechuan-Rhine TCM Hospital, Shanghai 201103, China. [*]Equal contributors and co-first authors.

Received August 14, 2022; Accepted November 28, 2022; Epub December 15, 2022; Published December 30, 2022

Abstract: Objective: To detect mRNA and protein expression of meiosis-specific genes in human umbilical cord mesenchymal stem cells (hUMSCs) in an *in vitro* co-culture microenvironment with mouse primordial germ cells (PGCs), and to further explore the effective potential of hUMSCs to differentiate into PGCs. Methods: HUMSCs were obtained from human Wharton's jelly fragments by adherent culture. PGCs were derived from 12.5 days post-coitum (dpc) BalbC mice. Then hUMSCs were co-cultured with PGCs in Matrigel, inside or outside of a culture chamber, respectively. The changes in morphology and cytogenetic characteristics were observed. SCP3 and DDX4 expression in hUMSCs were detected and analyzed using immunofluorescence staining. Oct-4, Stra8, Zp3 and Dmc1 gene expressions in PGCs, hUMSCs, and hUMSCs after co-culture with PGCs were analyzed by real time reverse transcription-polymerase chain reaction. Results: Both hUMSCs and PGCs expressed Oct-4 at different degrees. After co-culture with PGCs, hUMSCs became rounded and showed AKP activity. HUMSCs suspension-cultured in Matrigel or adherent cultured with cell chamber significantly expressed Stra8, DMC1, SCP3 and DDX4 genes. Conclusion: HUMSCs can be induced to express PGC-specific genes Stra8 and DMC1, spermatogonium/oogonium-specific genes SCP3 and DDX4 that predict directed differentiation potential into early germ cells at a molecular level.

Keywords: Human umbilical cord mesenchymal stem cells, induced differentiation, primordial germ cells, specific gene expression

Introduction

Infertility has been implicated in a series of physical ailments along with psychological stress in 15% of couples worldwide [1]. Premature ovarian failure (POF) is an important cause of female infertility, and its etiology involves genetic, immunologic, metabolic, infectious, environmental and iatrogenic factors [2, 3]. With a high incidence of 1% in the normal population, POF is characterized by anovulation, sustained secondary amenorrhea, infertility, sex steroid deficiency or perimenopausal syndrome in women under the age of 40 [3, 4]. The long-term sequelae of continuous estrogen deficiency (FSH>40 IU/L) as maternal aging, atherosclerosis, blood clots, heart disease or osteoporosis may adversely affect the lives of women [4, 5]. Treating anovulatory infertility at cellular and molecular levels, and with microenvironment control is expected to fundamentally improve the prognosis.

Transplanting pluripotent stem cells (PSCs) or autologus mesenchymal cells for tissue regeneration and wound repair is a new therapeutic approach in recovery ovarian function and fertility accepted by the majority of researchers [6]. With the ability to spontaneously differentiate into gametes *in vitro*, PGCs (natural precursors to gametes) are located in the basal layer of seminiferous epithelium in the testis and in the

ovary surface epithelium and survive in various adult organs including the gonads [6]. By physical and chemical *in vitro* methods (4 mT SMF, 25 ng/mL BMP-4), Javani et al. implemented bone marrow mesenchymal stromal cell (BMSCs) differentiation to PGCs, with moderate increase of Mvh expression (from 7.9% to 30.9%), homologous genes of VASA (DMC1), and significant decrease of Oct-4 expression (from 82.0% to 25.5%) [7]. Abd-Allah et al. injected bone marrow stem cells of male rabbits intravenously into CTX-induced POF animal model, and found that the number of follicles, follicular structure, FSH, E2 and VEGF showed no notable difference within 2 weeks compared with normal controls [8]. Multipotent mesenchymal stromal/stem cells (MSCs) in clinic trials extensively display immunomodulatory properties, suppression of inflammation and release of growth factors and chemotactic cytokines, thus contributing to folliculogenesis along with hormonal secretions among patients with degenerative diseases, infertility or POF [6, 9, 10].

Germline stem cells (GSCs) are the basis of cell totipotency, which can divide by meiosis to make sperm and eggs for fertilization to create a new individual [9]. To produce healthy gametes and offspring, scientists are continuously exploring new experimental technological platforms to create healthy micro-ecological environments that would enable PSCs derived from primordial germ cell-like cells (PGCLCs) to enter into meiosis and produce healthy gametes [11]. At present, therapeutic effect of transplanting human MSCs and cord blood mononuclear cells (HCMNCs) in animal experiments and clinical applications has attracted extensive attention [2]. The studies advanced our understanding how stem cells differentiate and provided insight into possible mechanism how to stimulate tissue repair.

As a noncontroversial source of stem cells for transplantation without ethical concerns, human umbilical cord MSCs (hUMSCs) provide an ideal seed cell type to help regenerate or repair damaged tissues, but the question remains, can PGCs trigger the co-cultured hUMSC to reprogram or trans-differentiate into a kind of human PGCs other than GSCs for reproductive health?

In this study, we analyzed the bioactivity of hUMSCs and the genomic information related to the prophase and early stages of meiosis after co-culture with PGCs in niche microenvironment, with an aim to explore the potential and conditions for germline-specific differentiation of hUMSCs.

Materials and methods

Isolation and labeling of hUMSCs

Umbilical cords from healthy infants at a gestational age of 37-40 weeks were collected from Obstetrics, Southwest Hospital, Army Medical University (Chongqing, China) and put in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) containing antibiotics (100 units/mL penicillin-streptomycin). HUMSCs were obtained by direct adherent culture of Wharton's Jelly minced into small pieces within 4 h after collection in DMEM/F12 medium containing 10% fetal bovine serum (FBS) in a 37°C humidified incubator with 5% CO_2. Every 2 to 3 days the medium was refreshed, and the cell concentration of hUMSCs was detected every other day using Cell Counting Kit-8 (CCK-8). Upon 80-90% confluency cells migrated using 0.25% Trypsin-EDTA, were passaged, and morphologically observed with Olympus CKX41 inverted fluorescent microscope.

This research project was approved by the Ethics Committee of Southwest Hospital, and was performed in line with the Ethical Principles for Biomedical Research Involving Human Subjects (Ministry of Health of the People's Republic of China) and the Declaration of Helsinki principles. Written informed consent was obtained from parents of all infants who participated in the study.

In order to avoid the sex-determining genes (Sry) that could affect primordial follicles to grow and develop to secrete hormones, green fluorescent protein (GFR) was transferred into the hUMSCs from male infants by lentivirus, and red fluorescent protein (RFP) into the hUMSCs from female infants [12]. GFP MSCs and RFP MSCs were selected using a flow cytometer, and cells from passages 3 to 8 (P3-8) were utilized for the experiment after routine G banding chromosome analysis to ensure genome integrity. The expression characteristics of surface antigens CD29, CD31, CD44, CD45, CD73, CD90, CD105, HLA-DR and SSEA-1 of hUMSCs were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA),

with mouse IgM PE, monoclonal mouse IgG2b K PE, IgG1 K FITC as Isotype control immunoglobulin. The pluripotency of hUMSCs was identified using adipogenic, osteogenic and chondrogenic differentiation.

Culture and identification of PGCs

Primordial ovaries were obtained from 12.5-dpc balbc mouse embryos from Experimental Animal Center of Army Medical University. Animal housing and research procedures were conducted in full compliance with Directive 2010/63/EU and in accordance with Chongqing Ethics Committee of Medical Research (SYXK-PLA-20120031). Primordial gonad and mesonephros were separated out under an anatomical microscope (LEICA M125) and adherent cultured directly in 0.1% gelatin-coated plate with M199 medium and 10% FBS, by adding 10 ng/mL rh-LIF (Recombinant Human Leukemia Inhibitory Factor), 10 ng/mL rh-bFGF (basic fibroblast growth factor) and 2 μM Forskolin to obtain PGCs. The primary PGCs or PGCs at passage 1 were used for the experiment. PGCs were identified by alkaline phosphatase (AKP) staining. Mouse primordial gonads, adult mouse testis identified by immunohistochemical staining and PGC slides were used as immune positive controls to detect gene expressions of Stra8, Dmc1, SCP3 and DDX4, and human foreskin dermal fibroblasts (HDF) of 3-5 passages from the Institute of Combined Injury of Army Medical University as negative control. Meanwhile, reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used for determining expression of Oct-4, SCP3, Stra8, Zp3 and Dmc1 in the germ cells.

Co-culture of hUMSCs and PGCs

HUMSCs at passage 3-5 and PGCs at passage 1 were respectively grown inside or outside of a diffusion chamber. A 24-well plate was coated with 0.1% gelatin outside of the diffusion chamber. Mixed MSCs at passage 3-5 and PGCs at passage 1 in the ratio of 1:3 were directly cultured with the adherent method into 0.1% gelatin-coated 12-well plate and cell slides, or in suspension culture preconditioned for cell aggregates with 1:1 diluted plant lectin, in Matrel diluted 1.5 times with knockout DMEM medium.

In the first 5 days, a mixture of hUMSCs and PGCs was co-cultured in M199 complete medium by adding 10 ng/mL rh-LIF, 10 ng/mL rh-bFGF, 2 μM Forskolin, 10 μM RA (all-trans retinoic acid; Sigma, Taufkirchen, Germany), 10 ng/mL stem cell factor (SCF), 2 mM glutamine, 0.1 mM non-essential amino acid and 0.1 mM β-mercaptoethanol. From the 6^{th} to the 11^{th} day, the mixture was co-cultured in αMEM medium (an improved MEM medium with L-glutamine, deoxyribonucleosides and ribonucleosides; TransGen Biotech, Beijing, China) with 10% FBS, 1% insulin-transferrin-selenium (ITS), 100 mIU/mL FSH (follicle-stimulating hormone), 100 μg/mL GDF (growth differentiation factor), 0.05 mg/mL EGF (epidremal growth factor) and 0.23 mM sodium pyruvate. On day 12, the co-culture of entire αMEM media was added with 1.5 U/mL HCG and 5 ng/mL EGF. After 6-48 h, the morphology of PGCs, red fluorescence-labeled MSCs on slides, as well as inside and outside of a diffusion chamber in suspension culture were observed under a fluorescence microscope. The cell aggregates in Matrigel were collected with BD™ Cell Recovery solution and adhere-wall cultured. AKP activities of all cells cultured in the different conditions were identified using AKP staining.

Immunofluorescence and RT-PCR assay

Expression of germline-specific genes SCP3 and DDX4 (1:100 dilution) in hUMSCs before and after co-culture were detected by indirect immunofluorescence assay. While RT-PCR was used to quantitatively analyze the expression of Oct-4, Stra8, Zp3 and Dmc1 genes in hUMSC before and after inducing differentiation using GAPDH as control and a housekeeping gene. Primers were designed through the NCBI/Primer-BLAST Web site and synthesized by Invitrogen Company (Shanghai, China). **Table 1** lists the gene primer sequences and expected DNA band for Oct-4, Stra8, Zp3, Dmc1 and GAPDH.

Real-Time quantitative PCR (q-PCR) with SuperGreen Fluorescence q-PCR Current Kit (SYBR®Premix Ex Taq™) was run on a Roche Lightcycler™ automatic qPCR analyzer with a final volume of 20 μL. Optimal PCR cycling parameters were set for 95°C (5 min) and 35 cycles of 94°C (45 s), 60°C (45 s) and 72°C (45 s). The relative gene expression were calcu-

Table 1. RT-PCR sequence primers were designed to detect gene expression at transcription level on PGCs and hUMSCs

Gene Primer	Primer sequence	Product size (bp)
Oct-4	Forward: 5'-CAAAGCAGAAACCCTCGTGC-3'	219
	Reverse: 5'-TGTGCATAGTCGCTGCTTGA-3'	
Stra8	Forward: 5'-CTGAAGAAAACAGCAATCCCC-3'	187
	Reverse: 5'-TGCCACTTTGAGGCTATGAG-3'	
Zp3	Forward: 5'-CAGAAGATGTGGTCAGGTTTG-3'	292
	Reverse: 5'-GCGTTCCAGTTCTCCTCCAT-3'	
Dmc1	Forward: 5'-TCGATTCAATAATGGCACTTTTTCG-3	157
	Reverse: 5'-GGATCGGCAGTCATTTGATTGGT-3'	
GAPDH	Forward: 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3'	240
	Reverse: 5'-CATGTAGGCCATGAGGTCCACCAC-3'	

Note: RT-PCR, reverse transcriptase-polymerase chain reaction; PGCs, primordial germ cells; hUMSC, human umbilical cord mesenchymal stem cells.

lated using the formula 2^-ΔΔCt (CP GAPDH-CP target gene) by normalizing Ct (cycle threshold) values, where CP is the crossing point in the LightCycler. PCR amplification products were further analyzed by agarose gel electrophoresis, and photographs were taken using a UV analyzer.

Statistical analysis

All the q-PCR experiments were carried out in triplicate to detect gene expression. Data were expressed as mean and standard error ($\bar{x}$ ± se). The homogeneity variance and a one-way analysis of variance with Tukey's post-hoc test were used for processing data in three groups. $P<0.05$ was considered statistically significant. The extent of variation of gene expression in hUMSCs before and after induction was expressed in percentage.

Results

Morphologic, cytogenetic and undifferentiated characteristics of hUMSCs

HUMSCs were long spindle shaped with fibroblast-like morphology and grew in colony-like cluster (**Figure 1A**). Male hUMSCs transfected with GFR (**Figure 1B**) and female hUMSCs transfected with RFP (**Figure 1C**) were selected by flow cytometry, which showed high potential of proliferation (**Figure 1C**).

Molecular phenotype of hUMSCs at passage 3 to 8 analyzed by flow cytometry showed common features of MSCs with lower immunogenic properties, but did not have characteristics of PGCs with weak expression of SSEA-1 molecule (0.946%, **Figure 2E**). They showed high expression of MSC markers CD90 (expressed in 77.6% hUMSCs, **Figure 2A**), integrins CD29 (95.5%, **Figure 2B**), as well as adhesion molecules CD73 (62.9%, **Figure 2C**), CD44 (81.5%, **Figure 2D**), and CD105 (78.2%, **Figure 2A**), while negative expression of HLA-DR (0.042%, **Figure 2D**), negative hematopoietic markers CD45 (0.186%, **Figure 2F**), and endothelium-specific marker CD31 (0.221%, **Figure 2G**), while the negative controls' expression levels of IgG 2b.k (PE) and IgG1 Kappa (FITC) were 0.968%, obviously low (**Figure 2H**). All the results suggested the purity and pluripotency of hUMSCs.

HUMSCs are able to maintain self-renewal since the growth curve showed a time-effect on the proliferation of hUMSCs (**Figure 3A**). Analyses of hUMSC chromosomes at passage 7 to 8 excluded chromosomal aberrations (**Figure 3B** and **3C**) which also have multi-lineage differentiation potential *in vitro*. In the adipogenic, osteogenic and chondrogenic induction medium, osteoblasts were stained positively with Alizarin Red-S (**Figure 4A**), adipocytes with Oil Red-O (**Figure 4B**), and chondrocytes with Alcianblue at the appropriate day (**Figure 4C**).

PGCs morphology, AKP activity and cytogenetic characteristics

Genital ridge and mesonephros in 12.5-dpc fetal mice were observed under an inverted microscope (**Figure 5A**), which showed fine-grained dots on the primordial ovarian surface, and big and round primordial follicles incubated in the surface of 6-well plate coated with gelatin (**Figure 5B**). After cultured and expanded with germ cell medium *in vitro*, germ cells were identified with high AKP activity (**Figure 5C**) and showed active proliferative capacity.

DMC1 and Stra8 protein expression were detected on routine paraffin sections of adult

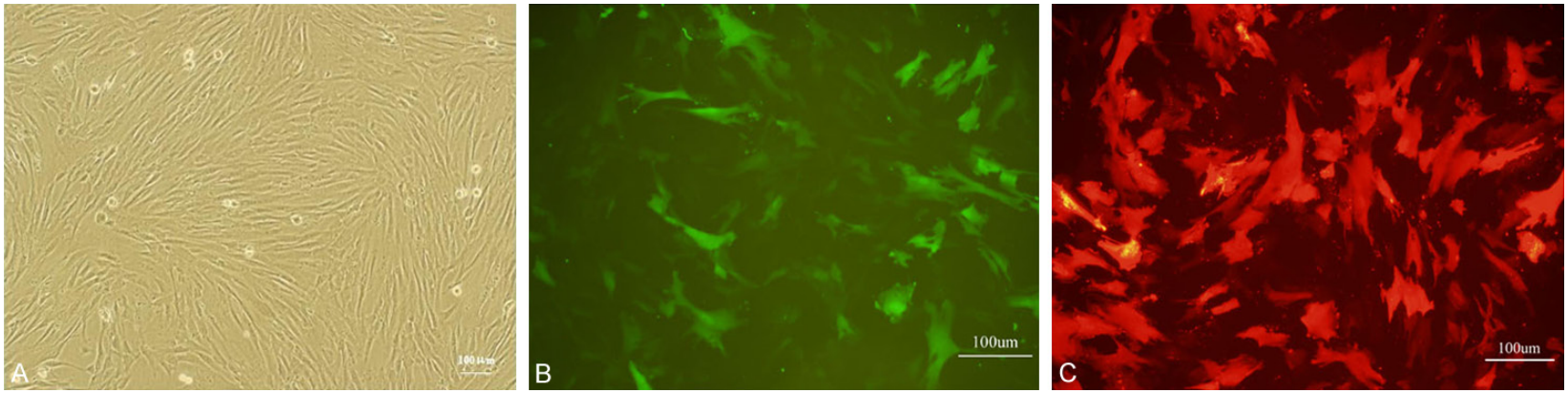
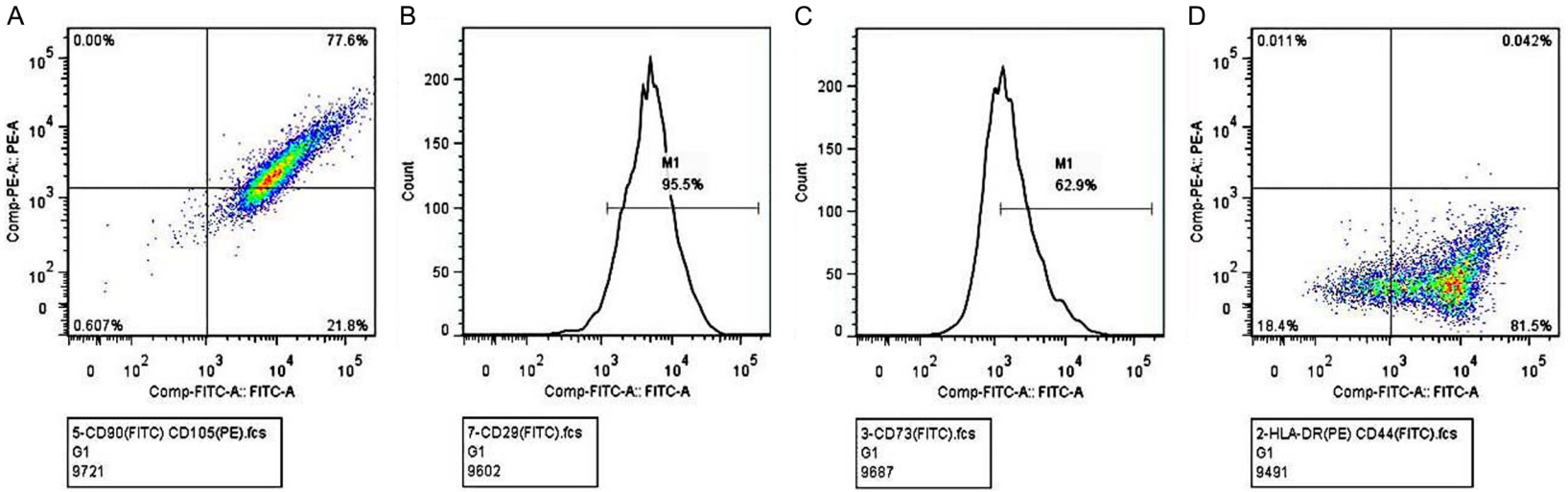


Figure 1. Morphological characteristics of hUMSCs before and after transfection of GFP and RFP were observed under Olympus inverted fluorescence microscope. A: Non-transfected hUMSC P3. Magnification (100×); B: RFP MSC P5. Magnification (150×); C: GFP MSC P5. Magnification (150×). hUMSCs, human umbilical cord mesenchymal stem cells.



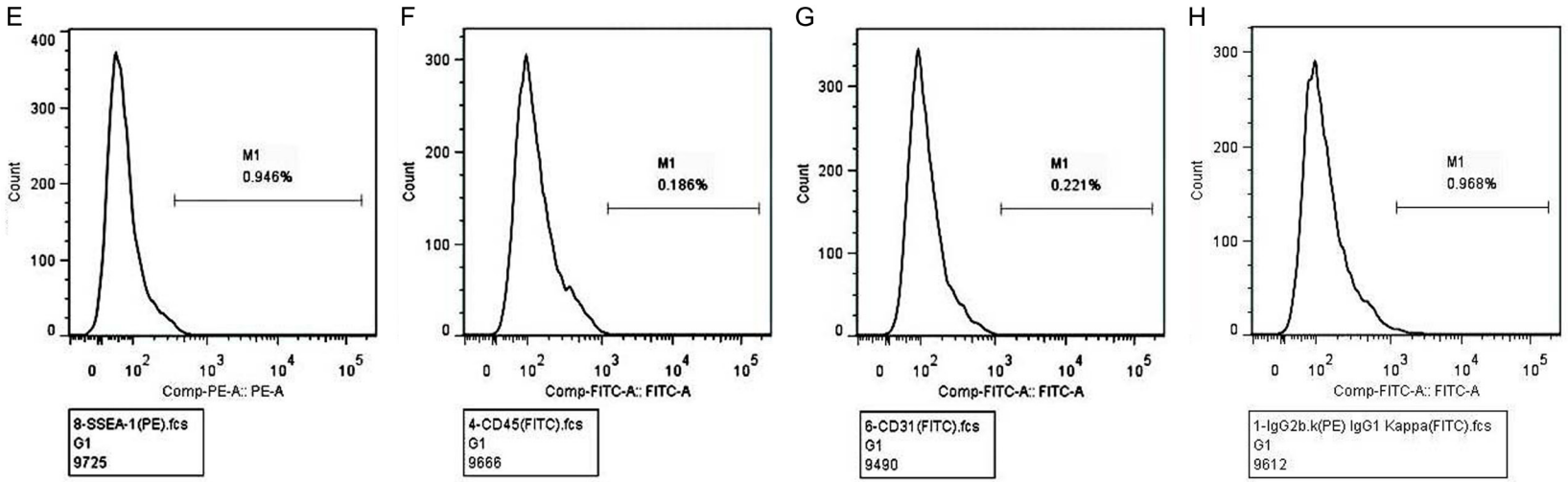


Figure 2. Expression features of CD90, CD105, CD29, CD73, HLA-DR, CD44, SSEA-1, CD45 and CD31 on the surface of hUMSCs were analyzed by indirect immunofluorescence staining and flow cytometry. A: Expression rates of CD90, CD105 were 77.6% and 78.2% respectively; B: High expression of CD29 molecule (95.5%); C: Positive expression of CD73 (62.9%) in hUMSCs; D: Negative expression of HLA-DR (0.042%) and strongly positive expression of CD44 (81.59%); E: Negative expression of SSEA-1 (0.946%); F: Negative expression of CD45 (0.186%); G: Negative expression of CD31 (0.221%) in hUMSCs; H: Negative Control IgG 2b.k (PE) and IgG1 Kappa (FITC) (0.968%). hUMSCs, human umbilical cord mesenchymal stem cells.

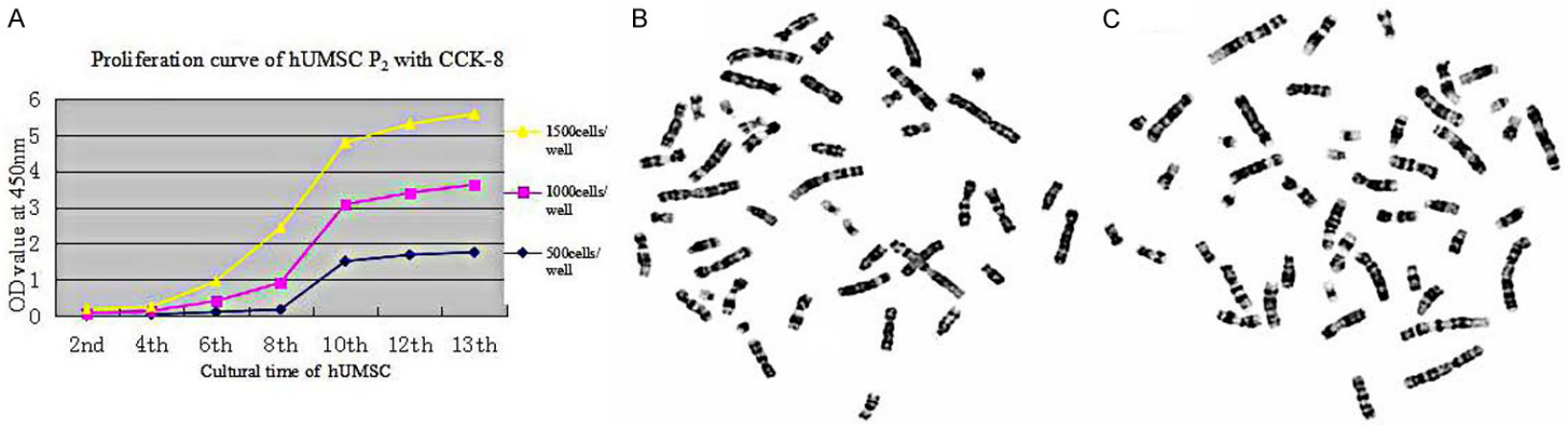


Figure 3. Tests of hUMSC viability and growth measured by CCK-8, without the number and structural aberration of the euchromosomes and sex chromosomes by classic cytogenetic analysis. A: The growth curve of hUMSCs at different concentrations showed "S" type; B: The right number and structure of chromatosomes in hUMSCs of female infants; C: Normal autosomes and XY chromosomes in male infants' hUMSCs. hUMSCs, human umbilical cord mesenchymal stem cells.

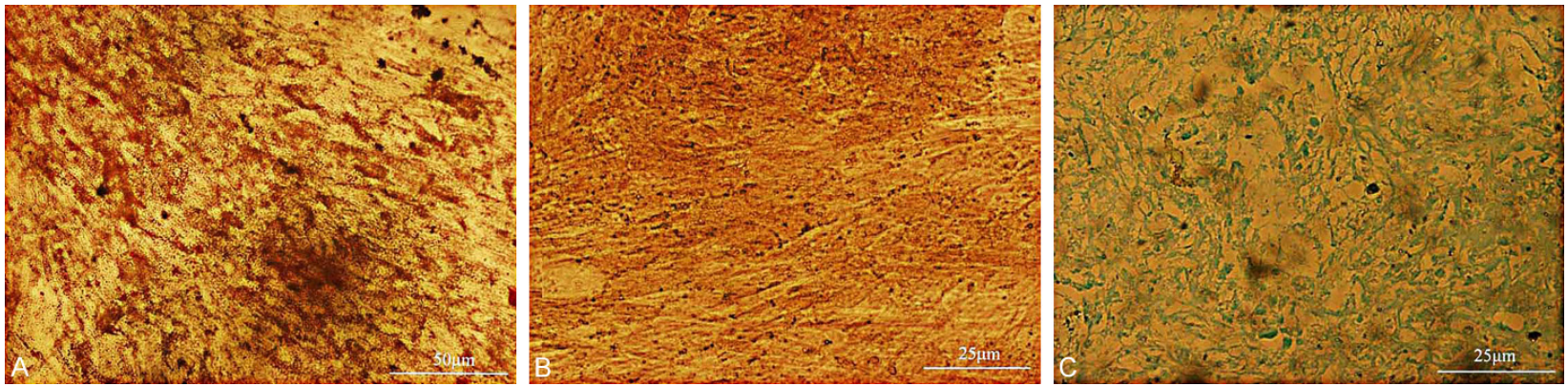


Figure 4. Activity of hUMSCs was identified using adipogenic, osteoplastic and chondrogenic differentiation. A: hUMSC P2 with Oil-red O staining at the 31st day of adipogenic induction. Magnification (200×); B: hUMSC P2 with Alizarin red staining at the 22nd day of osteogenic induction. Magnification (100×); C: hUMSC P6 with Alcianblue staining at the 21st day of chondrogenic differentiation. Magnification (400×). hUMSCs, human umbilical cord mesenchymal stem cells.

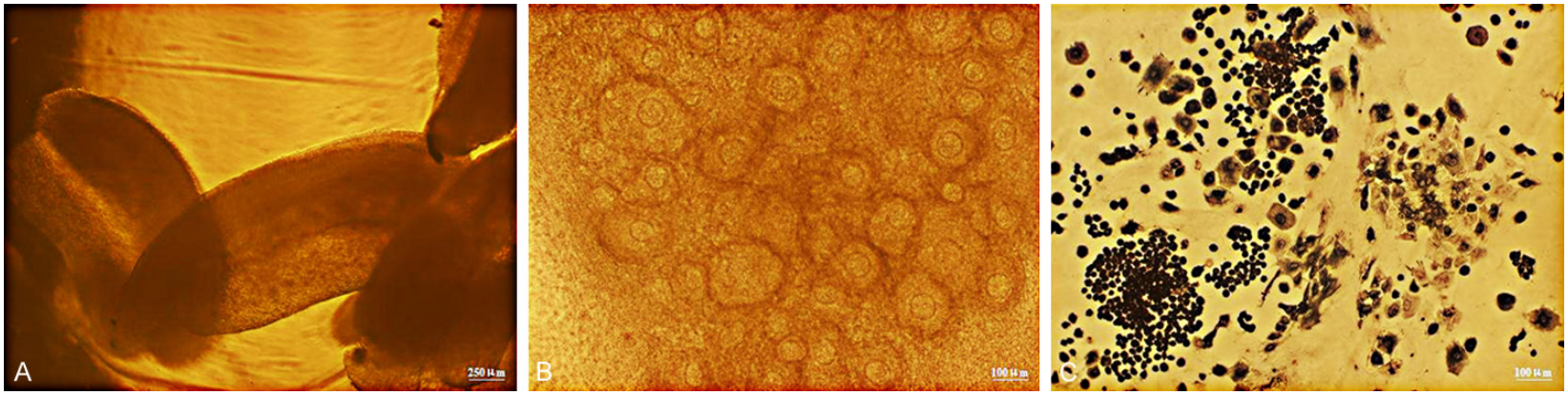


Figure 5. Morphological and biologic characteristics of female primordial gonads from 12.5-dpc fetal mice, where there are large amounts of germ cells during the first meiotic prophase. A: 12.5-dpc female genital ridge containing primordial ovary on the inner side and mesonephros on the outside. Magnification (40×); B: Primordial follicles were observed growing in the primordial ovary by direct adherent culture in 24-well plate after adding RA10 μM for 7 days. Magnification (100×); C: Follicile cells showed positive alkaline phosphatase staining on 9th day of culture. Magnification (100×).

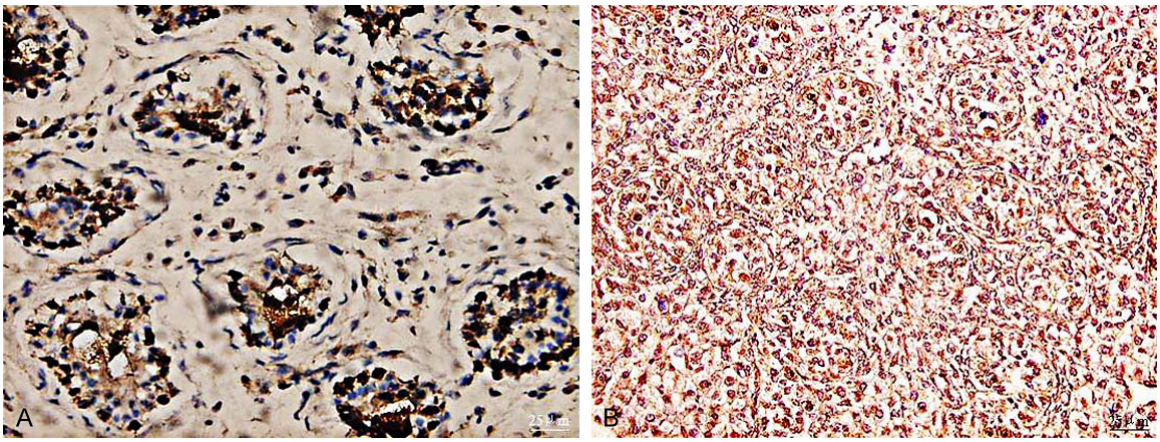


Figure 6. Immunohistochemical analysis using DMC1 and Stra8 antibodies showed specific and high expression in mouse mature and primordial gonads. A: DMC1-positive expression in adult mouse testis. Magnification (200×); B: *Stra8* being highly expressed in primordial gonad from 12.5-dpc Balbc mouse embryos. Magnification (400×).

mouse testis and primordial gonads from 12.5-dpc mouse embryos under a light microscope (**Figure 6**), which marked the existence of universal pre-meiosis-specific genes during meiosis and other processes unique to germ cells. At the sensitive and critical stage of meiosis prophase, germ cell-specific expression of DMC1 (**Figure 6A**), Stra8 (**Figure 6B**) was confirmed to have undergone meiosis to getting sperm or oocyte in primordial or mature gonad.

Alterations in morphology, cytogenetic characteristics of hUMSCs after co-culture with PGCs

HUMSCs from female infants grew alongside PGCs, proliferated and differentiated outside or inside of a diffusion chamber in induction culture medium with RA 10 μM and Forskolin. In appearance, hUMSCs were trending to aggregate and differentiate into oocyte-like cells (**Figure 7A**). Some of them became larger and round (**Figure 7B**). AKP (ALP) activity cells grew in colony-like clusters in the co-culture with PGCs (**Figure 7C**). Morphological changes corresponding to differentiated state of hUMSCs around living PGCs may depend greatly on the meiosis-inducing growth medium.

Under a fluorescence microscope, PGC (12.5-dpc) and hUMSC aggregates in Matrigel suspension culture grew freely like a tissue microstructure with strong proliferation activities (**Figure 8A** and **8B**). After recovery from Matrigel by direct adherent culture, indirect immunofluorescence assay showed that the adherent cells also expressed DDX4 genes (**Figure 8C**).

At the early days of co-culture, hUMSCs developed briskly and differentiated morphologically (**Figure 9E** and **9F**) to PGCs (**Figure 9A** and **9B**). After 12 days, with the extending of co-culture time, both PGCs (**Figure 9C**) and hUMSCs (**Figure 9G**) appeared to have lower growth with decreased colonies and death (**Figure 9D** and **9H**). With continuously RA-induced differentiation and notably increasing RA concentration, cell aging could be speeded up (data not shown).

Expression of pre-meiosis-specific genes in PGCs and hUMSCs after co-culture

Expression of SCP3 positive staining took place in most hUMSCs after co-culture with 12.5-dpc PGCs on slides by indirect immunofluorescent assay. More significantly, DDX4 protein, which was specifically expressed in germline cells, was also positively expressed in co-cultured hUMSCs in RA-induced medium (**Figure 10A** and **10B**). The positive protein expression of SCP3 and DDX4 related to the initial stages of meiosis implied that the PGC growth microenvironment might play an important role in the proliferation, stimulating meiotic differentiation of hUMSCs with molecular and morphological changes induced by RA *in vitro*, whereas in the negative control of vigorous HDF P3-5, SCP3 and DDX4 expression were all negative (**Figure 10C**).

Expression of meiotic genes in hUMSCs analyzed by RT-PCR after co-culture with PGCs

PGCs (12.5-dpc) expressed the germline-specific gene Oct-4 and respectively expressed

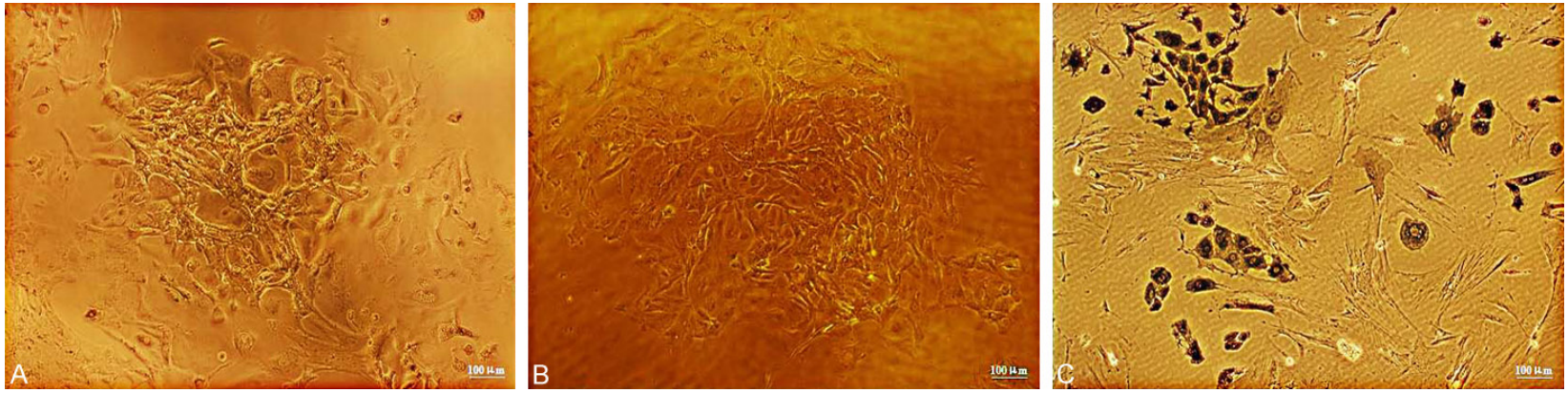


Figure 7. After induced by RA and incubated with PGCs outside or inside of a diffusion chamber, hUMSCs may realize PGC-like differentiation by morphological change of adherent cells and determination of alkaline phosphatase activity (AKP) under a light microscope. A: The shape of co-culturing hUMSCs grew by static adherence on the 5th day inside of a diffusion chamber. Magnification (150×); B: hUMSC on the 5rd day co-culturing outside of a diffusion chamber. Magnification (100×); C: hUMSC differentiated colonies showed separately, scatteredly weakly positive reaction to ALP staining on the 10th day in the microenvironment of live PGCs. Magnification (100×). hUMSCs, human umbilical cord mesenchymal stem cells.

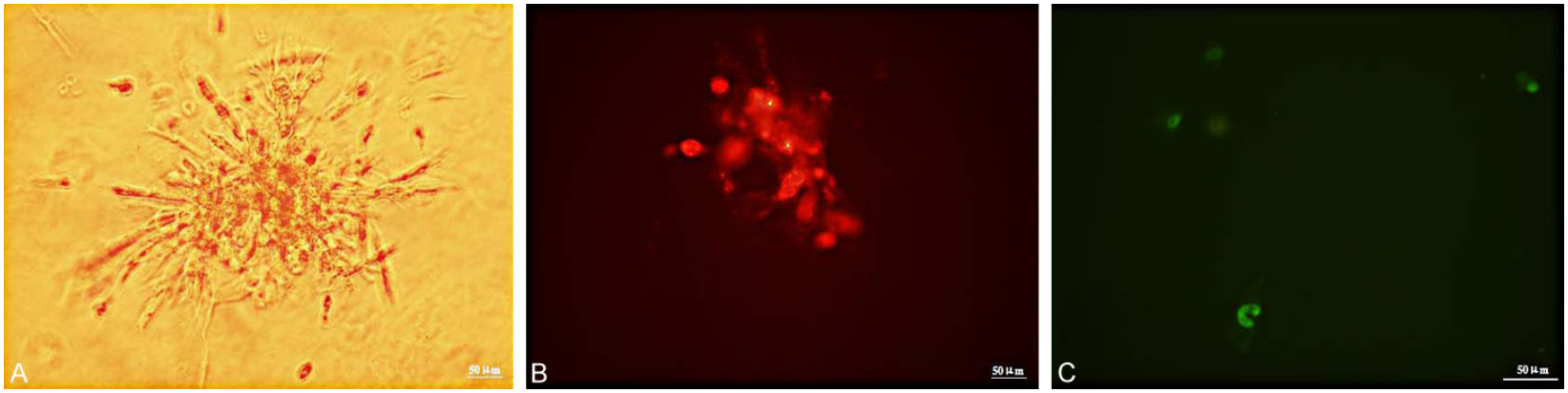


Figure 8. 12.5-dpc PGCs+hUMSCs in suspension culture in Matrigel grew well and continued to keep the meiotic competence. A: The growth of 12.5-dpc PGC+hUMSC aggregates in Matrigel-a three-dimensional environment for 6 d. Magnification (200×); B: RFP MSC in aggregates in Matrigel suspension culture for 12 d. Magnification (200×); C: The expression of protein DDX4 related to early meiosis was positive in the recovered 12.5-dpc PGC+RFP MSC from Matrigel of suspension culture for 15 d after adhesive culture for 8 h. Magnification (300×).

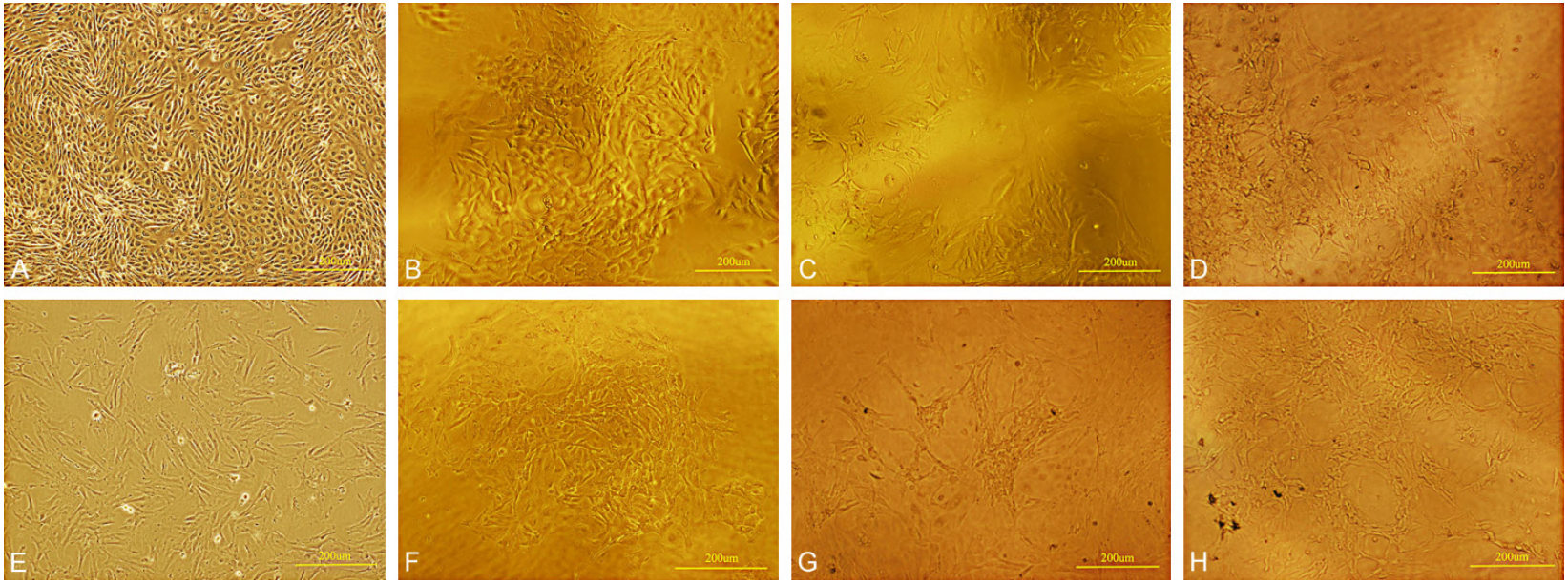


Figure 9. The morphological features of PGCs and hUMSCs before and after co-culture. A: Primary PGCs of 12.5 dpc BalbC mice; B: PGCs at passage 1 co-cultured with hUMSCs in a culture chamber on 4th-10th day; C: PGCs co-cultured on 13th day, showed decreased colonies and clusters; D: PGCs co-cultured on 15th day, dying; E: HUMSCs at passage 3; F: Co-cultured hUMSCs at passage 4 in a culture chamber on the 4th-10th day, with morphologic change similar to PGCs; G: Co-cultured hUMSCs on the 13th day, experienced decreased growth; H: Co-cultured hUMSCs on 15th day, dying.

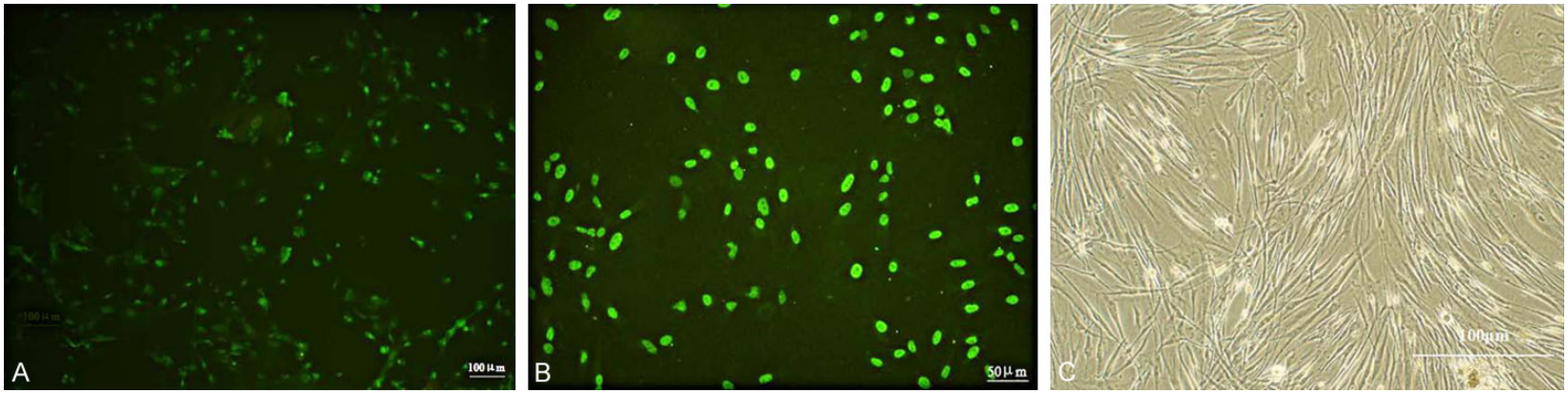


Figure 10. After indirect immunofluorescence cell staining, the expression in cell nucleus of SCP3 and DDX4 on the slide of RFP hUMSCs in co-culture with 12.5-dpc PGCs was observed. A: SCP3-positive cells, faintly visible at image upper left are RFP hUMSCs. Magnification (100×); B: DDX4-positive cells. Magnification (200×);

C: Human foreskin dermal fibroblasts at passage 3 in a good state with negative staining of SCP3 or DDX4. Magnification (100×). hUMSCs, human umbilical cord mesenchymal stem cells.

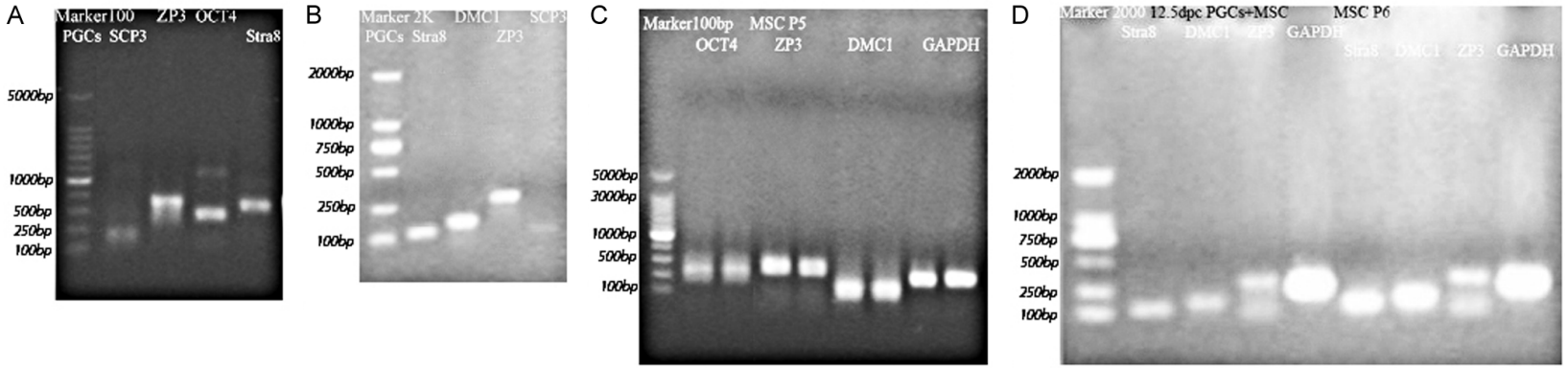


Figure 11. RT-PCR analysis confirmed the expression of SCP3, Zp3, Oct-4, Stra8 and the housekeeping gene GAPDH in PGCs and hUMSCs by 2% agarose gel electrophoresis and visualized by UV light. A: 12.5-dpc PGCs expressed SCP3-Zp3-Oct-4-Stra8; B: 12.5-dpc PGCs expressed Stra8-Dmc1-ZP3-SCP3; C: hUMSCs P5 expressed Oct-4-ZP3-DMC1-GAPDH before meiosis inducing culture; D: hUMSCs co-cultured with 12.5-dpc PGCs on slides and hUMSCs after induced differentiation inside or outside of a chamber expressed Stra8-Dmc1-ZP3-GAPDH. hUMSCs, human umbilical cord mesenchymal stem cells.

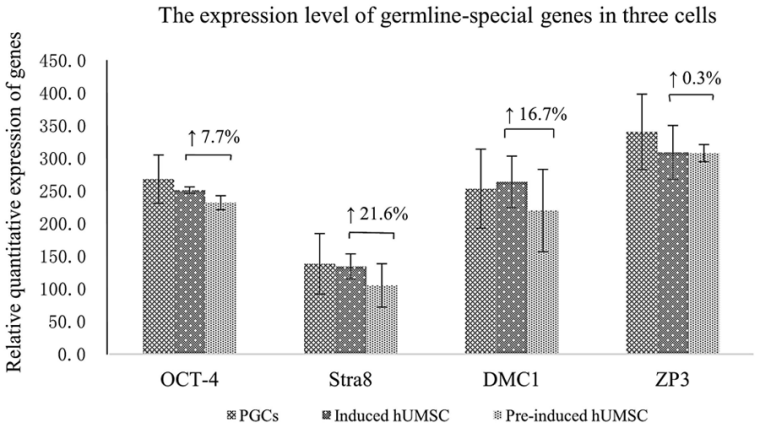


Figure 12. Several germline-specific genes of Oct-4, Stra8, DMC1, ZP3 expression profile in PGCs, hUMSCs before and after differentiating into PGC-like cells, with increasing expression of Oct-4 gene by 7.7%, Stra8 by 21.6%, DMC1 by 16.7%, and ZP3 by 0.3% in hUMSCs after differentiation. hUMSCs, human umbilical cord mesenchymal stem cells.

meiosis-related genes SCP3, Stra8, DMC1 and ZP3 (**Figure 11A** and **11B**) which are involved in the early stages of meiosis. Meanwhile, hUMSCs before meiosis-inducing culture also expressed genes Oct-4, ZP3 and DMC1 to some degree (**Figure 11C**). Furthermore, hUMSCs co-cultured with PGCs outside or inside of a diffusion chamber still expressed the Oct-4 gene, even oocyte-like cell marker ZP3, and obviously expressed meiosis-specific genes Stra8 and DMC1 (**Figure 11D**), compared with pre-induced hUMSCs ($P<0.01$). Relative quantitative expressions of the four genes in hUMSCs after differentiation showed increasing expression of the Stra8 gene by 21.6%, DMC1 by 16.7%, while the Oct-4 gene increased only by 7.7% and the ZP3 gene by 0.3% (**Figure 12**). There was no significant difference of Oct-4 and ZP3 expression in hUMSCs before and after inducing culture *in vitro* ($P>0.05$).

Discussion

Increasing studies are addressing multipotent stem cells-induced differentiation into GSCs, transplanted GSCs and gametogenesis for producing germ cells *in vitro*. However, little is known about the transdifferentiation efficiency and therapeutic potential of undifferentiated MSCs for infertility. High expression of CD29, CD73, CD44, CD90, CD105 and other MSC markers in hUMSCs may provide an ideal cell model for the research of committed stem cells' differentiation and gametogenesis *in vitro*. With the characteristics of being non-invasive, easy to obtain and high biosafety, our results confirmed that hUMSCs highly expressed MSC markers and possessed more proliferation potential and differentiation capacity compared to adult tissue-derived MSCs. They can secrete a wide range of bioactive molecules including growth and antiapoptotic factors, chemokines, interleukin and neurotrophic factors involved in hematopoiesis, neurodevelopment, cardiovascular development and growth by exposure to lineage-specification surroundings [10, 13]. Cho et al. showed that hUMSCs could secrete a high level of basic fibroblast growth factor (bFGF), as feeder cells and a pool of trophic factors, and even enabled undifferentiated human embryonic stem cells (ESCs) subculture to the 30th generation [14]. An important question is whether or not to initiate hUMSC specific differentiation in the meiosis microenvironment and produce homologous recombination like PGCs, oogonia, or home into injured ovaries to restore the fertility of infertile patients? Dissanayake et al. transdifferentiated hUMSCs into male germ cells using all-trans RA and Sertoli cell-conditioned medium [15]. HUMSCs showed good potential prospects in disease rehabilitation and wound repair through activating Wnt, TGF-β, MAPK and other signaling pathways [16].

GSCs may be derived from blood or marrow stem cells and migrate to the ovaries to promote follicular growth in POF [8, 17]. Mouse fetal ovaries can grow like primordial follicles cultured in conditioned medium and in the presence of FBS, ITS, LIF, Forskolin and FSH (**Figure 5B**), which have been confirmed by numerous PGCs with AKP activity, Oct-4 immunofluorescence-positive cells and ZP3 gene expression (**Figures 5B**, **10A** and **11B**). In 12.5-dpc mouse embryos, PGCs migrated into the genital ridge via dorsal mesentery and began to respond to RA generated by mesonephric duct and then went into meiosis [12]. RA regulated by the Cyp26b1 enzyme is the most common activator during germ cell di-

fferentiation, up-regulating the expression of Stra8, Mvh (DDX4) and other meiosis-specific genes through Smad1/5 signal-pathway [15, 18]. When hUMSCs grow with 12.5-dpc PGCs, intercellular communication, paracrine effects and the influence of different growth regulators (rh-LIF, rh-bFGF, Forskolin, SCF, ITS, RA, etc.) which can induce hUMSCs to differentiate into GC-like cells at gene expression levels [10, 12, 19]. So the micro-ecological environment with PGCs, RA, LIF, ITS, Forskolin, SCF and other signaling molecules is crucial for gene expression of SCP3 and DDX4 (**Figure 10A** and **10B**) and will eventually affect hUMSC proliferation, survival and differentiation [10, 20]. Bigger and round hUMSCs with morphological features like PGCs with AKP activity (**Figure 7C**) may result from contact with PGCs. The cell reprogramming could involve gene expression changes, RNA silencing, specific signaling pathway alterations and epigenetic modifications [20, 21].

Gene expression corresponding to biological activity may contribute to biological processes such as modulating primordial follicular development and maturation, thereby maintaining gonadal function [6, 22]. In the living microenvironment of numerically superior PGCs (3:1), by adding RA inducer, signaling molecules such as bFGF, EGF and FSH, hUMSCs have showed high sensitivity to the niche microenvironment with the expression of germ cell-specific genes OCT4, Stra8, Dmc1 and ZP3 (**Figures 11D** and **12**). Playing pivotal regulatory roles in meiosis initiation and haploid formation, Stra8, DMC1, SCP3 and DDX4 expression revealed transdifferentiation of hUMSCs into PGC-like cells *in vitro*, in spite of microenvironment signals with 10% heat inactivated FBS in DMEM/F12 medium in some cases (**Figures 11** and **12**). Weak expression of SSEA-1 protein (**Figure 2E**) indicated that hUMSCs do not behave like embryo stem cells (ESC), even though someone confirmed transdifferentiation of MSCs into germ cells undergoing meiosis under appropriate culture conditions [15, 23]. Oct-4 gene, a transcriptional enhancer, which regulates pluripotency in early germ cells, showed an enhanced expression in hUMSCs after differentiation [24]. DDX4 (VASA) gene, which may be implicated in germline epigenetic reprogramming, is specifically expressed in PGCs during post-migration to post-meiosis, spermatogonium/oogonium, and encodes RNA helicase and promotes meiotic progression [17]. Stra8 (stimulated by retinoic acid gene 8) is indispensable for premeiotic DNA replication and subsequent entry into the prophase of meiosis I [17]. SCP3, Stra8, DMC1 representatively mark the transition from mitosis to meiosis [12, 20, 25]. In our study, the significant increase of Stra8 and DMC1 in induced hUMSCs may imply the ability of differentiation into premeiotic germ cells or putative germ cells *in vitro*, and, more importantly, with the MSC secretome (paracrinic substances) or the microvesicular fraction in terms of protecting organs from acute and chronic damage [26].

Co-cultured and induced with PGCs, biomarker expression of germ cells in hUMSCs showed an interesting source for therapeutic use like other MSCs to mediate tissue-protection in a predominantly indirect manner, rather than substituting GSCs [26]. So induced hUMSCs after transplanting may provide a healthy niche in repairing the injured reproductive system, restoring ovogenesis with a normal endocrine or paracrine support to evoke responses from resident stem cells, decrease ovarian cell apoptosis and treat some incurable diseases including POF. In addition, germline chimeras may increase genetic diversity after being injected into induced pluripotent stem cells (iPSC), though the efficiency of iPSC induction still remains low [17, 27]. However, germ cell development involves complex process of mitosis and meiosis with accurate regulatory mechanisms. It is not determined whether reprogrammed MSCs could complete meiosis and form functional gametes [17]. The transdifferentiation of hUMSCs into PGCLCs are still in the stage of exploration.

In conclusion, easily harvested hUMSCs would be a potential source for cell therapy in infertile women that can respond to adjacent PGC signaling. However, germ cells have a longer growth cycle with a complex meiosis process to produce haploid germ cells and need a specific stem cell niche, microenvironment, critical growth factors and hundreds of genes to complete the first and second meiotic division [20]. The mechanism of meiotic recombination has not been fully understood yet. The probability of induced hUMSCs as germline supplemental cells to address ovarian aging is only theore-

tical upon morphological features and early molecular events in ovogenesis, but it still might encourage further research in developing future regenerative medicine.

Disclosure of conflict of interest

None.

Address correspondence to: Zhiqing Liang, Department of Obstetrics and Gynecology, The First Affiliated Hospital (Southwest Hospital), Army Medical University, No.30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-13320332902; E-mail: zhiqingliang-0903@126.com; Qifeng Tang, Department of Anesthesiology, Shanghai Hechuan-Rhine TCM Hospital, No.3071 Hechuan Road, Minhang District, Shanghai 201103, China. Tel: +86-15862511382; E-mail: ably.tang@qq.com

References

[1] Fonseca DJ, Patiño LC, Suárez YC, de Jesús Rodríguez A, Mateus HE, Jiménez KM, Ortega-Recalde O, Díaz-Yamal I and Laissue P. Next generation sequencing in women affected by nonsyndromic premature ovarian failure displays new potential causative genes and mutations. Fertil Steril 2015; 104: 154-162.

[2] Dang J, Jin Z, Liu X, Hu D and Wang Z. Human cord blood mononuclear cell transplantation for the treatment of premature ovarian failure in nude mice. Int J Clin Exp Med 2015; 8: 4122-4127.

[3] Ebrahimi M and Akbari Asbagh F. The role of autoimmunity in premature ovarian failure. Iran J Reprod Med 2015; 13: 461-472.

[4] Akawatcharangura P, Taechakraichana N and Osiri M. Prevalence of premature ovarian failure in systemic lupus erythematosus patients treated with immunosuppressive agents in Thailand. Lupus 2016; 25: 436-444.

[5] Wang S, Yu L, Sun M, Mu S, Wang C, Wang D and Yao Y. The therapeutic potential of umbilical cord mesenchymal stem cells in mice premature ovarian failure. Biomed Res Int 2013; 2013: 690491.

[6] Bhartiya D, Anand S, Patel H and Parte S. Making gametes from alternate sources of stem cells: past, present and future. Reprod Biol Endocrinol 2017; 15: 89.

[7] Jouni FJ, Abdolmaleki P, Behmanesh M and Movahedin M. An in vitro study of the impact of 4mT static magnetic field to modify the differentiation rate of rat bone marrow stem cells into primordial germ cells. Differentiation 2014; 87: 230-237.

[8] Abd-Allah SH, Shalaby SM, Pasha HF, El-Shal AS, Raafat N, Shabrawy SM, Awad HA, Amer MG, Gharib MA, El Gendy EA, Raslan AA and El-Kelawy HM. Mechanistic action of mesenchymal stem cell injection in the treatment of chemically induced ovarian failure in rabbits. Cytotherapy 2013; 15: 64-75.

[9] Kouchakian MR, Baghban N, Moniri SF, Baghban M, Bakhshalizadeh S, Najafzadeh V, Safaei Z, Izanlou S, Khoradmehr A, Nabipour I, Shirazi R and Tamadon A. The clinical trials of mesenchymal stromal cells therapy. Stem Cells Int 2021; 2021: 1634782.

[10] Boukelmoune N, Chiu GS, Kavelaars A and Heijnen CJ. Mitochondrial transfer from mesenchymal stem cells to neural stem cells protects against the neurotoxic effects of cisplatin. Acta Neuropathol Commun 2018; 6: 139.

[11] Asgari HR, Akbari M, Abbasi M, Ai J, Korouji M, Aliakbari F, Babatunde KA, Aval FS and Joghataei MT. Human Wharton's jelly-derived mesenchymal stem cells express oocyte developmental genes during co-culture with placental cells. Iran J Basic Med Sci 2015; 18: 22-29.

[12] Rodriguez KF, Brown PR, Amato CM, Nicol B, Liu CF, Xu X and Yao HH. Somatic cell fate maintenance in mouse fetal testes via autocrine/paracrine action of AMH and activin B. Nat Commun 2022; 13: 4130.

[13] Nartprayut K, U-Pratya Y, Kheolamai P, Manochantr S, Chayosumrit M, Issaragrisil S and Supokawej A. Cardiomyocyte differentiation of perinatally-derived mesenchymal stem cells. Mol Med Rep 2013; 7: 1465-1469.

[14] Cho M, Lee EJ, Nam H, Yang JH, Cho J, Lim JM and Lee G. Human feeder layer system derived from umbilical cord stromal cells for human embryonic stem cells. Fertil Steril 2010; 93: 2525-2531.

[15] Dissanayake D, Patel H and Wijesinghe PS. Differentiation of human male germ cells from Wharton's jelly-derived mesenchymal stem cells. Clin Exp Reprod Med 2018; 45: 75-81.

[16] Lavoie JR and Rosu-Myles M. Uncovering the secretes of mesenchymal stem cells. Biochimie 2013; 95: 2212-2221.

[17] Zhang YL, Li PZ, Pang J, Wan YJ, Zhang GM, Fan YX, Wang ZY, Tao NH and Wang F. Induction of goat bone marrow mesenchymal stem cells into putative malegerm cells using mRNA for STRA8, BOULE and DAZL. Cytotechnology 2019; 71: 563-572.

[18] Chen W, Jia W, Wang K, Zhou Q, Leng Y, Duan T and Kang J. Retinoic acid regulates germ cell differentiation in mouse embryonic stem cells through a Smad-dependent pathway. Biochem Biophys Res Commun 2012; 418: 571-577.

[19] Tan H, Wang JJ, Cheng SF, Ge W, Sun YC, Sun XF, Sun R, Li L, Li B and Shen W. Retinoic acid

promotes the proliferation of primordial germ cell-like cells differentiated from mouse skin-derived stem cells in vitro. Theriogenology 2016; 85: 408-418.
[20] Hu X, Wang H, Tian GG, Hou C, Xu B, Zhao X, Zhao Y, Fang Q, Li X, He L, Chen X, Li S and Wu J. Offspring production of haploid spermatid-like cells derived from mouse female germline stem cells with chromatin condensation. Cell Biosci 2022; 12: 5.
[21] van den Driesche S, Sharpe RM, Saunders PT and Mitchell RT. Regulation of the germ stem cell niche as the foundation for adult spermatogenesis: a role for miRNAs? Semin Cell Dev Biol 2014; 29: 76-83.
[22] Tang X, Zhang C, Jin Y, Ge C and Wu Y. Pro-proliferating effect of homologous somatic cells on chicken primordial germ cells. Cell Biol Int 2007; 31: 1016-1021.
[23] Schneider RK, Puellen A, Kramann R, Raupach K, Bornemann J, Knuechel R, Pérez-Bouza A and Neuss S. The osteogenic differentiation of adult bone marrow and perinatal umbilical mesenchymal stem cells and matrix remodelling in three-dimensional collagen scaffolds. Biomaterials 2010; 31: 467-480.
[24] Li Y, Hong WX, Lan B, Xu X, Liu Y, Kong L, Li Y, Zhou S, Liu Y, Feng R, Jiang S, He Q and Tan J. PDGF mediates derivation of human embryonic germ cells. Differentiation 2013; 86: 141-148.
[25] Rebscher N, Zelada-González F, Banisch TU, Raible F and Arendt D. Vasa unveils a common origin of germ cells and of somatic stem cells from the posterior growth zone in the polychaete platynereis dumerilii. Dev Biol 2007; 306: 599-611.
[26] Patschan D, Buschmann I, Ritter O and Kribben A. Cell-based therapies in acute kidney injury (AKI). Kidney Blood Press Res 2018; 43: 673-681.
[27] West FD, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, Gallegos-Cardenas A, Pratt SL and Stice SL. Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. Stem Cells 2011; 29: 1640-1643.