

Human Wharton's jelly-derived mesenchymal stem cells express oocyte developmental genes during co-culture with placental cells

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ABSTRACT

Objective(s): The present day challenge is how to obtain germ cells from stem cells to treat patients with cancer and infertility. Much more efforts have been made to develop a procedure for attaining germ cells *in vitro*. Recently, human umbilical cord-derived mesenchymal stem cells (HUMSCs) have been introduced with higher efficacy for differentiation. In this work, we tried to explore the efficacy of HUMSCs and some effective products of placental cells such as transforming growth factors. This study is aimed to optimize a co-culture condition for HUMSCs with placental cells to obtain primordial germ cells (PGCs) and reach into oocyte-like cells *in vitro*.

Materials and Methods: In this experimental study, HUMSCs and placental cells were co-cultured for 14 days without any external inducer *in vitro*. Then HUMSCs were assessed for expression of PGC markers; Octamer-binding transcription factor 4(OCT4), Tyrosine-protein kinase Kit (CKIT), Stage specific embryonic antigen 4 (SSEA4), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4(DDX4) and oocyte specific markers; Growth differentiation factor-9(GDF9), Zona pellucida glycoprotein 3(ZP3). The pertinent markers were assessed by immunocytochemistry and Q-PCR.

Results: Co-cultured HUMSCs with placental cells (including amniotic and chorionic cells) presented Oct4 and DDX4, primordial germ cells specific markers significantly, but increment in expression of oocyte-like cell specific markers, GDF9 and ZP3 did not reach to statistically significant threshold.

Conclusion: Placental cell supplements Transforming growth factor (TGF α , β) and basic fibroblast growth factor (bFGF) in a co-culture model can provide proper environment for induction of HUMSCs into PGCs and expression of oocyte-like markers.

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Introduction

Based on World Health Organization reports, infertility is estimated as a common disease, which affected 14% of couples in fertile age in Europe. A national survey on family growth Americans in 2005 reported that there was a 20% increase in American couples experiencing infertility between 1995 and 2002. Another paper stated an increase from 42% to 48.5% from 1990 to 2010 that may be related to delayed motherhood in the third decade of life and consequently resulted in decrease of oocyte quality in females. The short reproductive period in women can be even shortened if oocytes or granulosa cells are damaged following cancer treatments and as a

result assisted reproductive techniques is required (1, 2).

In recent years, stem cells can be considered as the potential solution for infertility problems by producing germ cells from stem cells. Stem cells are undifferentiated cells that have the potential of self-renewal and can give rise to specialized cells like germ cells that can be used for therapeutic purpose and in regenerative medicine (3). Recent studies explained that PGCs and gametes were derived from different types of stem cells such as embryonic stem cells (4-6), somatic stem cells like bone marrow and adipose tissue stem cells (7-10). However invasive methods for isolation of bone marrow, and

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embryonic stem cells and ethical considerations for application of embryonic stem cells, provoked researchers to find alternative sources. Recently, scientists have found a new source of stem cells from birth-associated tissues like umbilical cord. Contrary to bone marrow and embryonic stem cells, isolation of HUMSCs from the umbilical cord and placenta of a newborn (11, 12) is non-complicated, without any risk of contamination, ethical issues and invasive methods (13, 14).

HUMSCs can differentiate into cells of three embryonic layers, such as neuron (15-17), muscle, cartilage, hepatocyte, bone and cardiomyocyte (17). These cells and other neonatal associated tissues have a high efficacy to differentiate in comparison with other mesenchymal stem cells. In addition to their multipotent features, which are between that of adult and embryonic stem cells, it seems that these cells have high potential to differentiate into germ cells (19, 20). Therefore, establishment of an umbilical cord bank will be useful in the future for the preservation of newborns from cancer and infertility (21). They also have immunosuppressive and immunoprivilege properties owing to expression of HLA-G6, and these properties make them sufficient for allograft and even xenotransplantation of germ cells or gametes for infertility, cancer and chemotherapy in different stages of life (16, 19).

Huang *et al* in 2010 tried to obtain HUMSC-derived PGCs by using some inducers (20). Other scientists used growth factors, testicular conditioned medium, follicular fluid and gonadotropins and retinoic acid for various types of stem cells (8, 10, 11, 22). In this work, we used placental cells, which some scientists named them human body "spare parts" (23) or universal feeder cells (24). Some works based on placental cells have shown some positive properties like expressing growth factors (TGF α , TGF β , bFGF) (25-27), which are all important factors for a micro-environment needed for cell proliferation, tissue regeneration, cellular differentiation, cellular transformation and regulation of the immune system (28, 29). These cells may provide proper condition for transplantation as they did not show tumorigenicity and adverse immunological responses (19). Human placental cells decrease the risk of zoonosis in culture location (30). So, from the above mentioned properties, placental cells including amniotic and chorionic cells can provide an appropriate micro-environment for induction of HUMSCs. The aim of this study was to investigate the expression of PGC and oocyte like cell markers in HUMSCs. To accomplish this objective, we used placental cells to induce HUMSCs and to identify whether these cells were able to generate primordial germ cells and express principal oocyte-like cell markers *in vitro*.

Materials and Methods

Isolation and expansion of HUMSCs

All the sampling procedures were performed according to ethical guidelines of Tehran University of Medical Sciences. Samples (umbilical cord and placenta) were utilized after informed written consent of parents. Samples of full-term and healthy female newborns after cesarean delivery were transferred to laboratory in HBSS (Hanks Balanced Salt Solution) on ice. All cell culture reagents were purchased from GIBCO, except those specified. HUMSCs were isolated from umbilical cord using explant method (11). Amniotic membrane, arteries and vein were dissected out and the remaining Wharton's jelly minced into small pieces. Wharton's jelly fragments were plated in Dulbecco's modified essential medium-F12 supplemented with 10% FBS (Fetal Bovine Serum) and 100 units/ml penicillin-streptomycin and maintained in 37°C humidified incubator with 5% CO₂. Cultures were allowed to migrate for 3 weeks. Migrated cells were sub-cultured by 0.25% Trypsin-EDTA and morphologically evaluated with inverted phase contrast microscope (Olympus CKX-41). Cells upon 80 to 90% confluency were passaged and the medium was refreshed every other day. Cells from passages 3 to 4 were utilized for differentiation studies.

Differentiation into osteoblasts

HUMSCs were treated with osteogenic medium (Gibco-Invitrogen) for 21 days with twice a week medium change. The flasks were assessed every 3 days for osteogenesis. Osteogenesis progress was evaluated with Alizarin-red S staining. The cells were fixed with 4% paraformaldehyde and stained with 1% Alizarin-red S (Sigma-Aldrich) solution for 10 min.

Differentiation into adipocytes:

HUMSCs were treated with adipogenic medium (Gibco-Invitrogen) for 21 days and the medium was refreshed every 3 days. The cells were assessed every 3 days. Adipogenesis was evaluated by Oil Red-O staining. Briefly, the cells were fixed with 4% paraformaldehyde and stained with Oil Red-O (Sigma-Aldrich) for 10 min and observed with inverted phase contrast microscope.

Flowcytometry analysis

Superficial markers of isolated cells in third passage were assessed. To this end, the cells were detached by 0.25% Trypsin-EDTA and centrifuged at 1000 RPM for 5 min. Cells were resuspended with phosphate buffered saline (PBS) to 10⁶ cells per ml, and different aliquots were incubated with monoclonal antibodies against CD34-PE, CD45-FITC, CD29-PE, CD3-FITC, CD73-PE, CD90-FITC, CD105-FITC and human leukocyte antigen (HLA-FITC) for 30 minutes at 4°C in the dark. Isotype antibodies conjugated to FITC or PE were used for cell surface staining. Finally, cells were fixed with 1%

paraformaldehyde solution and analysed by Partec PAS III flowcytometry system, under Flomax software.

Isolation and expansion of placental cells (chorionic and amniotic cells)

After thoroughly wash in PBS, amniotic membrane was separated from chorionic plate, and tissues were separately incubated with 0.25% Trypsin-EDTA for 30 min followed by PBS wash. Afterward, enzymatic digestion was followed with 0.05% collagenase 1 and 4 for 30 min and Dispase 2 (2.5 mg/ml, Invitrogen) for 7 min. To remove tissue debris, cell suspension was filtered through 100 μ m cell strainer. Cells were cultured in DMEM-F12 supplemented with 10% FBS and 100 units/ml penicillin-streptomycin and maintained in 37°C humidified incubator with 5% CO₂.

Co-Culture method

To prepare for co-culture process, placental cells (included amniotic and chorionic cells) were inactivated by mitomycin-C (Sigma) for 3 hr and cultured in their medium overnight before further use. HUMSCs (passage 3) were cultured in the 6-well plates and placental cells (passage 3) plated on the 0.4 μ m cell culture inserts (ThinCert - greiner) which provide a reciprocal and indirect co-culture system. Co-culture medium contained DMEM-F12 supplemented with 5% FBS (FBS - GIBCO). Half of the medium was refreshed every 2 to 3 days and the co-culture was continued for 14 days. HUMSCs, which were treated with 1 μ m all-trans retinoic acid (Sigma-Aldrich) for 6 days and cultured in DMEM-F12 and 2% FBS were served as control group for gene expression studies.

Immunocytochemistry

For immunofluorescent assay of germ-cell markers in HUMSCs, 14 days after co-culture, cells were washed with PBS and fixed in 4% paraformaldehyde at 4°C for 10 min. Then, cells were washed 3 times with PBS and incubated in 0.1% Triton X-100 for 10 min. Non-specific binding sites were blocked with 5% goat serum for 45 min without washing followed by incubation with anti-C-kit (abcam, Rabbit polyclonal anti-human, 1:100), anti-SSEA4 (abcam, Mouse monoclonal anti-human, 1:100), anti-DDX4 (abcam, Rabbit polyclonal anti-human, 1:100), anti-GDF9 (abcam, Rabbit polyclonal anti-human, dilution 1:200) antibodies for overnight at 4°C. Then cells were washed with PBS and incubated with FITC-conjugated Goat Anti-Rabbit, (abcam, 1:50) or Goat Anti-Mouse (abcam, 1:200) for 1 hr at room temperature. Nuclei were counter-stained with DAPI (Sigma) for 5 min and observed by fluorescent microscope (Olympus, IX71).

Quantitative PCR

To isolate total RNA, Tri Pure reagent (Roche) was directly added to cells and processed per manufacturer

instruction. Genomic DNA contamination was digested using RNase-free DNase I (Thermo Scientific, Waltham, MA) for 30 min at 37°C. RNA concentration and purity were determined by spectrophotometric method (WPA spectrophotometer, Biochrom, UK). RNA was reversely transcribed by random Hexamer and 1000 ng of DNA-free RNA using transcriptor first strand cDNA synthesis kit (Roche). TaqMan® Gene Expression Assays (Life Technologies) were recruited to study the expression of SSEA4, DDX4, GDF9 and ZP3, which normalized against 18 sec expression as a housekeeping gene. PCR reaction mixture components for a final volume of 20 μ l were as follows: 10 μ l TaqMan® Universal Master Mix, 1 μ l TaqMan® Assay reagent, 0.5 μ l (25 ng) cDNA and 8.5 μ l distilled water. PCR cycling parameters were set for 10 min at 95°C (polymerase activation) and 40 cycles of 95°C for 15 second and 60°C for 1 min using a Rotor-Gene Q instrument (Qiagen). Using $\Delta\Delta$ Ct method, relative expression of targets was calculated by normalizing Ct values of targets against 18 sec.

Results

Cell morphology

Primary isolated cells from Wharton's jelly fragments after first passage were spindle-shaped and revealed fibroblast-like morphology. HUMSCs possessed some long and short processes with high potential of proliferation (Figure 1, a-e).

Flow cytometry findings

HUMSCs at passage 3 were assessed for their purity. They were mostly positive against mesenchymal stem cell markers, CD73, CD90, and CD105 and minimally reacted with CD34, CD45 (hematopoietic markers), CD14 (monocyte differentiation antigen),

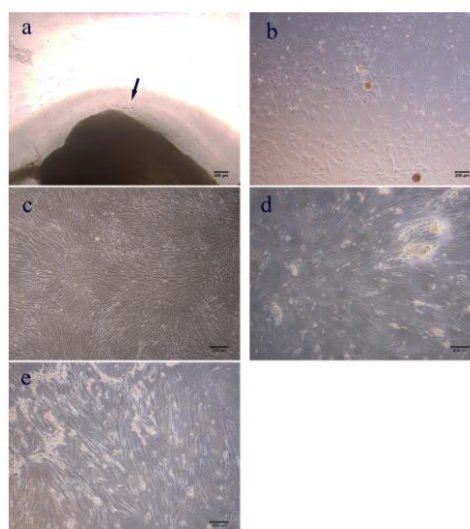


Figure 1. A-D: The appearance of human umbilical cord derived mesenchymal stem cells. (a) The cells are migrating out from a piece of Wharton's jelly (Black Arrow). (b) The cells after first passage forming their colonies. (c) The cells after third passage fill the bottom of flask. (d) Passage 4 co-cultured cells during differentiation at day 6. Magnification (40)

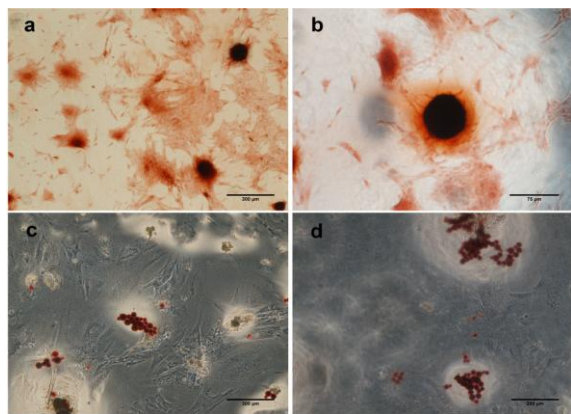


Figure 2. Osteogenic and adipogenic differentiation of human umbilical cord derived mesenchymal stem cells, (A, B) Cells in the osteogenic induction, prepared with Alizarin red staining after 21 days of induction. (C) Cells in the adipogenic induction, prepared with Oil-red O staining after 21 days of induction. Magnification (20×, 40×)

CD3 (T-cell co-receptor) and HLA (human leukocyte antigen). HUMSCs were 99.33%, 94.30%, and 87.22% positive for CD90, CD73 and CD105, respectively. The cells also showed negligible presence for CD45 (0.42%), CD34 (7.37%), CD14 (10.75%), CD3 (4.04%) and HLA (11.61%) (Figure 2, a-h). These results confirmed the purity of HUMSCs and ruled out the hematopoietic origin of isolated cells.

Adipocyte and osteoblast differentiation

HUMSCs after 21 days treatment with osteogenic and adipogenic differentiation medium stained positively with Alizarin Red-S for osteoblasts (Figure

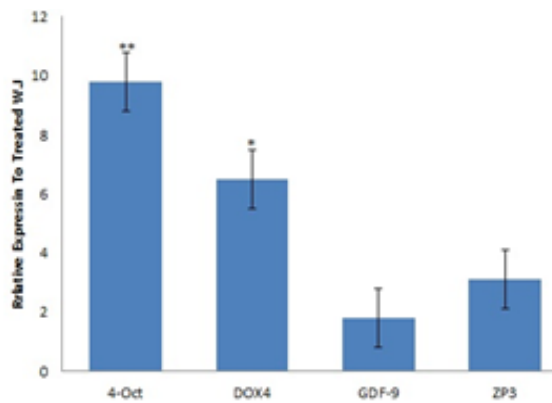


Figure 4. Human umbilical cord mesenchymal stem cells expression of PGC and oocyte markers in co-culture condition relative to all-trans Retinoic acid treated human umbilical cord mesenchymal stem cells. OCT4 and DDX4 (PGC markers) revealed significant increase in co-culture condition (P -value<0.01 and P -value<0.05, respectively). GDF9 and ZP3 (oocyte marker) were up-regulated in co-culture but did not achieve statistical significance threshold

3-a, b) and Oil Red-O for adipocytes (Figure 3-c). In addition, morphological changes corresponding to differentiated state of cells were detected.

Immunocytochemistry findings

HUMSCs after 14 days co-culture with placental cells presented strongly positive signal for PGC markers (SSEA4, C-kit and DDX4), marker of oocyte-like cells and GDF9, (Figure 5, a-h) in immunofluorescent studies. Cells in control group also expressed the markers but with considerably weaker signal (data not shown).

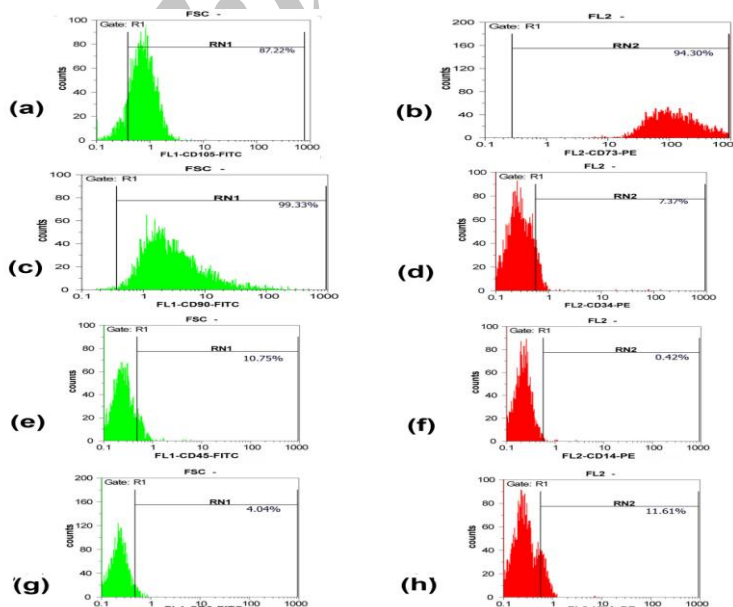


Figure 3. Characteristics of human umbilical cord derived mesenchymal stem cells, Flow cytometric analysis of surface-markers expression. The cells after passage 3 were labelled with PE or FITC-conjugated antibodies. Cells were positive for CD105 (a), CD73 (b), CD 90 (c), and negative for CD34 (d), CD45 (e), CD 14 (f), CD3 (g) and HLA (h). Data were representative of three independent experiments

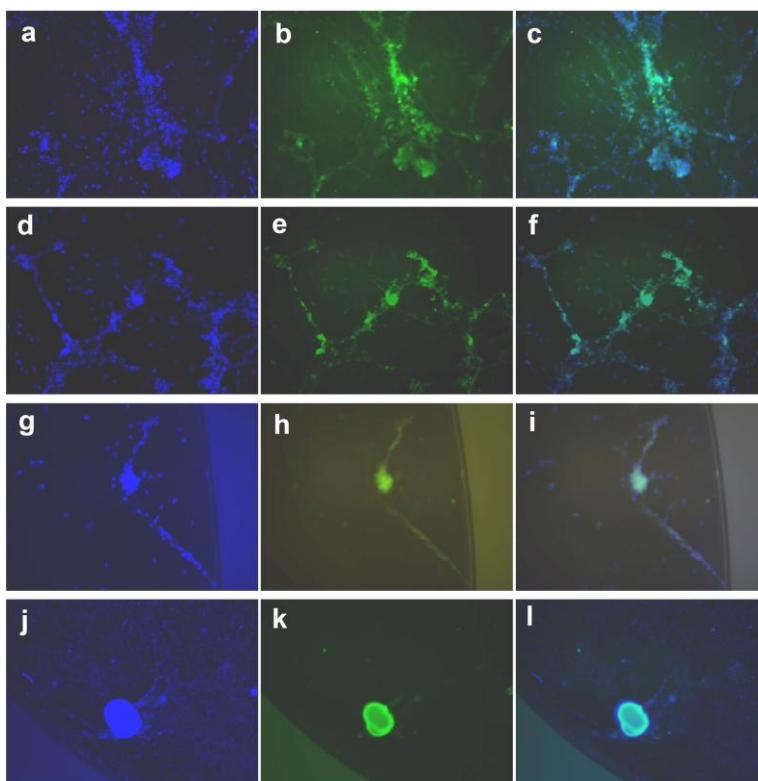


Figure 5. Immunocytochemistry analysis of human umbilical cord mesenchymal stem cells after 14 days co-culture with placenta cells (including amniotic and chorionic cells) for PGC specific markers; SSEA4 (a) nuclei in blue DAPI, (b) FITC positive cells, (c) merged view of SSEA4 positive cells. OCT4 (d) nuclei in blue DAPI, (e) OCT4 positive cells marked with FITC, (f) merged view of OCT4 positive cells. DDX4 (g) nuclei in blue DAPI, (h) DDX4 positive cells marked with FITC, (i) merged view of DDX4 positive cells. GDF9 (j) nuclei in blue DAPI, (k) GDF9 positive cells marked with FITC, (l) merged view of GDF9 positive cells. Magnification: 40×

Expression of primordial germ cells and oocyte-like cells genes

HUMSCs after 14 days co-culture with the human placental cells (including chorionic and amniotic cells) compared to retinoic acid treated HUMSCs. So, RNA extraction and quantitative PCR for cells were performed. The cells were significantly increased the expression of primordial germ cells specific markers, OCT4 and DDX4, but the up-regulation of oocyte-like cell specific markers, GDF9 and ZP3, did not achieve to statistical significance threshold. Expression of Oct4 and DDX4 in co-cultured group was 9.8 and 6.5 times higher compared to retinoic acid treated group, which showed significant trend P -value<0.01 and P -value<0.05, respectively. However, 1.7 and 3.1 times increment in expression of oocyte markers (GDF9 and ZP3) were not significant comparing to control group (Figure 4).

Discussion

In the present study, we reported that an innovative co-culture of placental cells can induce transition of HUMSCs into PGCs associated with expression of oocyte-like cells within 14 days by displaying special PGC markers such as OCT4, SSEA4, C-kit, DDX4 (VASA), and expression of oocyte-like

cell special markers including GDF9 and ZP3 *in vitro*. To the best of our knowledge, this is the first study that determined the expression of above markers by induction of HUMSCs within 14 days of *in vitro* co-culture with placental cells (including amniotic and chorionic cells) without any external inducer. It has been demonstrated that mesenchymal stem cells have more capability to differentiate than any other type of stem cells, but in our work HUMSCs from birth associated tissues was used, which has been reported to have a high efficacy to differentiate than any other mesenchymal stem cells from adult sources (19).

HUMSCs showed high expression of OCT4, Nanog and SOX2 same as embryonic stem cells and also expressed CD29, CD44 and CD59 markers such as pluripotent adult stem cells (19, 33). It has been demonstrated that HUMSCs have a high proliferative potential in first passages, and they have 24 hours doubling time that preserved for 80 passages (19, 34). It has been shown that HUMSCs were protected from immune rejection responses and their ability for immunocytes (T, B and neutral killer cells) activation suppressed; therefore, they have been considered as a suitable cell source for germ cell generating, cell therapy and transplantation (19).

These properties discriminated HUMSCs from other stem cells. Our flowcytometry findings showed acceptable purity, and HUMSCs potential characterized in osteocyte and adipocyte differentiation. HUMSCs presented appropriate potential for differentiation into PGCs and expression of PGC markers such as SSEA4, OCT4, C-kit and DDX4 alongside with oocyte specific genes like GDF9 in immunocytochemistry findings. On the other hand, we showed that feeder cells from placenta including amniotic and chorionic stem cells isolated from neonatal associated sources have higher differentiation potential and contain group of pluripotent stem cells that express OCT4, SOX2, SSEA4, and Nanog (19). Some researchers have reported that placental cells produce substances such as transforming growth factors α , β_1 , β_2 , β_3 and basic fibroblast growth factor (bFGF) in their supplement (25). It has been shown that bFGF is capable to support the PGCs survival and motivates female pathway in reproductive system (26, 35). Also, three members of the TGF β family (β_1 , β_2 , β_3) are highly similar, and they are efficient proteins in cell proliferation, tissue regeneration, and cellular differentiation in most cells alongside with anti-apoptotic and anti-oxidative effects (36, 37).

Our results based on recent studies revealed that human placental cells including amniotic and chorionic cells as a group of feeder cells have enough capability to induce differentiation of HUMSCs into germ cells and expression of oocyte like cells in co-culture condition. This capacity of placental cells was indicated in Q-PCR results by obvious increase of PGC markers expression such as OCT4 and DDX4 and a lower expression of oocyte markers like GDF9 and ZP3 in comparison with retinoic acid treated group.

In immunocytochemistry findings, the expression of SSEA4, C-kit, DDX4 and GDF9 protein markers were recorded. In our study, expression of genes and synthesis of protein markers in different developmental stages of germ cells were analyzed. SSEA4 (Stage specific embryonic antigen) is a pluripotency marker that also presents in human germ cells. OCT4 (Pou5f1) is a germ line-specific transcription factor, essential for primary stage of germ cell diagnosis and promotes germ cell survival and pluripotency (38, 39). C-kit is a migration and survival marker of PGCs that highly expressed by them, and a cell surface marker which binds to stem cell factor. DDX4 (VASA or DEAD Box Protein 4) is a pre-meiotic germ cell specific marker, which is an important protein in post migratory stage of primordial germ cells (22). GDF9 (growth differentiation factor-9) is known as the specific marker for oocyte recognition. ZP3 (zona pellucida producing gene- 3) is critical for oocyte construction; ZP proteins are markers of female germ cells and are detected in post-meiotic development (40). Recent works took the advantage of some inducers for induction of somatic stem cells to germ cells, utilizing

retinoic acid. RA signaling was activated in bone marrow stem cells and induced meiosis and differentiation into spermatogonial-like cells (7). In the same line, follicular fluid and gonadotropins influenced fetal porcine skin stem cells and created oocyte-like cells (8), while others applied testicular cell conditioned medium for induction of embryoid bodies and produced immature oocyte-like cells (5). In another strategy, ovarian granulosa cells co-cultured with embryoid bodies for generating oocyte like cells (35). Based on the above findings, we used retinoic acid as an inducer for our treated group in Q-PCR, and applied co-culture method as a functional and useful method for induction of HUMSCs (35, 41). We showed that co-cultured HUMSCs with placenta cells have higher capability of expressing PGC and oocyte-like cell markers. However, direct co-culture with cell-cell interaction may improve the expression of markers. Further studies should investigate HUMSCs derived PGCs features in more detail and appraise them in transplantation settings. We revealed that placental cells including amniotic and chorionic cells possessed effective role in differentiation of HUMSCs into PGCs and directed them toward oocyte-like cells. Parallel to previous studies, we thereby strengthen the fact that human umbilical cord mesenchymal stem cells have the capabilities to differentiate into germ line cells.

Conclusion

Finding novel inducers for differentiation of stem cells into germ cells is challenging. Herein, we introduced a co-culture system to induce HUMSCs into germ cells by secretory substances from placental cells, which may open a new avenue for induction methods in this interesting context.

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