Bone morphogenetic protein 15 induces differentiation of mesenchymal stem cell derived from human follicular fluid to oocyte like cell

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Background. To study the effect of Bone morphogenetic protein 15 on differentiation potential of mesenchymal stem cell derived from human follicular fluid to oocyte like cell. **Methods**. Human FF derived cells were collected from 78 women in assisted fertilization program, and cultured in differentiation medium containing human recombinant BMP15 for 21 days. Mesenchymal stem cells and OLCs were characterized by real-time PCR and immunocytochemistry (ICC) staining. **Results.** MSCs expressed germ line stem cell markers, such as OCT4 and NANOG. After 15 days, OLCs formed and expressed zona pellucida markers (ZP2, ZP3), and reached 20 – 30 µm in diameters. Ten days after induction with BMP15, round cells remarkably developed, and the maximum size of OLCs reached 115 µm. Finally, a decrease ranging from 0.04 to 4.5 in the expression of pluripotency and oocyte specific markers was observed in the cells cultured in BMP15 supplemented medium. Our work demonstrates, FF derived MSCs have an innate potency to differentiate into OLCs, and BMP15 is effective in stimulating the differentiation of these cells, which may give an *in vitro* model to examine human germ cell development.

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Abstract

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Methods. Human FF derived cells were collected from 78 women in assisted fertilization program, and cultured in differentiation medium containing human recombinant BMP15 for 21 days. Mesenchymal stem cells and OLCs were characterized by real-time PCR and immunocytochemistry (ICC) staining.

Results. MSCs expressed germ line stem cell markers, such as OCT4 and NANOG. After 15 days, OLCs formed and expressed zona pellucida markers (ZP2, ZP3), and reached 20 – 30 µm in diameters. Ten days after induction with BMP15, round cells remarkably developed, and the maximum size of OLCs reached 115 µm. Finally, a decrease ranging from 0.04 to 4.5 in the expression of pluripotency and oocyte specific markers was observed in the cells cultured in BMP15 supplemented medium.

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Introduction

Over the past decades, there has been debate among the reproductive scientists about the origin of ovarian germ cells and oogenesis during life span(Bukovsky 2011). However, there have been two main hypotheses in animal oogenesis. One theory is that the process of oogenesis occurs periodically throughout the reproductive life. Another one states that the oogenesis takes places during fetal period without mitotic division of oogonia within puberty(Hübner et al. 2003). In fact, new observations demonstrated the neo-oogenesis in mouse ovary and adult human(de Souza et al. 2017). In the early of 21th century, studies on rodents questioned the notion of definite ovarian reserve being endowed during perinatal period.(Johnson et al. 2005)According to numerous approaches, deductions showed that adult mice ovaries contain scarce oogonial

stem cells (OSCs), which produce oocytes, resembling to spermatogonial stem cells in mature testes (Brinster 2007). These cells rapidly grow *in vitro* for weeks, and automatically produce naïve oocytes in culture medium(Pacchiarotti et al. 2010).

Recent evidence indicated the components of ovaries for new primary follicles in the adult human beings, meaning continuous differentiation of germ cells and primitive granulosa cells from mesenchymal progenitor cells (MPCs) residing in the ovarian tunica albuginea (Bukovsky et al. 2005). Nevertheless, the MPCs can result in creation of epithelial cells as the granulosa cells. Surveys such as the one conducted by Heng et al. (2005) imply to a potent stem cell niche into immature ovarian follicles. Previous studies on the multipotency in the follicular antrum cells showed that some of which possess characteristics of mesenchymal stem cells (MSC) (Kossowska-Tomaszczuk et al. 2009b). Three ovarian functional somatic cell kinds are necessary for the follicle growth and antrum expansion. Three ovarian functional somatic cell kinds are necessary for the follicle growth and antrum expansion. These include granulosa cells (GCs), theca cells, and ovarian surface epithelium(OSE)(Rodgers & Irving-Rodgers 2010). Among these cells, OSE is a multipotential epithelium containing stem cell features, which play key roles in oogenesis and tumorigenesis (Auersperg et al. 2001).

Additionally, Zou et al. (2009) confirmed that the cultured ovarian stem cells transplantaion might produce new oocyte in sterilized mice ovary. Eventually, Woods & Tilly (2013) approved the existence of mitotically active germ cells in mature mice and human ovary, both *in vitro* and *in vivo* can differentiate into oocytes). The female germ cell generation from embryonic stem cells was demonstrated previously (Hübner et al. 2003), but the *in vitro* generation of somatic germ cells can present an interesting model for recognizing factors concerned with forming and differentiating germ cell. Therefore, many authors tried to appoint if murine or human embryonic stem cells(ESCs) have the ability for differentiating into PGCs or OLCs *in vitro* or not (Hübner et al. 2003; Kee et al. 2006). Additionally, the authors announced that germ cell-like cells are attainable *in vitro* from human adult ovaries, as well as in other species(Bukovsky et al. 2005; Dyce et al. 2010). Isolating and manipulating ovarian stem cells possess enormous medical, veterinary, and animal production applications(Mooyottu et al. 2011). Spontaneous oocyte and germ cell differentiation in the *in vitro* condition may cause spreading novel bio-technologies to explain infertility issues in humans and raising reproductive potentials of the genetically superior animals or vulnerable species. Nowadays, infertility scientists are seeking for a better method to identify the characteristics of oocyte-like cells, and more differentiation of such cell into mature oocytes.

On the other hand, studies indicate that, there is a bidirectional communication between the oocyte and GCs in the ovary, which includes the exchange of nutrients and signal molecules. This connection in turn stimulates the expression of mRNAs that controls development and differentiation of ovarian follicles by an intricate regulatory network of autocrine, juxtacrine, and paracrine factors(Belli & Shimasaki 2018). The BMPs as the members of the transforming growth factor-beta (TGF-β) super-family are found in ovary, regulate cell proliferation, migration, and stem cell differentiation(Varga & Wrana 2005; Zhang & Li 2005). Some of which are required for germ cell specification, departure, preservation, and follicle formation(Bayne et al. 2016; Chakraborty & Roy 2015; Pangas 2012). Evidence indicated that some members of BMPs play an important role for stromal cells in boosting primordial to primary follicle

transition and enhance follicle survival(Nilsson & Skinner 2003). Among these factors, BMP15 and GDF-9 are expressed in each stages of follicular growth, and are involved in controlling proliferation and steroidogenesis of granulosa cells(de Castro et al. 2016). BMP15 is another oocyte-derived factor, which is detected in oocytes of primordial and primary follicles of different species, and has an important mitogenic effect on the granulosa cells (de Castro et al. 2016; Lima et al. 2010). It has been shown that BMP15 regulates proliferation of undifferentiated granulosa cells in a FSH-independent manner, so it has a unique role in the regulation of folliculogenesis and GC activities (Otsuka et al. 2001). Evidence demonstrated that BMP15 is responsible for prevention and low prevalence of apoptosis within cumulus cells(Hussein et al. 2005). Although species alteration is obvious in the role of oocyte-derived BMP15 and its counterpart (GDF-9) in follicular formation(Knight & Glister 2006; Yu et al. 2014). Experimental data showed that BMP15 can cause expanding *in vitro* matured bovine cumulus oocyte complex through provocation glucose metabolism toward producing hyaluronic acid and monitoring gene expression in ovulatory cascade(Caixeta et al. 2013).Therefore, BMP15 is one of the major principal growth factors involved in synchronizing granulosa cells proliferation and normal reproductive physiology differentiation. The present research was organized for determining the impact of BMP15 on differentiation capacity of MSCs obtained from human follicular fluid to oocyte-like cells (OLCs) and expression of markers indicative of *in vitro* oocyte development.

Materials & Methods

Chemical reagents

Sigma Chemical Co., St. Louis, MO was selected to purchase each chemical reagent. Moreover, abcamInc.,1 Kendall Square, Suite B2304 Cambridge, MA 02139-1517 USA was chosen to buy antibody.

Ethical consideration

IRCCS Bioethics Committee (code of ethics of IR.AJUMS.REC.1396.433, approval date 15-07-20017) approved our study protocol. A written consent was employed to apply derived cells and surplus follicular liquids.

Sampling the ovarian follicular fluids

The human ovarian follicular fluids (~10-15mL) was obtained from 78 infertile patients treated with controlled ovarian hyperstimulation for in vitro fertilization (IVF)from the center of reproductive medicine of AJUMS general hospital. Briefly, the samples were gathered from women under IVF therapy during oocytes retrieval through ultrasound-guided aspiration needle (Lai et al. 2015; Riva et al. 2014). To get better quality control, oocyte retrieval process was conducted by two operators. According to the protocol (Magli et al. 2008), after identifying COC(cumulus oocyte complex) by the first operator for IVF purposes, when no more oocytes was observed by the second operator, follicular fluid was pooled in a conical 50 mL Falcon tube

containing two drops of heparin. Hypo-osmotic lysis technique was used to enrich the follicular cells and elimination of red blood cells from FF (Lobb & Younglai 2006). Thus, freshly follicular aspirates were centrifuged at 300*g* for six minutes. Then, aspirating supernatant was conducted, and cell slurry was carried into a 15 mL Falcon tube. In the next step, 9.0 mL of the sterile distilled water was poured into the cell slurry, and the tube was blended. After 20 s, 1.0 mL of 10X concentrated PBS (phosphate buffered saline, pH=7.2) was appended to tube, followed by mixing well.

The tubes were centrifuged at 150*g* for three minutes again. Finally, the cell pellet was poured into DMEM [Dulbecco's Modified Eagle's Medium, 0.5 mL] (Sigma–Aldrich, USA). Counting aliquots was performed for gaining the cells numbers and viability in 0.2% trypan blue on a hemocytometer (Lobb & Younglai 2006; Moore et al. 2005; Otsuka et al. 2000).

Cell culture condition

FF aspirated cells were seeded on 4-well plates [BD Biosciences] at 1×10^5 and 1×10^6 cells concentration, respectively. Growing the separated cells was done in DMEM in the presence of fetal bovine serum (FBS, 15%), 2 mM- Glutamine and penicillin/streptomycin [1%, Gibco, Grand Island, NY, USA]. Non-adherent cells were thrown away after 48 hours, and specimens were incubated for 3 weeks in a CO_2 humidified atmosphere at 37℃ and monitored daily. The medium was renewed every three days. Samples were cultured without (control group) and with 100 ng/mL of human recombinant BMP15 (R&D Systems, Minneapolis, MN, catalog no. 5096-BM; derived from Chinese hamster ovarian cells (Kedem et al. 2011).

DNA synthesis by BrdU incorporation

To estimate the newly synthesized DNA strands, actively proliferating cultured cells were treated with 10μM bromo-deoxyuridine (BrdU, Sigma) for 24 h. Briefly, according to the kit protocol [Chemicon, Millipore, CA, USA] PBS was used to wash the samples and remained constant in 70% ethanol. Then, 0.1% Triton X-100 was used to permeabilized it for 10 min at the ambient temperature. Afterwards, the blocking solution was applied to block the cells for 30 min, followed by incubation in the presence of anti-BrdU detector Antibody (mouse anti-human at a ratio of 1:200, EMD Millipore Co.) for 60 minutes. Cells were scored followed by streptavidin-HRP conjugate and DAB substrate via an Inverted Microscope [ZEISS Axio Vert.A1 – Carl Zeiss, Germany] (Lai et al. 2015). When the reaction ended, samples were considerably re-washed in PBS and counter-stained by Haematoxylin for 1-5 minutes. Not less than 500 cells were counted for all conditions, and all tests were repeated 3 times.

ELISA for estradiol assays

The collection of spent culture medium was done at each weekly medium renewal, kept then at a temperature of -80˚C for analyzing estradiol secretion. A specific estradiol ELISA kit was used to check estradiol concentrations in the culture supernatant (Catalog no. 1920) (Alpha Diagnostic International, San Antonio, TX, USA). The analysis was adjusted according to the manufacturers' instructions.

In vitro osteogenic and adipogenic differentiation

The multipotency capability of the FF aspirated cells was evaluated by their differentiation into osteoblast and adipocytes. The osteogenic differentiation was induced by culturing the cells for 14 days in the routine common osteogenic differentiation medium, which contains DMEM low glucose, FBS, dexamethasone, L-glutamine, β-Glycerophosphate, L-ascorbic acid 2-phosphate[Sigma], and penicillin/ streptomycin(Kossowska-Tomaszczuk et al. 2009a). The culture media were changed three times per week. To prove osteogenic differentiation and calcium deposition, Von Kossa staining protocol was used to culture cells, then they monitored under inverted microscope. As mentioned earlier, an induction medium was employed to promote adipogenic differentiation(Stimpfel et al. 2012). Culturing the cells was performed in a medium containing DMEM complemented with insulin [10 µg/mL, Sigma–Aldrich], 1 µM Dexamethasone, 10% FBS, 60 µM indomethacin, and 0.5 mM 3-isobutilmethylxantine [Sigma–Aldrich]. The differentiation medium was replaced every 3 days. After 2 weeks, to assess the intracytoplasmic lipid droplets, which were stained red in the cultured differentiated cells, Oil Red O staining [Sigma–Aldrich] was conducted.

Immunocytochemistry staining

For mesenchymal and morphological assessment of FF derived MSCs, the cells grown on plates and that were treated with BMP15 differentiation medium for 7 and 21 days. Then, PBS was used to wash it, and 4% ice-cold paraformaldehyde was applied to fix it for 10 to 15 minutes. When it was re-washed three times with PBS, the permeabilization of the cells was done by 0.1% Triton X-100 at room temperature for 10 minutes (Riva et al. 2014). Afterwards, the blocking solution (1% BSA,1x PBS) was used to block non-specific binding of the antibodies for 30-45 min, and incubation was done with anti-vimentin (rabbit antihuman at a ratio of 1:200; Santa Cruz), anti-OCT4 (rabbit antihuman, 1:200; Santa Cruz), anti-NANOG (rabbit antihuman, 1:200; Santa Cruz), anti-ZP2 and anti-ZP3 (mouse monoclonal,1:100; Santa Cruz) antibody at 4℃ overnight. Once the cells were rinsed with PBS three times, they were incubated by rabbit anti-mouse or goat anti-rabbit FITC-conjugated antibodies (Sc2012; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted at a ratio of 1:500 in PBS-1x at room temperature for one hour. Then, it was washed three times with PBS, and DAPI was used to stain nuclei. Finally, visualization was performed using a fluorescence microscope (Leica M205 FA; Leica Microsystems)(Hu et al. 2015a).

Extraction of RNA and analysis of real-time quantitative PCR (qPCR)

The real-time PCR analysis were performed on freshly follicular fluid aspirated cells collected at time 0 h and cell culture gathered at 7 and 21days. The separation of total RNA was conducted using RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Then, 500 ng of total RNAs underwent reverse transcription into cDNA through Superscript II Reverse Transcriptase kit (Fermentas Life Sciences, Schwerte, Germany) according to the company's guidelines (Lai et al. 2015). The qPCR process was performed by SYBR-Green mix kit via the ABI Prism 7900 sequence detector. Next, the synthesized cDNA (2.0 µl) was appended into SYBR-Green mixture (12.5 µl) in the presence of forward and reverse primers (0.3 µM), added by water to reach 25-µl final

volume. The set program for 40 cycles was as follows: 95 ˚C for 15 seconds, 56 to 62 ˚C for 30 seconds (depending on primer-), 72 ˚C for 30 seconds, and 75 ˚C for 30 seconds for final cycle. TABLE 1 reports the primer sequences for NANOG, OCT4, ZP2, ZP3, and GAPDH. The formula of $2^{-\Delta\Delta Ct}$ (comparative threshold cycle method) was used to determine the melting curve for all PCR products so that outputs showed modifications in expressing genes in the cells generated for differentiating corresponding to the un-differentiated cells (controls), as mentioned earlier (Livak & Schmittgen 2001).Each test was done three times.

Statistical analysis

Attained data were statistically analyzed by SPSS (SPSS Inc.; Chicago; IL: USA) and GraphPad Prism6 (GraphPad Software Inc.; San Diego CA: USA). Differences in expressing gene and percent of the proliferating and positive cells for marker expression between the cells generated for differentiating, and analysis of the controls was performed by the one-way analysis of variance, represented as mean± SD (standard deviation) at statistically significance level of P-value less than 0.05.

Results

Morphological analysis

The adherent small mesenchymal cells showed spindle-like shapes within the first two days after culturing, morphologically as undifferentiated fibroblast-like cells. However, after3 days, the cells or clusters of cells disclosed a heterogeneous cell population, represented the extended fibroblast-like morphology (Fig. 1A), exhibiting big cuboidal or round epithelial-like feature (Fig. 1B). Nearly some days after developing in minimal culture medium, neural-like morphology cells were formed with two or more obvious cytoplasmic processes resembling to dendrites and axons (Fig. 1C). Approximately 15 days after cell culturing, the MSCs increased in sizes and differentiated spontaneously into OLCs (Fig. 1B, E). OLCs with different sizes(20-30µm), were distributed dispersedly among the fibroblastic-like cells. In the BMP15 treated group, after 10 days of culturing, round cells remarkably developed with apparent nucleus and cytoplasmic changes (Fig. 1B, E, F). BMP15 not only increased the size of round cells and OLCs in the culture medium compared to the control group (115µm), but also caused obvious cytoplasmic enlargement of fibroblastic-like and epithelial cells (Fig. 1E, F).

DNA synthesis and cell proliferation

Dual immunofluorescence staining to detect BrdU disclosed proliferating cells in the cultures. Immunofluorescence analysis demonstrated numerous BrdU-positive cells during the 1stweek in minimal culture medium (Fig. 2A, B). A comparison was made between the percentage number of proliferating cells (in S phase) and total cells that showed a form over 59%, which slowly reduced. A decrease in the number of the proliferative cells was observed at the second week of culture so that a reduction in proliferative value equaled 16.5 %($P < 0.05$) (Fig. 2 B).

Production of estradiol

On the second and third days, there were high estradiol levels in the undifferentiated cell medium (control groups). This can be due to the presence of some follicular fluid in the culture medium. While the estradiol level from the day 7-21 showed a decreasing trend, the levels of estradiol hormone significantly decreased in the group under treatment with BMP15 compared to the control group. Before day 17, there were higher estradiol level in the medium of control (117.49±1.35[day4],106.84±3.04[day7],46.63±0.67[day17]), while the estradiol levels in the treated group were lower compared to the controls group (98.83±2.5[day4],54.67±1.51[day7]37.32±0.60[day17]) ($P<0.05$) (Fig. 3). On the other hand, estradiol concentration in the treated group (25.40±1.23) had no significant difference with the controls on day 21 (23.38±1.08).

Adipogenic and osteogenic differentiation potential

The multipotency ability of follicular fluid derived MSCs were evaluated by examining adipogenic and osteogenic differentiation capacity after culturing for 3 weeks (Fig 4). The ability of FF derived MSCs to undergo adipogenic differentiation shown by staining with Oil Red O dye, and appeared with red stained lipids (Fig. 4A, B). Von Kossa staining was used to examine the osteoblastic potential of FF aspirated cells. Morphology of FF derived MSCs in the presence of osteoinductive medium, slightly changed, shrunk, and experienced intense cytoplasm brown-red staining (Fig. 4C, D).

Immunocytochemistry staining for markers related to pluripotency and oocyte maturation

All adherent MSCs derived from FF indicated a cytoplasmic immune-staining for vimentin after three days seeding (Fig. 5A-F). All data were achieved from 15 specimens, and written as mean ±SD. Fluorescent light intensity was interpreted by Image J software (Fiji 1.46). As shown in Fig. 5G, the level of vimentin marker detection on day 21 after BMP15 treatment showed a significant reduction (9.91± 3.19) compared to the day 3(22.08±8.5) and day 21 of the control groups (21.96± 8.5) ($P<0.05$). This value decreased on day 21 compared to day 3 in control groups, although this was not statistically significant.

Immunolocalization for pluripotency of MSCs were performed by detecting specific markers, including OCT4 and NANOG. Immunofluorescence study demonstrated an intense cytoplasmic positivity staining for OCT4 and NANOG in day 3 of culturing (Fig. 6 A-F). A few epithelial cells in culture medium were stained by OCT4 and NANOG. However, OCT4 but not NANOG proteins were detected in MSCs during same culture period (Fig.7).

To understand the possibility that MSCs from FF have the ability to differentiate into OLC, oocyte specific markers, ZP2 and ZP3 were examined in controls and BMP15 supplemented medium groups. ZP2 and ZP3 were detected in OLC of both groups after 3 weeks of culture (Fig.7). Fluorescent light intensity of ZP2 and ZP3 in OLCs significantly differed between the treated and control group ($P = 0.000$). Moreover, ZP3 protein was seen in the periphery of

cytoplasm in OLCs rather than uniform cytoplasmic distribution of ZP3 and ZP2 in OLCs (Fig. 7). It should be noted that ZP proteins were not detected in the cytoplasm of other epithelial and mesenchymal cells. Fibroblast like cells adjacent to epithelial cells were negative for oocyte marker in the controls and BMP15 induced groups.

RT-PCR analysis of specific markers of stemness and oocytes

RT-PCR analysis was carried out in order to further approve the ICC results. Initially, real-time PCR examined FF derived MSCs with various culturing intervals. Cell markers were analyzed on day 0, 7 and 21 after culture. Then, the gene expression level was measured and compared between control and BMP15 treated groups (Fig. 8). Nonetheless, fold changes in BMP15 treated cells in comparison with the control groups for each the markers suggested specific dynamic modifications during the induction process($P<0.05$). Pluripotent genes expression for OCT4 and NANOG was higher in earlier days of culturing (week 1), down-regulated, and decreased on day 21 in both treated and control groups ($P <0.000$) (Fig. 8A, B). The expression of OCT4 and NANOG (on the day 7) showed a different and sinusoidal behavior after treatment with BMP15 ($P <0.05$) (Fig. 8 A, B). On day 7 of the treatment, gene expression levels of OCT4 but not NANOG up-regulated by approximately 3-fold, when compared to the control groups. In the treated groups, gene expression levels of OCT4 and NANOG from day 7-21 showed down-regulation approximately 0.041-fold and 0.40-fold, respectively ($P= 0.000$) (Fig. 8 A, B).
To determine OLCs development in control group from day 7, the gene expression levels of ZP2 increased in comparison with the levels achieved on day 0(3-fold), and gradually reduced (2-fold) on day 21. ZP3 gene expression levels increased 1-fold and 5-fold from day 7 – 21 in comparison with the level observed on day 0 ($P <0.05$). From day 0 to 21 of treatment with BMP15 where OLCs grew, levels of gene expression of ZP2 and ZP3 showed down-regulation and decreased approximately 6-fold and 4-fold ($P =0.000$) in comparison with the levels achieved on control groups ($P< 0.05$) (Fig. 8 C, D). Regarding the BMP15-treated groups, ZP2 and ZP3 levels increased by approximately 4.45-fold and 2.17-fold ($P=0.000$) from day 7-21(Fig. 8 A-D). In addition, OCT4, ZP2 and ZP3 proteins were present in OLCs during the same culture time (Fig. 7).

Discussion

Today, biology of the stem cell and principally investigation on the adult human stem cells are continuously emerging. Due to plasticity, accessibility, and responses to *in vitro* gene manipulations, MSCs are the most favorable between these cells (Bobis et al. 2006). The results of this investigation indicated that, germ cell precursors correlated with OLCs are established according to the criteria below: 1. morphologic changes, 2. Expression features of markers at the mRNA and protein levels, and 3. Secretion of estradiol from cumulus oocyte complex structures.

Routinely, FF are thrown away after isolation of COCs, in the infertility clinics. Our observations indicated that this fluid, despite the presence of granulosa and thecal cells(Honda et al. 2007; Kossowska-Tomaszczuk et al. 2009b), consist of heterogeneous cell populations involving a cell collection exchanging fibroblast-like morphology and other characteristics of MSCs (Kucia et al. 2005). Feasible presence of human ovarian follicle-derived MSCs was published formerly, and these research findings illuminate previous inquiries (Kossowska-Tomaszczuk et al. 2009b; Riva et al. 2014). Vimentin, which is a specific protein of cytoskeleton intermediate filaments of mesenchymal cells, was chosen to determine cell populations isolated from human FF(Riva et al. 2014). Immunofluorescence showed an intense cytoplasmic staining for vimentin in MSC at different times of culture. However, the amount of vimentin in the differentiation medium (which will be referred to below) showed a significant decrease. Our study illustrated multipotency of a cell population via inducing differentiation in adipogenic and osteogenic culture medium. In fact, there was adipogenic and osteoblastic differentiation after three-week culture in the differentiative medium, which are counted as the initial marker of adipogenesis and osteogenesis. Therefore, this research provided more confirmation on the opinion of the human FF MSCs stemness. The interesting point is the direct differentiation of FF MSCs into neural and hepatocyte-like cells that are not found within ovarian tissues (Lai et al. 2015).

Analyzing the synthesis of DNA examined by incorporation of BrdU revealed an effective rapid growth of each cell population throughout first week in culture. Afterwards, a descending trend was observed after ten days, which could be the result of slowing down proliferative property during a 10-day interval. One possible explanation for this might be the proliferative phase cessation and arrival in the differentiation phase in these cells. These findings confirmed earlier works by Rive et al (Riva et al. 2014).

A number of epithelial cells are another cell population derived from FF Lai et al. (2015) initially isolated this cell population. The origin of these epithelial cells is ovarian surface epithelium (OSE), a prominent human ovary structure, which is known as the source of neo-oogenesis(Motta & Makabe 1986). Lately, Germline stem cells (GSCs) were substantially separated from ovaries of neo-natal and adult mice and human ovaries so that it disputes central dogma that the ovarian oocytes are not renovated in post-natal period of the female mammals (Bukovsky 2011; Johnson et al. 2004). Similar to what reported by Lai et al. (2015) our results revealed small number of the populations of such epithelial cells could synthesize pluripotent markers, such as OCT4 and NANOG. Fibroblast-like cells, despite of epithelial-like cells, could synthesize NANOG and OCT4 genes and proteins in the primary days of culture, although expression pattern of these genes reduced with increasing the culturing time. In our study, approximately from day 7 to 15 after differentiation of FF derived cells, the PGC-like cells resumed for forming structures with a morphologically identical to the oocyte like cells. Ultimately, OLCs spontaneously became visible during day 10 – 21 of culture.

This study showed the advantage of human recombinant BMP15 to the cultured human FF derived MSCs for the first time. BMP15 and its homologue (GDF9) are oocyte secretory factors which are expressed in an oocyte-specific manner from a primary developmental phase.

Members of the TGF-β super family, specifically BMP15 play an essential role in promoting follicle growth over the primary stage and in development to MII phase (Huang & Wells 2010). In the early stage of oocyte development (germinal vesicle breakdown[GVBD] and MI) BMP15 expression level highly increase, but it reduced at MII stage. These evidences indicated that synchronous expression of BMP15 and GDF9 can play a key role in mammalian oocyte maturation(Zhao et al. 2010). Furthermore, exogenous addition of BMP15 did increase OLC formation rate, gene expression alteration and protein formation. In line with the results of the present study, Lin et al. (2014) reported the effect of BMP15 and GDF9 on the *in vitro* maturation of Porcine Oocytes. After assessment of spontaneous differentiation of FF derived cells into OLC in control groups, the effects of BMP15 were evaluated on the gene and protein expression pattern of OLCs. It was observed that BMP15 reduced duration of OLC formation culturing time compared with the control groups less than 10 days. In addition, BMP15 led to an increase in OCT4 expression 2.5-fold changes compared to the control groups. Then, we observed a reduction in its expression (Fig. 6A). Expression of the OCT4 transcription factor in cells occurred with the increase of differentiation potential, including PGCs spermatogonia type A, oogonia, and is essential for survival of primordial germ cells (Kehler et al. 2004; Zuccotti et al. 2009a). Indeed, down-regulating of OCT-4 is associated with the decline in pluripotency, which consequently causes cell differentiation(Babaie et al. 2007). From day 7 to 21 of BMP15 treatment, we observed a significant reduction in OCT4 expression, although its expression was observed in day 21 of culture in control groups, which can indicate the survival rate of these cells in culture medium. The analysis of OCT4 expression in all stages of folliculogenesis indicated the presence of this protein in primordial follicles, which disappeared in the primary oocytes and reappeared at the onset of the oocyte growth(Zuccotti et al. 2009a; Zuccotti et al. 2009b). The present study deduced that BMP15 leads to early differentiation of OLC in the day 7 of culture, which is in line with Zuccotti et al. (2009a) observations. Another transcription factor, NANOG, which belongs to the homeobox gene family, is necessary for preserving pluripotency and suppressing differentiation in embryonic stem cells (Mullin et al. 2008). It is also expressed in unipotent PGCs in mammal, where its exact role is still unknown (Murakami et al. 2016). In a study on embryo of female mice, Yamaguchi et al. (2005) showed that NANOG down-regulated in germ cells that had entered meiosis stage. In the study ahead, a temporary reciprocal expression of NANOG was observed. In a way that, increased expression was shown after second week of culture in control groups, but reduction was observed in treated groups, in which it is inferring that OLCs experienced meiosis. In ICC assay, NANOG protein was not detected in both the cytoplasm and nuclei of OLCs, which confirmed RT-PCR findings. It was also demonstrated that OLCs and PGC-like cells expressed genes and cytoplasmic proteins, such as ZP2 and ZP3(Fig. 7,8). The ZP (zona pellucida) refers to extracellular sulfated glycoprotein matrix around the oocytes in the mammals. Recent findings showed the presence of four ZP glycoproteins of ZP1, ZP2, ZP3 and ZP4 in human (Gupta 2018). Evidence indicated that in mice ZP1 is not vital, and ZP1 null animal form a ZP matrix which retains biological activity for fertilization and preimplantation development (Hoodbhoy et al. 2006). Moreover, lower ZP1

mRNA expression has been reported in human fetal and mature ovaries (Törmälä et al. 2008). It was also stated that ZP4 has a structural role in human oocyte and a minor function in sperm binding and induction of acrosome reaction(Canosa et al. 2017). Therefore, we decided to focus on ZP2 and ZP3 intracellular trafficking and expression in FF derived MSC and OLCs. ZP2 and ZP3 relative expression fold change increased during differentiation process (Fig. 8). This study outputs have consistency with Hu et al. (2015b) findings who confirmed that some of human umbilical cord-derived PGC like cells are prone to more growth into OLCs and express markers specific in oocytes, including ZP1, ZP2, ZP3, and so forth. In the day 21 of differentiation, ZP3 expression was much higher than ZP2, which could be due to ZP3 importance in development competence and fertilization process. In treated groups, BMP15 resulted in a significant reduction in expression pattern of ZP proteins (Fig. 8). This can be due to the differentiation of OLCs in BMP15 induced groups. Canosa et al. (2017) observed that expression of ZP proteins in mature oocyte significantly decreased compared to the early stage oocyte. Immunocytochemical examinations of our study confirmed the RT-PCR results, and showed the cytoplasmic ZP proteins staining. Hence, amplification of ZP gene expression in the third week was observed in OLCs of control groups, which could be caused by insufficient maturation of these cells. This is the first report of the expression pattern of ZP genes in FF derived OLCs on different times in both BMP15 induced and control groups. Moreover, a minimal amount of ZP2 and ZP3 gene expression was observed in the first days of culture, which could be due to heterogeneous nature of FF derived cells. Furthermore, it has been indicated that ZP elements are found in human fetal ovaries prior to forming follicle. Finally, the researchers inferred that ZP components are found at the initial stages of ovarian development (Törmälä et al. 2008). In our study, both OLCs and epithelial cells expressed ZP component. Nevertheless, it is important to notice that the exact position of ZP proteins in the mammalian ovary is disputable, and any general agreement does not exist regarding the synthesize of human ZP proteins in the granulosa cells, oocyte or both (Lefievre et al. 2004; Törmälä et al. 2008).

In addition, identification of estradiol production proofs the functional activity of OLCs and its surrounding cumulus like cells. Reduction of estradiol concentration over the controls and BMP15 treated groups on day 21 maybe due to the differentiation of these cells. Our observations correspond to Prapa et al. (2015) who found that BMP15 could reduce basal estradiol levels in human granulosa cells; however, Costa et al. (2004) showed that levels of estradiol considerably decreased in the follicles with mature oocytes compared to the follicles with immature oocytes.

We conclude that FF derived MSCs are a structural portion of the ovarian tissue, whether from OSE or from the cortical zone of the ovary. A rare number of cells showed the expression scheme of pluripotent stem cells and morphology of oocyte cells. However, BMP15 boosted OLC formation in the induction medium. This consideration supports more proof regarding *de novo* folliculogenesis and oogenesis feasibility in mature ovary of human, as opposite to central dogma in terms of the limit numbers of oocytes and follicles at birth(Zuckerman 1953). In the assisted reproductive technology, aspirated follicular fluids are usually discarded following COC

removal; hence, it might be applied as a potent source of stem cells. The ovarian follicular fluid-derived MSCs would prepare a favorable practical model for the *in vitro* maturation of ovarian follicles in human, and can be used in coming times in assisted regenerative and reproductive medicine.

Conclusions

Briefly, the present research showed that isolation and growth of MSCs from follicular fluid is feasible, which is routinely wasted during IVF operation. Based on this observation, we recommended that this fluid can be another source for MSCs, and can be as a valuable and reasonable biological tentative sample for the basic stem cell research after IVF procedure. The ovarian follicular fluid-derived MSCs can introduce a hopeful approach in the regenerative medicine and oogenesis in the future. In this way, our results indicated that BMP15 increases developmental ability of OLCs, and supplies a different *in vitro* model to examine the mechanisms, by which germ cell formed and differentiated.

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Table 1(on next page)

List of primers used in RT-PCR.

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Gene	Primers	Amplified size (bp)
OCT4	GGCCCGAAAGAGAAAGCGAACC	224
	ACCCAGCAGCCTCAAAATCCTCTC	
NANOG	GGGCCTGAAGAAAACTATCCATCC	400
	TGCTATTCTTCGGCCAGTTGTTTT	
ZP2	CAGAGGTGTCGGCTCATCTGA	110
	GCAGTCTTGTGCCCTTTGGT	
ZP3	GACCCGGGCCAGATACACT	110
	CATCTGGGTCCTGCTCAGCTA	
GAPDH	GGGAGCCAAAAGGGTCATCA	203
	TGATGGCATGGACTGTGGTC	

Figure 1

Primary cell culture of FF planted on plate at various time points in minimum conditions of cultivation. Heterogeneous cell density found in the colonies contains minimally two types of cells:

Figure 1: Primary cell culture of FF planted on plate at various time points in minimum conditions of cultivation. Heterogeneous cell density found in the colonies contains minimally two types of cells: (A) fibroblast-like shape and (B) cuboidal epithelial-like shape following four-day cultivation. After 72 hours, cytoplasmic protrusion and large spherical cell body were observed in some cells, typical for neural shape (C). (D) represents a neuronal-like cell that immunostaining by Vimentin. € shows the morphology of FF derived cells culture in medium supplemented with BMP15. The hollow arrows in (B and E) show different size of germ cell and OLCs developed spontaneously from human FF derived cells. (F) Large OLCs exhibit perinuclear accumulation of cytoplasmic organelles and eminent nucleus. Scale bars: 100µm, (D) Scale bar: 20µm.

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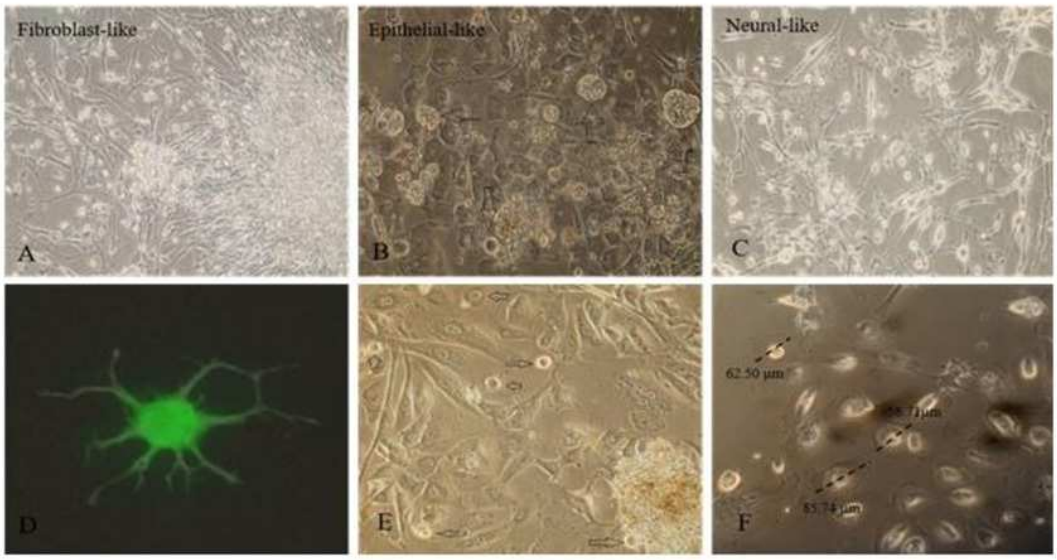

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Figure 2

Proliferation of human follicular fluid-derived cell population.

Figure 2: Proliferation of human follicular fluid-derived cell population, appeared on plates at various time points in minimum conditions of cultivation in the presence of bromo-deoxyuridine(BrdU) within 24 hours. (A) Proliferating cell immunostained (brown) for BrdU HRP with three-day cell analysis; the counterstained cell nucleus by Haematoxylin . (B) Mean percentage of proliferating cells when comparing with total cell count. Attained data from 10 samples are expressed as mean ± SD, standard deviation. (* = P <0.05). (A) scale bar: 20 µm.

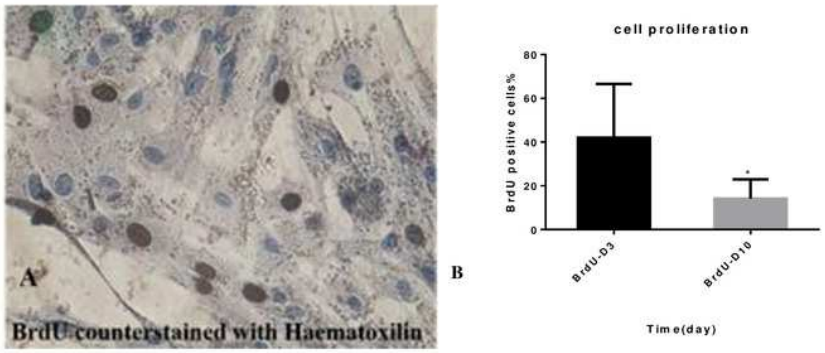

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Figure 3

Determination of estradiol production in medium within process of differentiation

Figure 3: Determination of estradiol production in medium within process of differentiation; a remarkable difference exists in the levels of estradiol between BMP15-induced group and control ($P<0.001$).

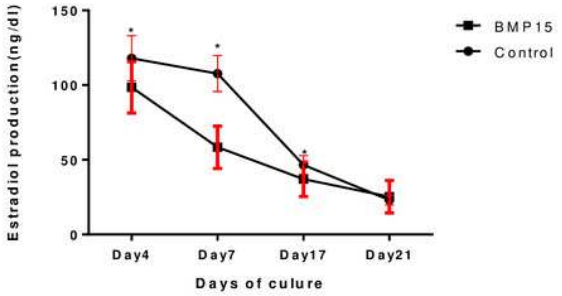

Figure 3: Determination of estradiol production in medium within process of differentiation; a remarkable difference exists in the levels of estradiol between BMP15-induced group and control ($P<0.001$).

Figure 4

Adipogenic and osteogenic differentiation of human FF cells.

Figure 4: Adipogenic and osteogenic differentiation of human FF cells. Oil Red O (A) and (C) Von Kossa staining of follicular fluid derived cells with differentiation medium during three weeks, under an optical microscopy. Red differentiated cells in adipogenic medium following the staining by Oil Red O counter stained with Haematoxylin, (B, D) show differentiated cell before staining procedure. Von Kossa exhibited osteogenic differentiation, with cytoplasm stained intensively in brown(C). Scale bars: 50 µm.

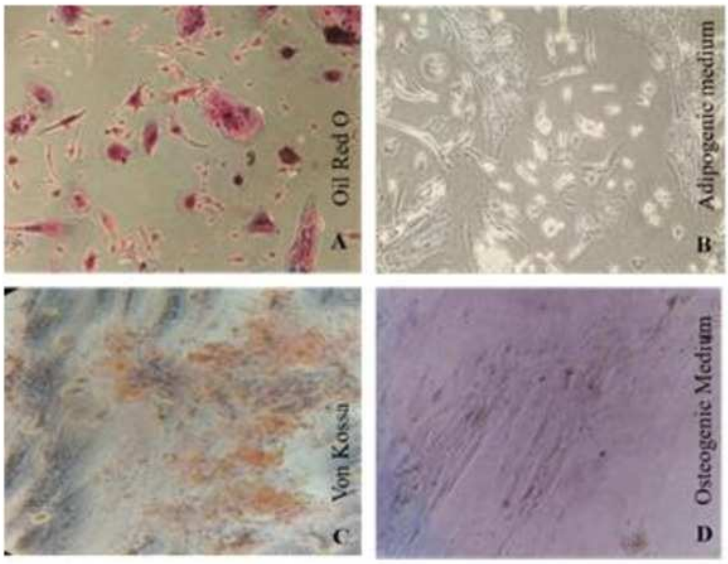

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Figure 5

Mesenchymal stem cells derived from human FF grown *in vitro* for 3 days (A-C)

Figure 5: Mesenchymal stem cells derived from human FF grown *in vitro* for 3 days (A-C). Cells show cytoplasmic immunostaining for vimentin. Nuclear deoxyribonucleic acid was stained with DAPI (B, E blue fluorescence). (D-F) showed vimentin immunostaining of FF derived adherent cells after 21 days of Culture in BMP15 differentiation medium. **G** shows the percentage of positive cells for vimentin at different time of growth (* = P <0.05). Fluorescent light intensity between day 3 and day 21 interpreted by Image J software. (A-F) Scale bar: 20 µm.

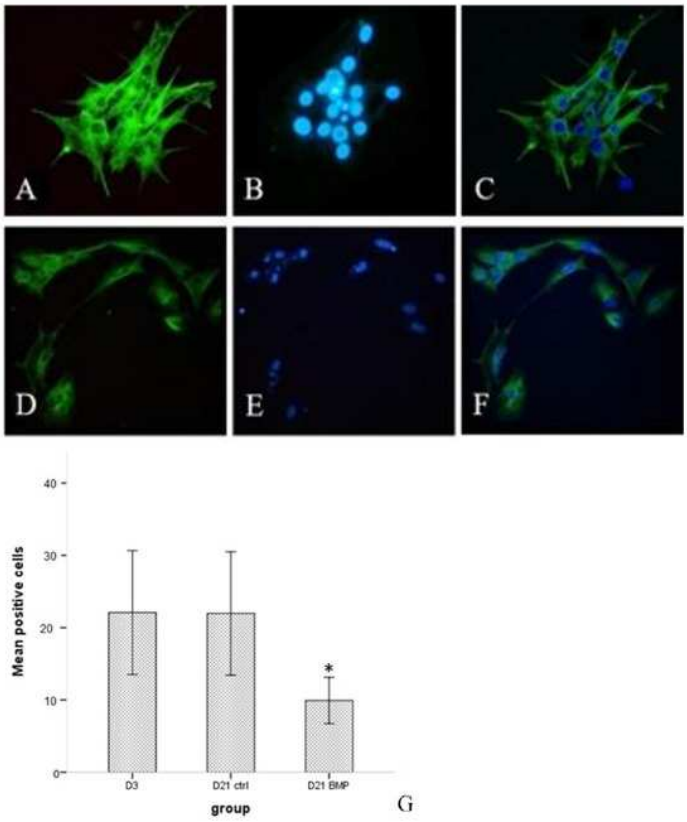

Figure 5: Mesenchymal stem cells derived from human FF grown *in vitro* for 3 days (A-C). Cells show cytoplasmic immunostaining for vimentin. Nuclear deoxyribonucleic acid was stained with DAPI (B, E blue fluorescence). (D-F) showed vimentin immunostaining of FF derived adherent cells after 21 days of Culture in BMP15 differentiation medium. **G** shows the percentage of positive cells for vimentin at different time of growth (* = P <0.05). Fluorescent light intensity between day 3 and day 21 interpreted by Image J software. (A-F) Scale bar: 20 µm.

Figure 6

Pluripotent activities of human FF-derived cells.

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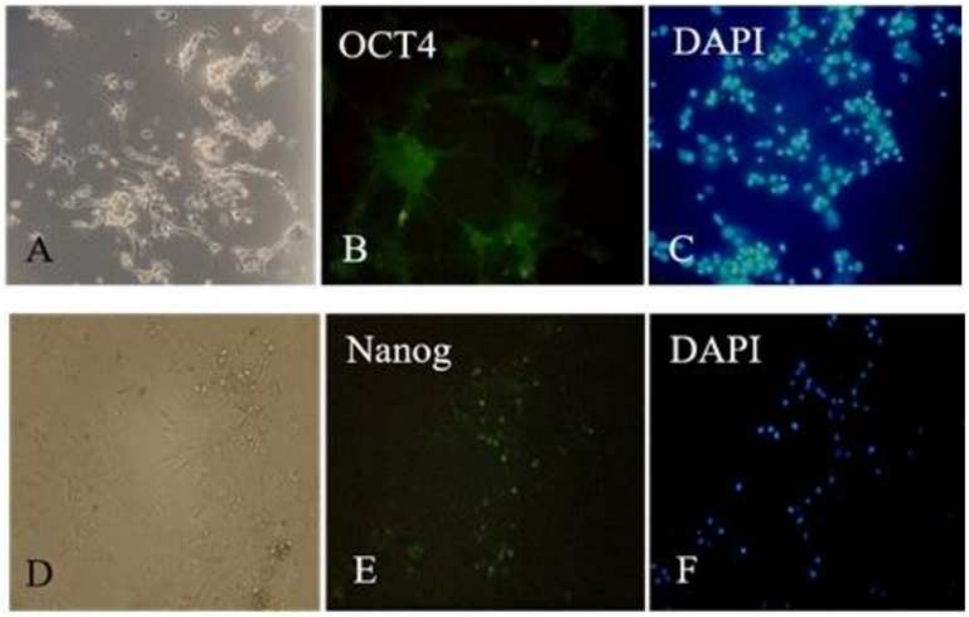

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Figure 7(on next page)

Oocyte-like cells characterized after treatment with BMP15 using germ cell marker immunolocalization when comparing with control groups as fluoresceinisothiocyante-green fluorescence.

Figure 7: Oocyte-like cells characterized after treatment with BMP15 using germ cell marker immunolocalization when comparing with control groups as fluoresceinisothiocyante-green fluorescence. The positively stained OLCs following three-week cultivation for ZP3 and ZP2 and OCT4; the nuclei were counterstained and observed by 4′,6-Diamidino-2-phenylindole (DAPI). Scale bar = 20µm.

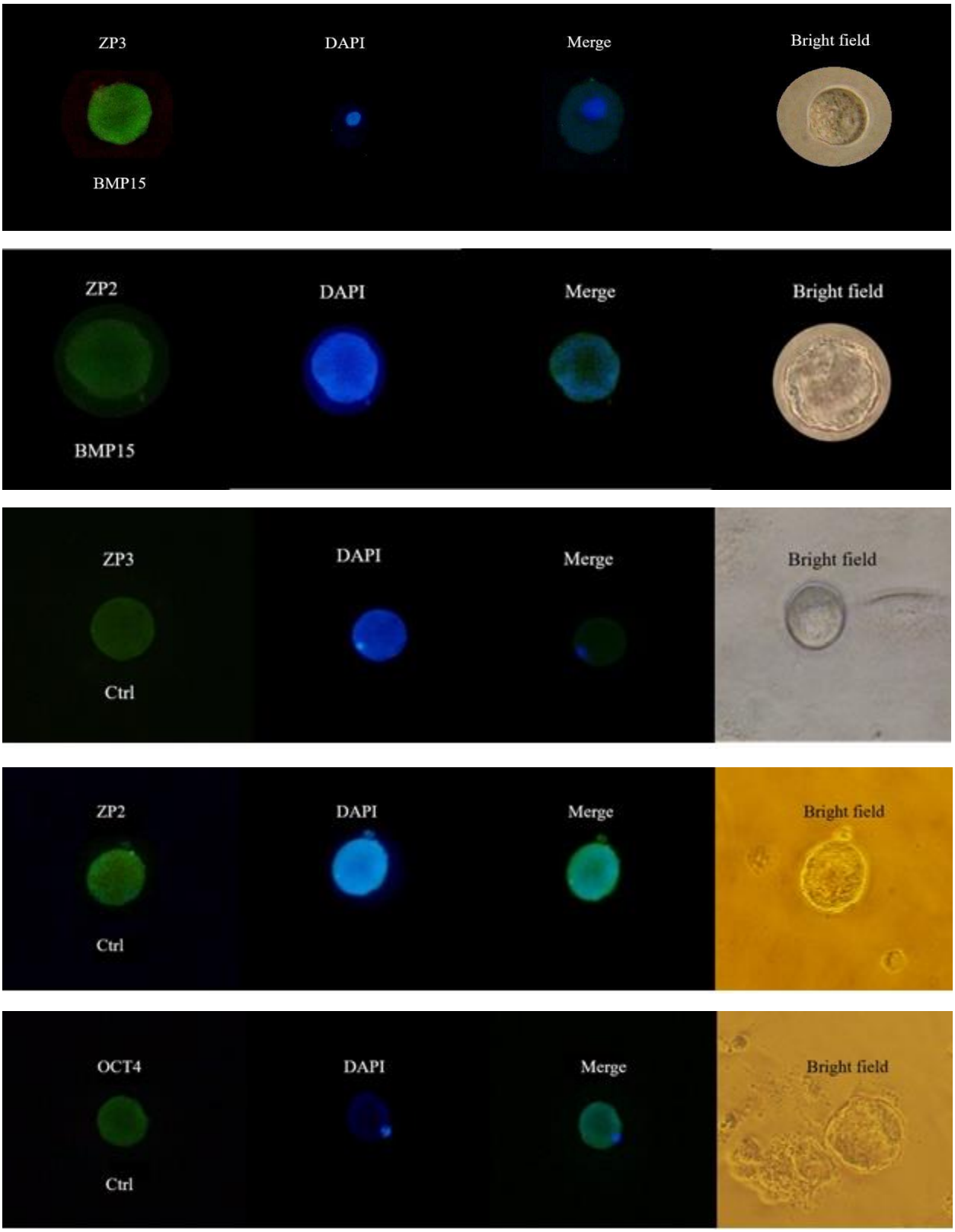

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Figure 8

RT-PCR of pluripotency and oocyte markers of human FF derived MSCs cultured for three weeks, and comparison after induction with BMP15.

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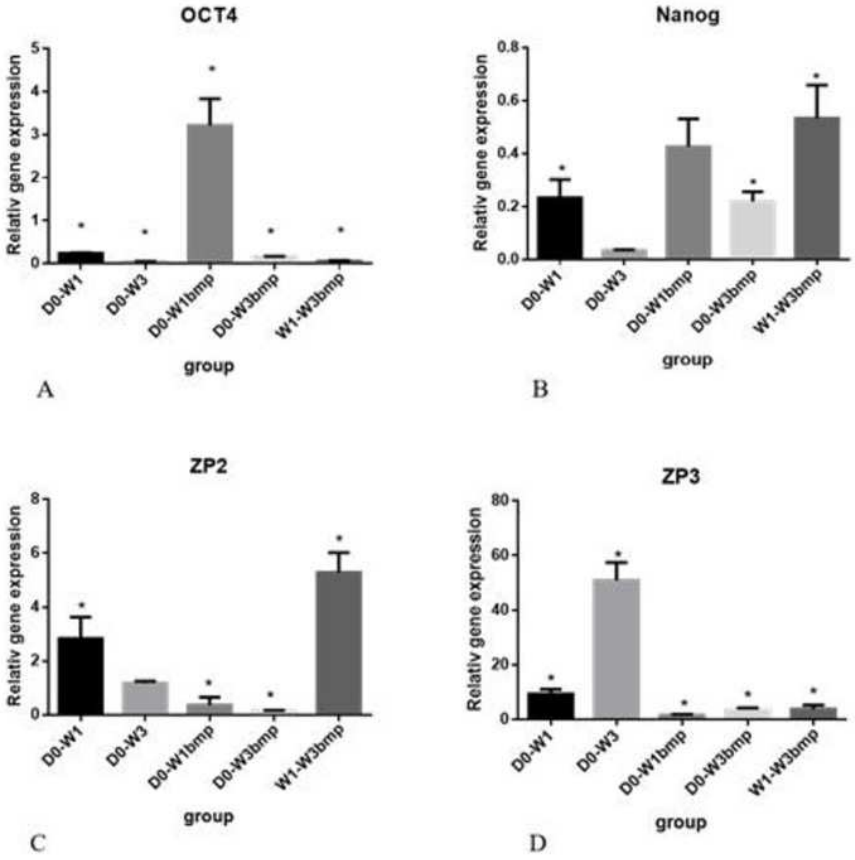
BMP15 increased the expression of ZP2 in the 3rd week of cell cultures compared to the control group, but decreased the ZP3 expression in the second and third weeks compared to ZP2. GAPDH was used as housekeeping control gene. Data demonstrate mean ± standard error (SE) obtained from independent tests performed in triplicate (* = $P < 0.05$).



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