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Original Article

Gender difference of Wharton's jelly-derived mesenchymal stem cells on differentiation potential into functional granulosa cells

Sang-Yun Lee[1], Dinesh Bharti[2], Young-Bum Son[3], Won-Jae Lee[4], Yong-ho Choe[1], Hyeon-Jeong Lee[1], Seong-Ju Oh[1], Tae-Seok Kim[1], Chae-Yeon Hong[1], Sung-Lim Lee[1,5*], Gyu-jin Rho[1,5*]

[1] College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Korea
[2] Division of Pulmonary, Critical Care, Sleeping and Occupational Medicine, School of Medicine, Indiana University, Indianapolis, IN 46202
[3] Department of Obstetrics, College of Veterinary Medicine, Chonnam National University, 300 Yonbongdong, Buk-gu, Gwangju 500-757, Republic of Korea
[4] College of Veterinary Medicine, Kyungpook National University, Daegu, 41566 Republic of Korea
[5] Research Institute of Life Sciences, Gyeongsang National University, Jinju 52828, Korea

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Abstract

The ovarian follicles consist of theca and granulosa cells, which play a crucial physiological role in sex hormone and cytokine secretion and provide an optimal induction microenvironment for oocytes. However, ethical concerns and the absence of a cellular model for investigating the molecular pathway in humans present challenges for research on granulosa cells. To address these challenges, differentiation induction into granulosa cells using mesenchymal stem cells (MSCs) could offer a novel approach to advancing granulosa cell research. In this study, the granulosa cell differentiation ability and hormone synthesis function of MSCs derived from male and female donors were investigated to identify gender differences. MSCs isolated from Wharton's jelly (WJ-MSCs) were successfully differentiated into granulosa cell-like cells, as evidenced by the expression of granulosa cell-specific markers at both the mRNA and protein levels. Differentiated WJ-MSCs into granulosa cell-like cells increased aromatase activity, which plays an important role in converting testosterone to estradiol, resulting in significantly increased estradiol levels in differentiated cells compared to undifferentiated WJ-MSCs. However, the activity in female-differentiated cells was significantly higher than in male-differentiated cells. The current study indicates that female-derived WJ-MSCs may represent a novel stem cell resource for understanding granulosa cells and could provide an excellent cellular source for studying various developmental stages and processes of human folliculogenesis.

Keywords: Differentiation, Gender difference, Granulosa cells, Mesenchymal stem cell

1. Introduction

Infertility is defined as the inability to conceive after one year of regularly unprotected sexual intercourse [1], and fertility rates are declining due to various factors in some countries. Several factors influence reproductive health risks, including lifestyle, health conditions, germ cell quality, and reproductive disorders [2], particularly environmental changes [3]. Despite numerous studies conducted to address this issue, a clear solution remains elusive. Currently, stem cell-based therapy is suggested as a promising solution.

Mesenchymal stem cells (MSCs), regarded as adult stem cells, are gaining interest in regenerative medicine due to their capacity for differentiation into mesenchymal lineages and ectodermal, endodermal, and mesodermal lineages as well [4], self-renewal, and immunomodulatory properties [5]. Among MSCs, Wharton's jelly-derived MSCs (WJ-MSCs) are considered superior candidates for clinical applications due to their higher proliferative capacity and lower immunogenicity compared to MSCs derived from other tissue [6]. Additionally, WJ-MSCs are characterized by low non-stem cell contamination, richness in stemness features, and a high potential for differentiation. These character make it possible to apply in variet of field, such as regenerative medicine, and tissue engineering [7, 8]. Consequently, WJ-MSCs are recognized as a cell model suitable for application and research in regenerative medicine.

The mammalian ovary is a dynamic tissue that undergoes continuous structural and functional changes, such as those observed in the ovarian cycle. The complex process of folliculogenesis involves the recruitment of primordial follicles into a group of developing follicles from which some antral follicles are selected to ovulate. Granulosa cell-derived factors that coordinate the recruitment of primordial follicles and the selection of the antral follicle as

* Corresponding author.
E-mail address: jinrho@gnu.ac.kr (G-J. Rho); sllee@gnu.ac.kr (S-L. Lee.
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it responds to follicle-stimulating hormone (FSH) and subsequently ovulation is crucial to this process [9]. Oocyte development is dependent on bidirectional signaling with ovarian somatic cells, including theca cells and granulosa cells, which comprise the supportive microenvironment. In each individual follicle, granulosa cells are located outside the zona pellucida and are crucial to the development of new follicles [10]. In addition, oocyte development requires the presence of granulosa cells since they can largely support oocyte metabolism such as amino acids, ions, and metabolites through gap junctions [11]. Granulosa cells can produce and secrete a variety of hormones and growth factors that control the development, differentiation, and maturation of theca cells and oocytes. Among them, the most important factor is estrogens. Granulosa cells act as the main sources of estrogen and progesterone as well [12]. The androgen produced by theca cells is aromatized into estrogen by granulosa cells [13]. Hence, the healthy function of granulosa cells is important for female reproduction and folliculogenesis.

Granulosa cells, which possess supportive cell functions, can be categorized into two types of populations, such as cumulus granulosa cells and mural granulosa cells. Crucially, each group of granulosa cells within the female gonad aids in the proliferation of theca cells, which secrete endocrine hormones and play a key role in follicle production [14]. For autocrine, paracrine, and endocrine functions, the granulosa cells secrete various growth factors, sex hormones, and cytokines. The cells provide an ideal microenvironment for oocytes [14]. Abnormalities in somatic supporting cells are linked to several gonadal diseases [15]. By maintaining normal function, the granulosa cells also play a crucial role in preventing diseases of the human ovary, such as polycystic ovary syndrome, premature ovarian failure, and granulosa cell tumors [16–18]. However, ethical concerns and the absence of a cellular model for investigating the molecular pathway in humans face challenges in research on granulosa cells [14]. Consequently, it is essential to develop a suitable cellular model to uncover intrinsic mechanisms crucial for understanding the pathophysiology and advancing the management of clinical infertility. Establishing granulosa cell lines derived from MSCs can be suggested as a promising approach to studying this purpose.

However, the gender of the tissue donor is considered an unclear but critical factor in stem cell research and clinical applications. Specifically, using gender-matched MSCs for inducing differentiation into cells with gender-specific functions is being considered, but the evidence supporting this approach is currently insufficient. Therefore, to identify gender differences in stem cell differentiation, it is necessary to perform a comparative analysis using the same differentiation protocol. Moreover, there is a lack of understanding regarding the necessity and advantages of using female-derived MSCs to induce differentiation into granulosa cells, which function in oocyte maturation and estrogen synthesis within the ovarian follicle.

In the present study, we investigated the granulosa cell differentiation ability and hormone synthesis function of MSCs derived from male and female Wharton's jelly. By comparing the performance and properties of MSCs from both genders, we aimed to better understand how gender differences may influence important aspects of their differentiation into cells with female-specific functions.

2. Materials and methods

2.1. Chemicals and media

All chemicals were purchased from Sigma (St. Louis, MO, USA) and media from Gibco (Gibco Life Technologies, Gaithersburg, MD, USA), unless otherwise specified.

2.2. Isolation of Wharton's jelly's mesenchymal stem cells in umbilical cord

Human female and male umbilical cord tissues undergoing either cesarean section or normal vaginal delivery were obtained from full-term births after taking informed donor's consent and approval from the Ethics Committee of the Gyeongsang National University Hospital (No. GNUH IRB-2012–09-004), and their MSCs were isolated from the tissues as previously studied [19]. In brief, after washing three times with Dulbecco's phosphate-buffered saline (DPBS) containing 1% penicillin/streptomycin (Pen/Strep, 10,000 IU and 10,000 µg/mL, respectively, Pen), the umbilical cord was cut into 2-3 cm lengths. To isolate Wharton's jelly from the umbilical cord, the tissues were removed two arteries and one vine. Then, slices of 1 mm pieces of Wharton's jelly were attached to a culture media-coated dish. The culture media consisted of advanced Dulbecco's modified Eagle's medium (ADMEM) with 10% fetal bovine serum (FBS), and 1% Pen/Strep. After 2 weeks, the tissues were detached from the dish, and cells were cultured in a humidified incubator at 5% CO_2 at 37°C. The culture media was changed every three days. At 70-80% confluence, the cells were sub-cultured using 0.25% trypsin-ethylene-diamine-tetra-acetic acid (Trypsin-EDTA).

2.3. Flow cytometry

MSCs derived from Wharton's jelly were evaluated for the expression of surface markers by BD FACS flow cytometer (BD FACS Calibur; Becton Dickinson, Franklin Lake, NJ, US). Briefly, MSCs (1×10^5) were fixed in a 4% paraformaldehyde solution for 1 hr at room temperature. The fixed cells were washed by DPBS 2-3 times and reacted with FITC-conjugated mouse anti-human CD34 (1:200; BD Biosciences, NJ, USA), FITC mouse anti-human CD45 (1:200; BD Biosciences), FITC mouse anti-human CD105 (1:200; BD Biosciences), FITC rat anti-human CD44 (1:200; BD Biosciences) and APC-conjugated mouse anti-human CD73 (1:200; BD Biosciences), APC mouse anti-human CD105 (1:200; BD Biosciences) for 1 hr at room temperature. After the reaction, the cells were washed by DPBS 2-3 times to remove unbounded antibodies. The standard was established by FITC mouse IgG1 (1:200; BD Biosciences) and APC mouse IgG1 (1:200; BD Biosciences). Data were analyzed by FlowJo v10 software. The antibodies used in flow cytometry are listed in Table 1.

2.4. Mesenchymal lineage differentiation

WJ-MSCs were differentiated into mesenchymal lineages following the previous study [19]. Adipogenic differentiation was induced for 3 weeks with DMEM containing 10% FBS, 100 µM indomethacin, 10 µM insulin, and 1 µM dexamethasone. The adipogenesis was confirmed by observing the accumulation of lipid droplets after staining with Oil Red O staining. Osteogenesis was induced for 3 weeks with DMEM supplemented with 10% FBS,

Table 1. List of antibodies and their amount in flow cytometry.

Antibody (clone)	Company	Amount
FITC, anti-CD34	BD Pharmingen™	0.5 mg/ml
FITC, anti-CD44	BD Pharmingen™	0.5 mg/ml
FITC, anti-CD45	BD Pharmingen™	0.5 mg/ml
FITC- isotype	BD Pharmingen™	0.5 mg/ml
APC, anti-CD45	BD Pharmingen™	0.5 mg/ml
APC, anti-CD90	BD Pharmingen™	0.5 mg/ml
APC, anti-CD73	BD Pharmingen™	0.5 mg/ml
APC, anti-CD105	BD Pharmingen™	0.5 mg/ml
APC-isotype	BD Pharmingen™	0.5 mg/ml

200 µM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 µM dexamethasone. The mineralization and calcium deposition were confirmed by Von Kossa. The MSCs were cultured for 3 weeks in STEMPRO chondrogenesis media (STEMPRO chondrogenesis differentiation kit, Invitrogen, CA, US). The differentiation into chondrocyte lineage was evaluated by the accumulation of proteoglycans using 1% Alcian blue solution staining.

2.5. *In vitro* differentiation into granulosa cell-like cells

WJ-MSCs were differentiated into granulosa cells under the culture conditions as described previously with minor modifications [20]. In brief, WJ-MSCs were differentiated into granulosa cells using induction media for 7 days. The induction media contained DMEM/F-12 (1:1) media supplemented with 10% FBS, 1% non-essential amino acids, 0.1 mM beta-mercaptoethanol, 25 ng/ml recombinant human follistatin (Peprotech, NJ, USA), 5 ng/ml recombinant human FGF-basic (Peprotech, NJ, USA), and 10 ng/ml recombinant human BMP-4. The media was changed every two days intervals.

2.6. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from un-differentiated and differentiated WJ-MSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After quantifying total RNA using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), complementary DNA (cDNA) was synthesized with 500 ng of RNA sample using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-qPCR was reacted with Rotor-gene (Qiagen, Hilden, Germany), and the reaction mix contained Quantinova SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany), 50 ng of cDNA, 0.7 µM forward and reverse primers. RT-qPCR was run in a Rotor-Gene Q qRT-PCR machine (Qiagen) following pre-denaturation, denaturation, and combined annealing/extension step. Pre-denaturation at 95°C for 2 min; 40 PCR cycles at 95°C for 10 sec, 60°C for 6 sec. The melting curve shows that temperature alters from 60°C to 95°C by 1°C/sec and cooling at 40°C for 30 sec. Rotor-Gene Q series software (Qiagen, Hilden, Germany) was used for the analysis of cycle threshold values (Ct values) values for each sample. In addition, the relative levels of gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. The primers used in this study are listed in Table 2.

2.7. Immunofluorescent staining

The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton-X for 10 min at room temperature. After blocking with 3% BSA solution for 1 hr, the cells were incubated with primary antibodies such as rabbit anti-aromatase (1:100; Invitrogen, MA, USA), goat anti-FOXL2 (1:200; Invitrogen, MA, USA), and rabbit anti-FSHR (1:200; LSBio, WA, USA) for overnight at 4°C. The was followed by incubation with FITC-conjugated mouse anti-rabbit IgG (1:200; Santa Cruz, TX, USA) or FITC-conjugated donkey anti-goat IgG (1:200; Santa Cruz, TX, USA) for 1 hr at room temperature. After being washed 2-3 times with DPBS, nuclei of cells were counterstained with 1 µg/ml 4′, 6–diamidino-2–phenylindole (DAPI) for 5 min. A fluorescence microscope (Leica, Wetzlar, Germany) was used to capture the images of the cells. The number of granulosa cell-like cells was deter-

Table 2. Lists of primers used in RT-qPCR analysis.

Gene	Primer sequence	Product size (bp)	Accession no.
FSHR (Follicle stimulating hormone receptor)	F: GGCCATCACTGGGAACATCA R: GAGATCAGCAAAGGCCAGGT	103	NM_000145
CYP19A1 (Cytochrome P450 family 19 subfamily A member 1)	F: GCAAGCTCTCCTCATCAAACC R: AATCAACTCAGTGGCAAAGTCC	178	NM_031226.3
FOXL2 (Forkhead box protein L2)	F: CTACTGGGCTCGTCTCTTTCC R: CACCGACCTGTGAGAGAAGC	128	NM_023067
AMHR2 (Anti-Mullerian hormone receptor type 2)	F: GAGGTCATGCAGTGGTTTGG R: GCCTGGAAGTTCGTACAATGC	119	NM_001164691.2
YWHAZ (Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta)	F: CCCACAGACTATTTCCCTCATCC R: AGACAATGACAGACCATTCAGG	114	NM_001135699.2

Table 3. List of antibodies used to detect granulosa cell-specific markers in immunocytochemical staining.

Antibody	Company	Amount
Rabbit anti-FSH IgG	LSbio	0.5 mg/ml
Goat anti-FOXL2 IgG1	Invitrogen	0.5 mg/ml
Rabbit anti-Aromatase IgG1	Invitrogen	0.5 mg/ml
Mouse anti-Rabbit IgG-FITC	Santa Cruz	0.5 mg/ml
Donkey anti-Goat IgG-FITC	Santa Cruz	0.5 mg/ml

mined using Photoshop CS6 software by counting FSHR, Aromatase, and FOXL2 positive cells in 3 randomly captured high-power fields. The antibodies used in immunofluorescent staining are listed in Table 3.

2.8. Aromatase activity assay

Undifferentiated WJ-MSCs or differentiated cells (1 x10^5 cells/well) were seeded on 24 well culture plates for 24 hrs. The cells were then cultured in serum-free DMEM/F12 (1:1) media supplement with 50 ng/ml testosterone for 24 hrs. After collecting cultured media, a 17-beta Estradiol ELISA Kit (Abcam, Cambridge, UK) was used for the evaluation of the estradiol concentration following the manufacturer's instructions.

2.9. Statistical analysis

Data analysis was performed using GraphPad Prism version 8. The statistical differences among experimental groups were estimated by Student's t-test and one-way ANOVA with Newman-Keuls post hoc in this study. Data were represented as mean ± standard deviation (SD) and $P<0.05$ was considered significant.

3. Results

3.1. Isolation and characterization of female and male MSCs derived from Wharton's jelly of the umbilical cord

Wharton's jelly-derived MSCs (WJ-MSCs) of umbilical cords from both females and males were successfully isolated and cultured. At passage 3, the female- and male-derived WJ-MSCs exhibited homogenous, culture plate-adherent, fibroblast-like morphology (Fig. 1A). Both WJ-MSCs were positively expressed MSC-specific markers such as CD44, CD73, CD90, and CD105, and negatively hematopoietic markers such as CD34 and CD45 by flow cytometry (Fig. 1B). The differentiation potential of female- and male-derived WJ-MSCs into mesenchymal lineages was assessed by specific cytochemical staining. All WJ-MSCs successfully differentiated into adipocytes, osteocytes, and chondrocytes over 21 days. Adipocyte differentiation was confirmed by Oil Red O staining, which showed the accumulation of intracellular lipid droplets. Osteocyte differentiation was validated by Von Kossa staining, which revealed the production of mineralized nodules. Chondrocyte differentiation was confirmed by Alcian blue staining, which demonstrated the accumulation of proteoglycans (Fig. 2).

3.2. The gender-dependent ability of WJ-MSCs to differentiate into granulosa cells

The differentiation potential of female- and male-derived WJ-MSCs into granulosa cell-like cells was evaluated using a previously described protocol with minor modification (Lan et al. 2013). After induction for 7 days, both WJ-MSCs exhibited slight morphological changes indicative of granulosa cells (Fig. 3A). Differentiated female cells showed a significant increase in granulosa

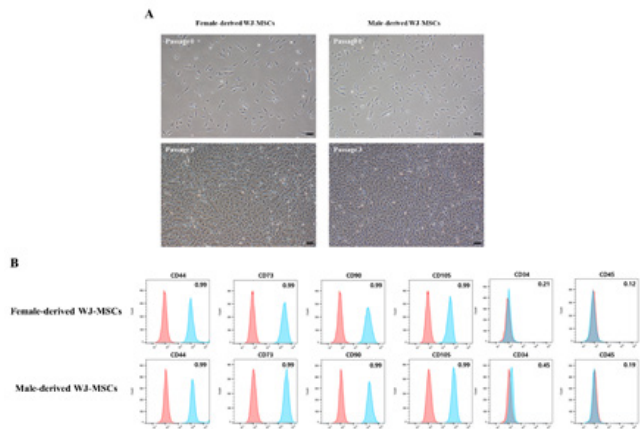

Fig. 1. Characterization of stem cells from female and male (A) MSCs derived from Wharton's jelly at passages 0 and 3 were observed under phase-contrast microscope. (scale bar = 100 µm). The fibroblast-like morphology was observed in both female- and male-derived WJ-MSCs. (B) Flow cytometric analysis of cell surface marker expression in WJ-MSCs. Red peaks show isotype IgG expression as a control. The expression of each CD marker is represented by blue peaks. Flow cytometry revealed that mesenchymal markers such as CD44, CD73, CD90, and CD105 were positively expressed while hematopoietic markers such as CD34 and CD45 were negatively expressed.

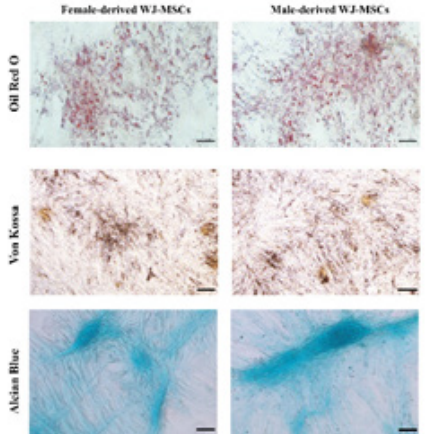

Fig. 2. Female- and male-derived WJ-MSCs differentiated into mesenchymal lineage. Mesenchymal lineage differentiation was confirmed by lineage-specific cytochemical staining. The accumulation of intracellular lipid droplets in adipocytes derived from WJ-MSCs was confirmed by Oil Red O. The production of mineralized nodules in osteocytes was confirmed by Von Kossa. The accumulation of proteoglycans in chondrocytes was confirmed by Alcian blue staining (scale bar = 100 µm).

cell-specific markers, including follicle-stimulating hormone receptor (FSHR, P<0.001), anti-Mullerian hormone receptor type 2 (AMHR2, P<0.001), forkhead box protein L2 (FOXL2, P<0.001), and aromatase (CYP19A1, P<0.001) compared to undifferentiated MSCs. Similarly, differentiated male cells significantly expressed granulosa cell-specific markers such as FSHR (*P*<0.001), AMHR2 (*P*<0.01), FOXL2 (*P*<0.001), and CYP19A1 (*P*<0.05) as measured by qRT-PCR. Although both female- and male-derived WJ-MSCs showed significant increases in granulosa cell-specific gene expression compared to undifferentiated MSCs, the levels of FSHR (*P*<0.001), AMHR2 (*P*<0.01), FOXL2 (*P*<0.001), and CYP19A1 (*P*<0.001) were significantly higher in female-derived WJ-MSCs compared to male-derived WJ-MSCs (Fig. 3B). Additionally, immunostaining confirmed that all differentiated cells expressed granulosa cell-specific proteins such as FSHR, CY*P*19A1, and FOXL2. However, the number of granulosa-specific protein-positive cells, including FSHR (*P*<0.05), CY*P*19A1 (*P*<0.01), and FOXL2 (*P*<0.001), was significantly higher in differentiated female-derived WJ-MSCs compared to male-derived WJ-MSCs (Fig 4).

3.3. Conversion of testosterone to estradiol by granulosa cell-like cells

Granulosa cell-like cells differentiated from female- and male-derived WJ-MSCs were evaluated for aromatase activity, a key granulosa cell function that converts testosterone to estradiol. Both WJ-MSCs differentiated into granulocyte-like cells exhibited active aromatase function, and were significantly (*P*<0.001) higher estradiol levels than undifferentiated WJ-MSCs. However, differentiated female-derived WJ-MSCs had significantly higher *(P*<0.001) estrogen levels compared to differentiated male-derived WJ-MSCs (Fig 5).

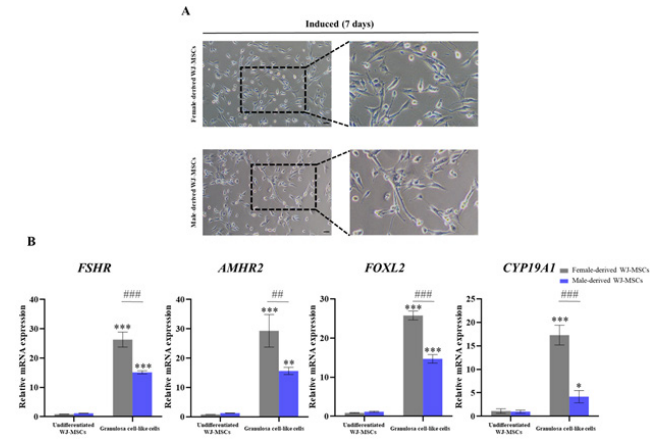

Fig. 3. Cellular morphologies and granulosa cell-specific gene expression levels of differentiated cells. (A) Granulosa cell-like cells induced from WJ-MSCs showed granulosa cell morphology (scale bar = 100 µm). (B) Expression of granulosa cell-specific genes FSHR, AMHR2, FOXL2, and CYP19A1 was analyzed by RT-qPCR. The differentiated cells significantly expressed granulosa cell-specific genes. The expression of genes in differentiated female-derived WJ-MSCs was higher than in differentiated male-derived WJ-MSCs. Data are represented by the mean ± SD. The significance of differences was determined using a one-way ANOVA with Newman-Keuls post hoc. * = P<0.05; ** = P<0.01; *** = P<0.001 between differentiated granulosa cell-like cells and undifferentiated WJ-MSCs. ## = P<0.01; ### = P<0.001 between differentiated female-derived WJ-MSCs and differentiated male-derived WJ-MSCs.

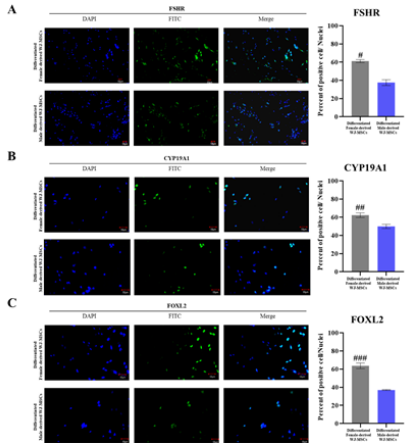

Fig. 4. Quantified immunocytochemical analysis of granulosa cell-like cells. Immunocytochemical staining showed positive expression of granulosa cell-specific markers. Nuclei were stained with DAPI (scale bar = 50 µm). (A, B, C) All marker proteins positive cells in differentiated female-derived WJ-MSCs showed a significant increase compared to differentiated male-derived WJ-MSCs. Data are represented by the mean ± SD. The significance of differences was determined using a Student's t-test. # = P<0.05; ## = P<0.01; ### = P<0.001 between differentiated female-derived WJ-MSCs and differentiated male-derived WJ-MSCs.

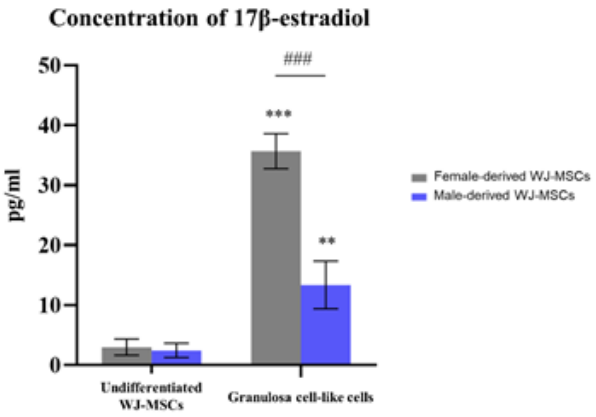

Fig. 5. The evaluation of aromatase activity in granulosa cell-like cells using an ELISA kit. The granulosa cell-like cells derived from WJ-MSCs exhibited aromatase activity. The estradiol levels in differentiated female-derived WJ-MSCs were higher than in differentiated male-derived WJ-MSCs. Data are represented by the mean ± SD. The significance of differences was determined using a one-way ANOVA with Newman-Keuls post hoc. ** = P<0.01; *** = P<0.001 between differentiated granulosa cell-like cells and undifferentiated WJ-MSCs. ### = P<0.001 between differentiated female-derived WJ-MSCs and differentiated male-derived WJ-MSCs.

4. Discussion

MSCs derived from the umbilical cord have in vitro culture properties and the potential for mesenchymal lineage differentiation [19], but due to their cell origin, interest has been drawn in their gender-specific characteristics. Characterization of both WJ-MSCs showed that the properties of MSCs, including fibroblast-like morphology, expression of cell surface markers, and mesenchymal differentiation potential, did not differ between female- and

male-derived WJ-MSCs. These results are consistent with previous studies [21]. Several studies have reported that granulosa cell-like cells can be generated from stem cells [20,22,23]. However, it remains unclear whether the differentiation potential of granulocyte-like cells in WJ-MSCs is influenced by gender. In this study, it was demonstrated that the male-derived WJ-MSCs didn't differentiate into granulosa cell-like cells as much as the same level of female-derived WJ-MSCs. Previous reports also demonstrated a similar result for the differentiation of MSC into oocyte-like cells [24]. Additionally, it appears that female MSCs might differentiate into oocyte-like cells more easily. The expression levels of the sex determination genes, receptors of sex hormones, and growth factors were different between female and male differentiated cells, suggesting that male-derived WJ-MSCs did not differentiate into granulosa cell-like cells as much as the same level of female MSCs. However, to provide direct evidence that elucidates the mechanism, studies investigating physiological aspects and sexual dimorphism will be necessary.

Granulosa cells secrete a range of growth factors, sex hormones, and cytokines in order to modulate autocrine, paracrine, and endocrine functions. Although granulosa cells are a crucial component of the ovary, studies on granulosa cells have been complicated. The stem cells could be a novel resource for understanding granulosa cells. Granulosa cells originate from the mesonephric region, which develops from the intermediate plate of mesoderm [25]. Some previous study was used to differentiate pluripotency stem cells into mesodermal lineage to generate granulosa cell-like cells [20,26]. Since it has been known that MSCs originate from a population of mesodermal lineage cells, the WJ-MSCs were differentiated directly into granulosa cells instead of mesodermal differentiation in the present study [27]. During the development of the urogenital system, the commitment of the mesoderm is significantly influenced by BMP4 and bFGF. Furthermore, bFGF plays a pivotal role in enhancing the proliferation of granulosa cells in vitro [28]. Moreover, the process of folliculogenesis is regulated by follistatin along with interaction of activins, growth differentiation factor 9 (GDF-9), and BMPs [29]. During the differentiation of stem cells, the germ layer of the target cells is a crucial factor to be considered. To differentiate into target cells, it established the specific signaling pathways and growth factors. Actually, it has been reported that germ layer origin influences epigenetic memory and differentiation into particular cell lineages[30]. Consequently, in order to enhance the reliable differentiation protocols, considering the germ layer of stem cells is essential for achieving the desired cellular phenotype.

In the present study, WJ-MSCs differentiated into mature granulosa cells were positive for granulosa cell-specific markers such as FSHR, CYP19A1, FOLX2, and AMHR2, and possessed granulosa cell function, including the conversion of testosterone to estradiol. FSHR is essential for follicle growth and ovulation in the ovary [31]. The expression levels of FSHR in granulosa cells were modulated in a manner dependent on the stage of follicular growth. Notably, mutations in FSHR within granulosa cells have been influenced by conditions such as premature ovarian failure, ovarian hyperstimulation syndrome, and infertility [32]. It was demonstrated that FOXL2, which was predominantly expressed in fetal and adult ovaries granulosa cells, was crucial for the formation of follicles [33]. CYP19A1, which converts androgen to estrogen, was specific to functioning granulosa cells. In porcine granulosa cells, CYP19A1 is necessary for the production of estrogen and for preventing apoptosis [34]. In the present study, differentiated WJ-MSCs expressed granulosa cell-specific markers. It is consistent with previous study, which showed that stem cells can differentiate into granulosa cells and express granulosa cell-specific markers [20,26]. The functional confirmation of granulosa cell-like cells derived from WJ-MSCs was demonstrated by the increased levels of the aromatase enzyme and the successful aromatization of testosterone to estradiol. These findings are consistent with previous studies of granulosa cell differentiation from other stem cells [20,22].

5. Conclusion

It was proven that both female-and male-derived MSCs using growth factors were directly differentiated into functional granulosa cells. However, female-derived WJ-MSCs exhibited a greater capacity for differentiation into granulosa cell-like cells with functional properties than male-derived WJ-MSCs. This finding will provide critical information for understanding human granulosa cell differentiation using MSCs, which can be clinically applied to infertility and hormone treatment.

Conflict of Interests

No potential conflict of interest was reported by the author(s).

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

Human female and male umbilical cord tissues undergoing either cesarean section or normal vaginal delivery were obtained from full-term births after taking informed donor's consent and approval from the Ethics Committee of the Gyeongsang National University Hospital (No. GNUH IRB-2012–09-004).

Authors' contributions

Sang-Yun Lee: conceptualization, software, validation, formal analysis, data curation and writing—original draft preparation; Dinesh Bharti: data curation, investigation and validation; Young-Bum Son: Methodology, software and investigation; Won-Jae Lee: software, validation and formal analysis; Yong-ho Choe: Methodology and investigation; Hyeon-Jeong Lee: Methodology and investigation; Seong-Ju Oh: visualization, methodology and investigation; Tae-Seok Kim: visualization, methodology and investigation; Chae-Yeon Hong: visualization, methodology and investigation; Sung-Lim Lee: conceptualization, validation, formal analysis, data curation, writing—original draft preparation, supervision, funding acquisition, Gyu-jin Rho: conceptualization, validation, formal analysis, data curation, writing—original draft preparation, supervision, funding acquisition.

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