



# Basal characterization and *in vitro* differentiation of putative stem cells derived from the adult mouse ovary

Masoumeh Majidi Zolbin, PhD<sup>1,2</sup> · Gulcin Sahin Ersoy, MD<sup>3</sup> · Fereshte Aliakbari, PhD<sup>4</sup> · Fardin Amidi, PhD<sup>2</sup> · Faezeh Daghigh, PhD<sup>5</sup> · Mehdi Abbasi, PhD<sup>2</sup> · Joshua Johnson, PhD<sup>6</sup>

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## Abstract

Lately, stem cell approaches have provided new information on reproductive organ function and additionally recommended novel treatment possibilities. The type(s) and differentiation potential of stem cells present in the mammalian ovary are largely unknown; while oogonial stem cells have been reported, we explored the possibility that multipotent stem cells may reside in the ovary and have wide differentiation potential. In this experimental study, homogenates of whole mouse ovaries were sorted using the stem cell surface markers stem cell antigen-1 and stage specific embryonic antigen-1/CD15. Viable double-positive cells 3–10 µm in diameter were evaluated immediately after sorting and after culture using differentiation conditions. Ovarian-derived stem cells were differentiated into the three main cell types: adipocytes, chondrocytes, or osteocytes. The subsequent culture was performed in media containing bone morphogenetic protein 4 (BMP-4) and/or retinoic acid (RA). RA, BMP-4 or the two agents in combination, consistently stimulated germ cell gene expression. RA treatment strongly stimulated germline gene expression and also the development of cells that were morphologically reminiscent of oocytes. The germ cell genes *Dazl*, *Ddx4*, *Figla*, *Gdf-9*, *Nobox*, *Prdm9*, and *Sycp-1* were all detected at low levels. Remarkably, treatment with BMP-4 alone significantly increased protein expression of the granulosa cell product anti-Müllerian hormone (AMH). We have shown that an inclusive isolation protocol results in the consistent derivation of multipotent stem cells from the adult ovary; these cells can be differentiated towards the germ cell fate (RA alone), somatic ovarian cell fate as indicated by AMH production (BMP-4 alone), or classical mesenchymal cell types. Taken together, these data suggest the presence of multipotent mesenchymal stem cells in the murine ovary.

**Keywords** Stem cells · Ovary · BMP-4 · Retinoic acid · Multipotent

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✉ Masoumeh Majidi Zolbin, PhD  
Masoumeh.majidizolbin@gmail.com

Gulcin Sahin Ersoy, MD  
gulcinsahinmd@gmail.com

Fereshte Aliakbari, PhD  
fereshtehaliakbari@yahoo.com

Fardin Amidi, PhD  
Famidi@tums.ac.ir

Faezeh Daghigh, PhD  
F\_daghigh@yahoo.com

Mehdi Abbasi, PhD  
abbasima@tums.ac.ir

Joshua Johnson, PhD  
Joshua.2.johnson@ucdenver.edu

- <sup>1</sup> Pediatric Urology and Regenerative Medicine Research Center, Section of Tissue Engineering and Stem Cells Therapy, Children's Hospital Medical Center, Tehran University of Medical Sciences, Tehran, Iran
- <sup>2</sup> Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Poorsina Street 16th Street Keshavarz BLV, Tehran, Iran
- <sup>3</sup> Kartal Dr. Lutfi Kirdar Educations and Research Hospital, Istanbul, Turkey
- <sup>4</sup> Infertility & Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
- <sup>5</sup> Department of Physiology, Islamic Azad University, Tabriz Branch, Tabriz, Iran
- <sup>6</sup> Departments of Obstetrics, Gynecology and Reproductive Sciences, University of Colorado-Denver, Denver, CO, USA

## Introduction

In recent years, stem cell approaches have provided new information on reproductive organ function as well as suggested novel treatment possibilities. Cells with stem cell and/or regenerative characteristics have been isolated from the female reproductive tract (Taylor 2004; Bhartiya and Patel 2018). For example, multiple studies demonstrated the isolation and characterization of endometrial mesenchymal stem cells from the uterus (EMSc) (Johnson et al. 2004; Du et al. 2012; Santamaria et al. 2018). Initially, the capacity for self-renewal and multilineage differentiation of EMSc was established, as well as their ability to differentiate to chondrocytes; more recently, it has been shown that EMSc have restorative ability when delivered to the brains of mice and non-human primates with induced Parkinson's disease, leading to increased dopamine production (Wolff et al. 2011; Wolff et al. 2015). Similarly the ability of endometrial stem cells demonstrated to produce insulin and treat diabetes in a murine model (Santamaria et al. 2011). Endometrial stem cells resemble bone marrow mesenchymal stem cells. Bone marrow-derived mesenchymal stem cells (MSC) have been delivered to both systemically and directly into the uterus. MSC were shown to engraft and differentiate into uterine cell types (Du et al. 2012; Du and Taylor 2007). Very recently, this approach was shown to improve uterine function in Asherman's syndrome, first in a mouse model (Alawadhi et al. 2014; Ersoy et al. 2017) and later in humans (Santamaria et al. 2016). Most of tissue-specific stem cell residents initiate from the inner cell mass of the blastocyst during primary embryogenesis and upon specific lineage commitment lose pluripotent potential and become multipotent (Oatley and Brinster 2012). Like stem cell, population in the testes are sustainable population; during neonatal and embryonic progress, which will persist undifferentiated throughout life, spermatogonial stem cells (SSCs), as unipotent stem cells, are responsible for the production of sperm during the male's maturation life (Koruji et al. 2012).

Most published studies describing ovarian stem cells have focused on the germ cell lineage and the potential for regeneration of the oocyte pool during adulthood (White et al. 2012). The concept of a fixed ovarian reserve of primordial follicles established before birth was defined in 1951 (Green and Zuckerman 1951). This question has been re-addressed by several groups, some of which have offered evidence that proliferative germline cells (labeled variously "female germline stem cells/FGSC," "germline stem cells/GSC," "oogonial stem cells/OSC," etc.) are present in the adult mouse ovary (Johnson et al. 2004; Bhartiya et al. 2013; Parte et al. 2013). Components for the new primary follicles, primitive granulosa and germ cells, are proposed to differentiate *de novo* from mesenchymal progenitor cells residing in the ovarian tunica albuginea. During differentiation into OSE cells, the

mesenchymal progenitor cells line either the ovarian surface or invaginated epithelial crypts. Mesenchymal progenitor cells would first contribute to the development of epithelial cells similar to granulosa cells, and these cells subsequently form epithelial nests descending into the deeper ovarian cortex. These cells may be a source of germ cells, which assemble together with nests of primitive granulosa cells to form primary follicles.

Other studies have instead provided strong evidence that these cells are not present in the adult mouse ovary (Zhang et al. 2012), and even if present, do not produce new oocytes under physiological conditions. Recently, two separate groups have isolated and characterized putative FGSC from adult human ovaries. First, White *et al.* (White et al. 2012) isolated the cells from human ovarian cortex biopsies, and more recently, Ding *et al.* were able to isolate putative FGSC from follicle aspirates collected during human *in vitro* fertilization cycles (Ding et al. 2016). These latter findings in human have re-focused the field on characterizing stem cells in the adult ovary.

Effects of different signaling molecules on ovarian stem cells have been evaluated *in vitro*. Parte *et al.* showed that bone morphogenic protein 4 (BMP-4) increases the number of FGSC and *in vitro*-derived oocytes in a dose-dependent fashion (Park et al. 2013). Separate studies showed that initiation of meiosis in ovary is dependent upon retinoic acid (RA) signaling, including evidence that RA treatment significantly elevates meiotic gene expression (Bahmanpour et al. 2015; Huang et al. 2010; Le Bouffant et al. 2010). Because of their reported potency and known relevance to germ cell development, we chose BMP-4 and RA as our experimental treatments.

Rather than attempting to isolate germline stem cells, this study was designed to isolate multipotent stem cells from the adult ovary. After isolation, we then characterized these cells and their differentiation potential. Our strategy began with fluorescence-activated cell sorting (FACS) of whole ovary single cell suspensions for the surface markers stem cell antigen-1 (Sca-1) (stem cell marker) and stage specific embryonic antigen-1 (SSEA-1)/CD15 (germ and general stem cell marker). This population of putative progenitor cells expresses the stem cell marker lymphocyte antigen 6 complex, locus A (LY6A), also known as stem cell antigen-1 (Sca-1). This population is regulated by at least two factors found in the follicular fluid at the time of ovulation, indicating an important role for these cells in facilitating ovulatory wound repair (Gamwell et al. 2012). Sca-1 expression has not been previously reported in oocytes, more ever there is no study to demonstrate these cells potential to differentiate to germ-like cells *in vitro* and this examination raises the fascinating opportunity of a connection between this stem-like cell population and neo-oogenesis. There are different ideas about the Sca-1, like the study that considered these cell populations as non-hematopoietic

mesenchymal stem cells, although Sca-1 in most studies is considered a stem cell marker for isolation of stem cell from different organs (Sudres et al. 2006; Zolbin et al. 2018). Sca-1 mice go through normal bone skeleton development but with age reveal dramatically diminished bone mass resulting in brittle bones (Bonyadi et al. 2003). Thus, defective mesenchymal stem or progenitor cell self-renewal may represent a previously uncharacterized mechanism of age-dependent disease. We then evaluated the effects of the addition of BMP-4 and RA separately and together, as well as defined mesenchymal stem cell differentiation media (adipocyte, chondrocyte, and osteocyte) to examine the germline and somatic differentiation potential of these cells.

## Material and Methods

### Animals

In this experimental study, six-week-old C57BL/6 female mice were maintained in the animal facility at the Tehran University School of Medicine under an approved institutional animal care and use committee protocol.

### Isolation of SCs from mouse ovaries

For isolation of stem cells (SCs), ovaries were collected, and the attached fat pad, bursa, and oviduct were carefully excised. Ovaries were then minced into very small pieces. Minced ovarian tissue was digested in 0.83 mg/ml collagenase type 2 (Worthington Biochemical Corporation NJ; #LS004174) in sterile Hank's Balanced Salt solution (HBSS; diluted from 10× stock, containing no calcium, no magnesium, no phenol red; Life Technologies CA; #14185-052) containing 3% bovine serum albumin (BSA), 1.23 mM calcium chloride, 1.03 mM magnesium chloride, and 0.83 mM zinc chloride for 15 min in a 37°C shaking water bath (160–180 rpm), and interrupted incubation with vigorously shaking by hand for 10–20 s after 10 min of incubation. The supernatant was separated from the pellet by centrifugation at 400×g for 3 min. The ovarian homogenate was re-suspended in 3 ml HBSS, 3% BSA wash buffer, and filtered through sterile 40 μm (BD Biosciences CA, USA; #352340) nylon mesh filters before antibody staining.

### Cell staining and FACS

Putative ovarian stem cells were sorted and characterized by FACS analysis; digested cells were stained with the following antibodies: CD15 PE (Biolegend#125605) (diluted 1:100 in HBSS with 3% BSA wash buffer) and LY-6A/E PE/CY5 (Sca-1) (Biolegend#108109) (1:500). Antibodies were diluted according to the manufacturer's recommendations in wash

buffer and the ovary pellet was re-suspended in antibody staining solution and placed on ice in the dark for 30 min. An excess of wash buffer was then added, and the stained cell suspension was centrifuged at 300×g for 3 min. The supernatant was removed, and the pellet was re-suspended in wash buffer and subsequently filtered through a 40 μm nylon filter prior to FACS. Isotype-matched negative controls were used to define background staining. Cell sorting was performed using the BD FACS Aria II software (BD Biosciences).

### Ovarian stem cell culture

After sorting, the cells were resuspended in primary culture medium (PMS) consisting of 40 ml DMEM-F12 (Gibco#11330-032) supplemented with 10% fetal bovine serum (FBS; Gibco#10437028), and 10<sup>3</sup> U ml<sup>-1</sup> leukemia inhibitory factor (LIF; amsbio#AMS-263) were plated in 12 well plates at a density of 2 × 10<sup>5</sup> cells per well in 500 μL culture media. Plates were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in air for 3 d. Plated cells were obtained after 3 d of incubation and then used for long term culture. Cells were trypsinized and replated onto mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layers (6 well plates; seeded in concentration of 5 × 10<sup>4</sup> cells/well). Culture media was maintained for 1 wk with 200 μl drops of fresh complete stem cell culture medium added every day. After 7 days, putative germline differentiation components were added to the cell culture as follows: BMP-4 200 ng/ml; RA 2 μM for either 1 or 2 wk. The initial culture medium was maintained for 1 wk while a 200 μl of fresh complete culture medium was added every other day. Germ cell marker gene expression was evaluated at the end of the culture period by RT-PCR, and production of AMH was evaluated by ELISA (details below).

### RNA extraction

RNA was extracted with TRIzol RNA Isolation Reagent (Sigma, St. Louis, Mo.) from all groups including vehicle-treated controls and those treated with RA, BMP-4 or BMP-4 + RA in triplicate. RNA was further purified with columns using the RNAqueous-Mini Kit (Qiagen). DNase enzyme and RDD digestion was performed to exclude genomic DNA contamination. RNA was quantified and assessed for integrity using Nano Drop ND 2000 spectrophotometer. Purified RNA was stored at -80°C until use.

### Quantitative real-time polymerase chain reaction (qPCR) analysis

Purified RNA was reverse-transcribed in 20 μl reaction mixture using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR (qPCR) was performed using SYBR Green (Bio-Rad) and optimized in the

MiyQ Single-Color Real-Time PCR Detection System (Bio-Rad). Primer sequences for all the genes are listed below. Gene expression was normalized to the expression of  $\beta$ -actin for each sample. Relative mRNA expression for each gene was calculated using the comparative cycle threshold (Ct) method, also known as the  $2^{-\Delta\Delta CT}$  method. All experiments were carried out in duplicate. Nuclease-free water instead of cDNA template as well as the reaction mix without reverse transcriptase were used as negative controls. Data were normalized using log transformation.

### Reverse transcription–polymerase chain reaction

Polymerase chain reaction was performed at first day, 1 wk, and 2 wk after differentiation using GO Taq Green ,Master Mix with synthesized cDNA and primer pairs of pluripotency related markers. Cycling parameters were as follows: 5 min initial denaturation at 94°C, then 33 cycles at 94°C for 30 s, corresponding to the annealing temperature of primer pairs for 30 s, and 72°C for 1 min. Polymerase chain reaction products were loaded on 2% agarose gel, stained with ethidium bromide, and visualized by UVITEC Cambridge Gel Documentation systems (Cambridge, CB4 1QB-UK England). Expression of  $\beta$ -actin as a housekeeping gene was evaluated as an internal control.

### Immunocytochemical staining

To estimate the germ cell marker expression, differentiated cells from each group were plated in four chamber glass sides and evaluated for following antibodies: DAZL (ab34139), (1:100); DDX4(ab13840–100), (1:100); and Fragilis (ab15592), (1:100). Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature and then washed three times in ice cold PBS, and the permeabilization in 0.1% Triton in PBS for 10 min blocking has been done in 5% BSA and 10% donkey serum for 1 h. Blocking buffer was removed, and after washing for 3 times with PBS, cells were incubated overnight with primary antibodies, and subsequently a day after, cells were stained with secondary donkey anti-rabbit antibody.

### Anti-Mullerian hormone (AMH) protein quantification of conditioned media by enzyme-linked immunosorbent assay

Detection of AMH protein concentration in the conditioned media from the cell culture was quantified in triplicate by enzyme-linked immunosorbent assay kit (ELISA; Cloud-Clone Corp., Houston, Tex), according to the manufacturer's instructions. The supernatants were collected after 2 wk of incubation with specified culture media, centrifuged to remove any cellular debris and subsequently frozen at  $-80^{\circ}\text{C}$

until use. Part of each sample was likewise diluted (1:2) to ensure that AMH concentration would fall within the detection range of the assay kit. A 96-well ELISA strip plate pre-coated with anti-AMH monoclonal antibody was incubated with varying concentrations of standard, blank, and samples for 1 h at 37°C. This set utilizes avidin conjugated to horseradish peroxidase (HRP) and tetramethylbenzidine (TMB) as substrate. Optical density was read in a microplate reader at 450 nm, and a standard curve was subsequently generated to extrapolate AMH concentration in the samples.

### Statistical Analysis

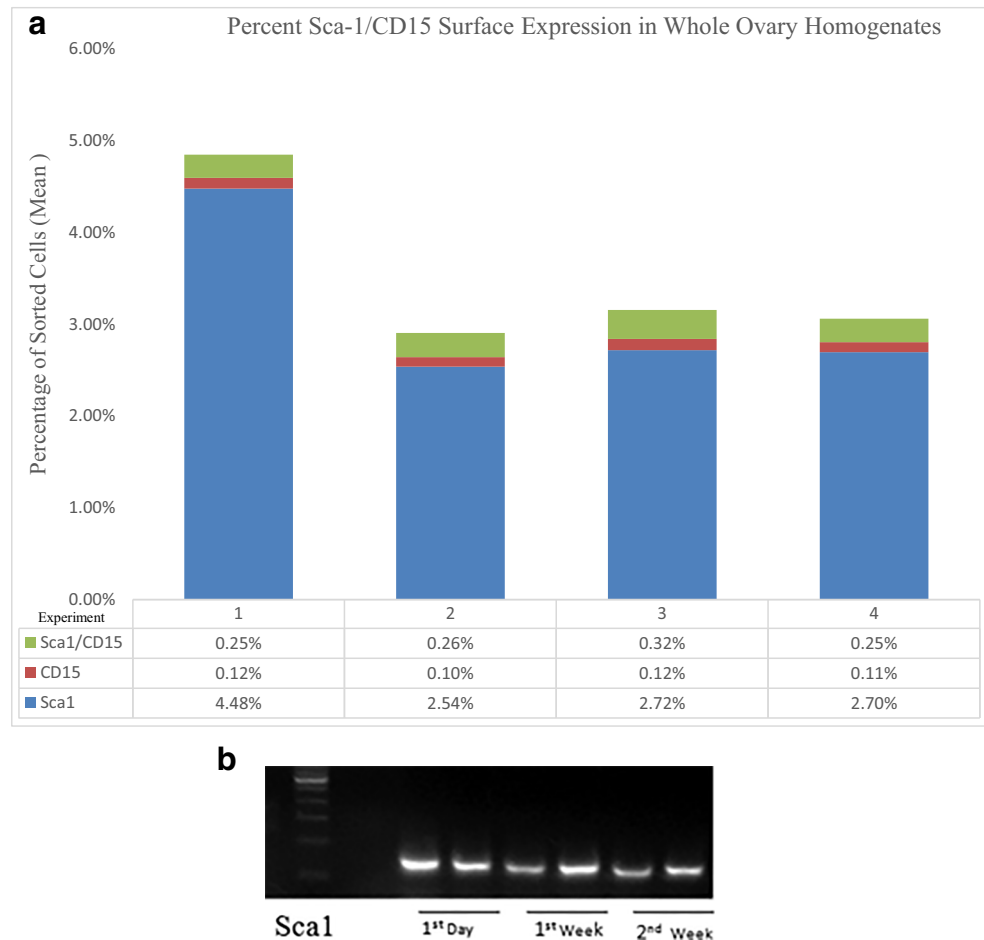
The Graphpad Prism 6 was used to perform all statistical analysis (GRAPHPAD software; [www.graphpad.com](http://www.graphpad.com)). All the experiments were performed with at least three replicates; raw data was normalized using log transformation where indicated. Two-way ANOVA was performed using Holm-Šidák multiple comparison test with  $p < 0.05$  to determine statistically significant differences between datasets.

### Results

Our approach began with the cell sorting of single cell suspensions prepared from whole adult ovaries. In keeping with prior studies, we stained and sorted cells using Sca-1 and SSEA-1/CD15 (Gamwell et al. 2012; Virant-Klun et al. 2008). We prepared 4 separate ovarian single-cell homogenates followed by cell sorting to produce cells positive for each single marker alone as well as double-positive cells. Figure 1A summarizes the percentage of cells of each category. In each experiment, approximately 93–95% of cells were not positive for either marker; 3–5% of cells were Sca-1-positive; 2.54% were SSEA-1-positive, and less than 1% of cells were SSEA-1/CD15-positive or double-positive. RT-PCR results showed the expression of Sca-1<sup>+</sup> stem cells in the sorted cell in culture in three different timeline of cell culture (1st day, 1st week, and 2nd week) which confirms that these cells preserved their stem profile during the cell culture process (Fig. 1B). Moreover, in order to characterize the starting population of cells, the expression of oocyte-specific genes along with Sca-1 marker in these cells prior to plating were evaluated with NOBOX gene marker. The RT-PCR results confirmed that these cells did not express germ cell gene marker, but they were remarkably expressing stem cell marker (Fig. S1). We performed subsequent experiments on double-positive cells, hypothesizing that cells positive for both markers would behave the most like stem cells.

We then attempted to differentiate Sca-1/CD15-positive cells into adipocytes, osteocytes, and chondrocytes by culturing freshly isolated cells in corresponding differentiation media.

**Figure 1.** Consistent fractions of ovarian cells stain for Sca-1 and/or CD15. **A** After staining and cell sorting, we summarized the fractions of single- and double-positive cells in each of 4 unique experiments and expressed them as percent of total cells isolated by FACS. **B** Expression of Sca-1 in the sorted cells by RT-PCR. These cells characterized by RT-PCR prior to culture with Sca-1 markers in three different time lines.



Additionally, the differentiation potential has been confirmed by Oil Red O and Safranin O staining as a specific staining for adipose and chondrocyte. In each case, we found that ovary-derived double-positive cells were consistently capable of differentiating into these three somatic lineages (data not shown).

We next focused on the capacity of the cells to differentiate into the germ lineage and into a key somatic lineage in the ovary, granulosa cells. We tested the effects of two agents known to be involved with germ cell development, BMP-4 and RA.

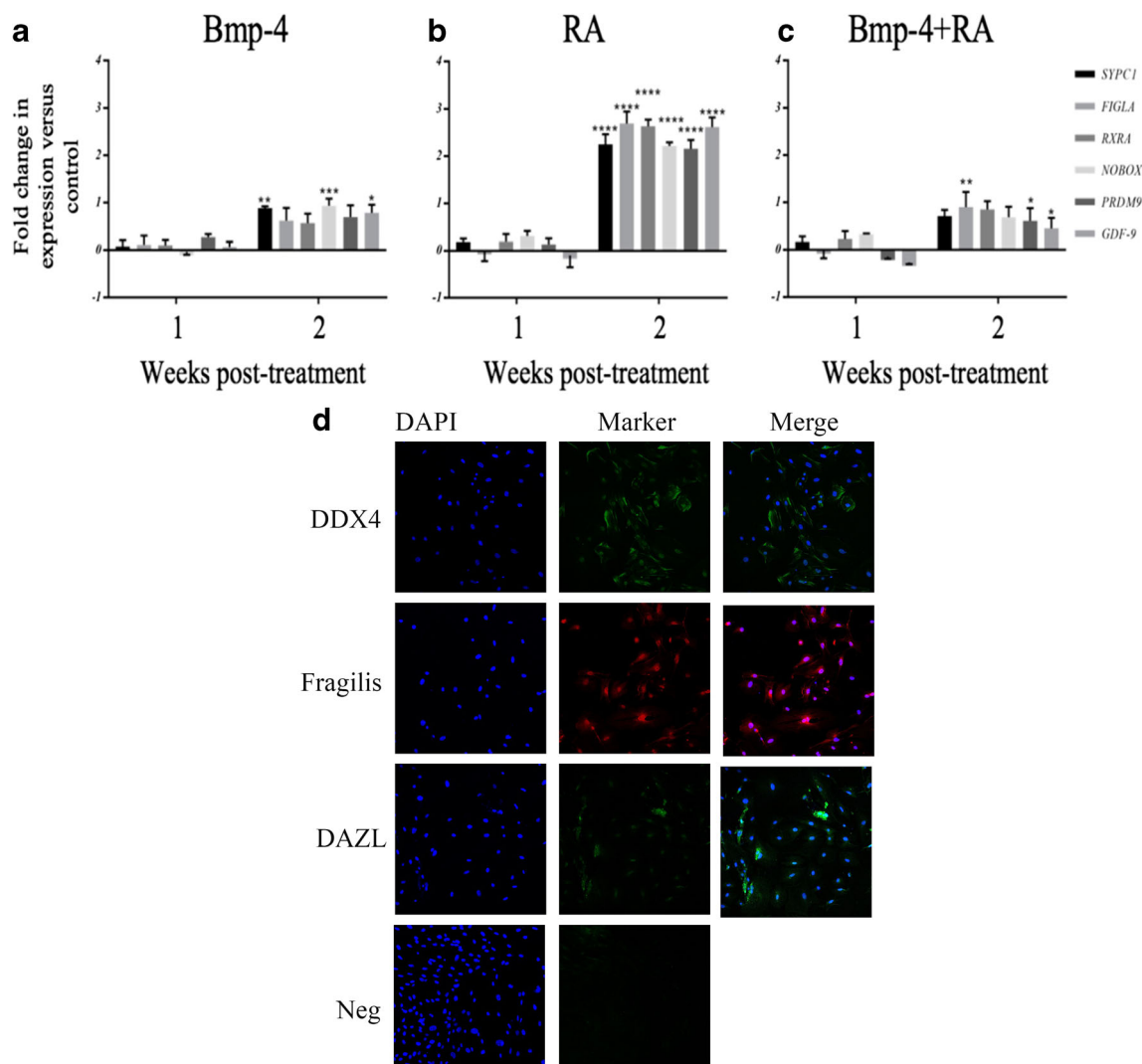
Our analysis of potential germ cell differentiation began with quantitative reverse-transcription PCR (qPCR) measurement of the expression of germ cell genes *Sycp1*, *Figla*, *Nobox*, *PRDM9*, and *GDF-9*, as well as the retinoic acid receptor (*RXR*) in response to culture with BMP-4, RA, or both agents (Fig. 2A-C). Interestingly, inclusion of either or both agents was associated with increased expression of each of the germ cell genes after 2 wk of culture. Inclusion of RA alone resulted in the significantly increased expression of *all* genes assessed, and BMP-4 largely abrogated the increased germ cell gene expression.

We followed the qPCR studies with the immunocytochemical staining of cells treated with RA for 2 wk for three separate germ cell markers, Dazl, Ddx-4, and Fragilis (Ifitm3) (Fig. 2D). A subset of cells was positive for each of these three

proteins *via* fluorescence detection, while exclusion of primary antibody resulted in no detectable signal (Fig. 2, lower right panels) under identical detection conditions.

Along with the gene expression changes suggestive of germline differentiation, we noted that the appearance of cells appeared to change depending upon which treatment with our two agents (alone or in combination) was applied. The development of spherical cells that detach from culture plates has been noted and interpreted as development of “oocyte-like cell” in prior publications (Wen et al. 2017). We quantified such cells (denoted by arrowheads) in each treatment group (Fig. 3). Consistent with the changes in gene expression, different numbers of spherical cells were found in each group. After plating identical numbers of cells at the start of each experiment, control wells contained an average of 97 detached spherical cells, BMP-4 treatment 286 cells, RA treatment, 362 cells, and BMP-4 + RA treatment, 244 cells. Here again, treatment with any agent increased the appearance of “oocyte-like” cells while RA treatment alone corresponded with the highest number, an effect that was abrogated by BMP-4 treatment.

Secreted AMH protein level in media would be strong evidence of granulosa cell identity within our cultures. We therefore performed ELISA to detect AMH in conditioned culture media from our treatment groups (Fig. 4). AMH



**Figure 2.** Analysis of germline differentiation of ovary-derived cells. Cells were either maintained in simple culture media or were supplemented with BMP-4, RA, or both agents for 1 or 2 wk. Cells were collected at the end of each time period and were processed for qPCR measurement of the expression of germ cell genes *Sycp1*, *Figla*, *Nobox*, *PRDM9*, and *GDF-9*, as well as the *retinoic acid receptor*. Data are presented as the fold change in expression versus controls. **A** BMP-4 treatment was shown to significantly increase the expression three of the six genes tested. **B** RA

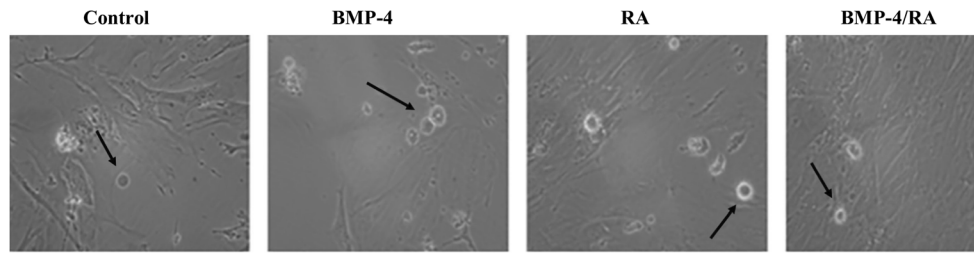
treatment significantly increased the expression of all six genes, while treatment with both BMP-4 and RA (**C**) resembled the effect of BMP-4 alone. \* $p = 0.05$ , \*\* $p = 0.04$ , \*\*\* $p = 0.03$ , \*\*\*\* $p = 0.001$  (**D**). Immunocytochemical staining of cells treated with RA for 2 wk identifying three germ cell markers, *Dazl*, *Fragilis*, and *Ddx-4*. A subset of cells was positive for each of these three proteins via fluorescence detection. Neg: Exclusion of primary antibody resulted in no detectable signal under identical detection conditions.

secretion was detectable in all groups, including control cells. Unlike the germ cell gene expression that was associated most with RA treatment, treatment with BMP-4 alone significantly increased AMH levels above than the detected in control cells (approximately two-fold). In this case, RA appeared to abrogate AMH production induced by BMP-4.

## Discussion

We were able to isolate a fraction of cells with stem cell characteristics using FACS sorting for Sca-1 and/or CD15 cell surface expression. Gene expression analysis at baseline

revealed low but consistent expression of a panel of six germ cell specific genes, all of which could be increased by treatment with BMP-4, RA, or the two agents in combination. Treatment with RA alone was associated with the greatest increases in the expression of germ cell/oocyte-specific genes and, accordingly, with the highest production of cells that lifted off of culture plates, morphologically resembled oocytes, and maintained germ cell gene expression. Interestingly, treatment with BMP-4 alone resulted in significantly increased AMH peptide hormone production; this effect was abrogated by RA. RA has many known roles in morphogenesis, proliferation, and differentiation during vertebrate spermatogenesis and organogenesis (Ma et al. 2018;



**Figure 3.** Spherical “oocyte-like cells” detach from culture plates. In all treatment groups, spherical cells are found. In control cultures, these cells tended to be dark and irregular (A; *arrowhead*). In contrast, BMP-4, RA,

and both BMP-4/RA treatment resulted in the generation of many such large detached cells with an intact, qualitatively shiny appearance. The greatest numbers were seen when treated with RA alone.

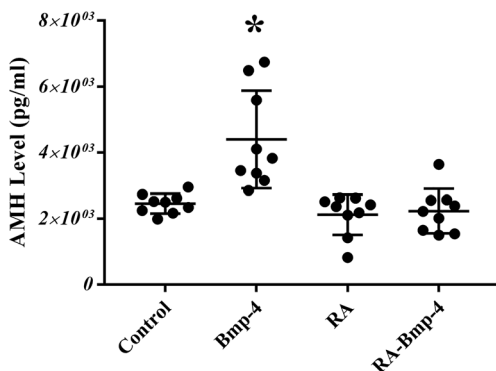
Ruivo et al. 2018). RA is responsible to check whether mouse fetal germ cells go into meiosis or not. Some studies reported that, although RA induces germ cells to enter meiosis in the ovary at around 13.5 d post coitum, its degradation protects germ cells from entering meiosis in males at that time; additionally, RA induces differentiation of embryonic cells and mesenchymal stem cells into male germ cells (Kashani et al. 2014). Our findings complement earlier studies and showed that exogenous RA added to mice ovarian stem cells in *in vitro* culture accelerates their entry into meiosis with upregulation of RXR which is a RA receptor and SYPC1 as a gene marker for meiosis initiation. Although, higher number of round cells in RA group confirm that RA has prominent role in growth of differentiated cells, but further investigation needed to point out the functional potential of these differentiated cells. The simplest interpretation of these findings is that RA treatment favors the development and/or survival of cells consistent with germline identity; BMP-4 favors somatic cell differentiation and development (including granulosa cells), and the agents antagonize each other in the specification of each cell lineage. The sex differentiation of germ cells is determined not by their chromosomal constitution but by cues from their environment. Regarding the morphology of the cells, since these cells are not supporting with the ovary cytoskeleton also, it is not a

natural niche for ovarian stem cells or oocytes so we could not expect them to be round like those that are located in the ovary; based on previous studies, we found that ovarian surface epithelium cells or stromal cells or stem cells have been reported with the same morphology in *in vitro* studies, and they were looking elliptical or spindle shape, or they are not round (Park et al. 2013; Bahmanpour et al. 2015; Yang-Hartwich et al. 2014; Silvestris et al. 2015).

### Conclusion

Infertility is a condition that affects 1 out of 6 couples. Women afflicted with poor oocyte quality, sometimes accompanied by diminished ovarian reserve (DOR), make up a large fraction of these infertility cases. Because assisted reproduction technologies (ART) cannot always help couples achieve a live healthy birth, there is significant incentive to intervene and improve ovarian function. In sum, our data presented here suggest that at least the murine ovary can be viewed as a reasonably rich source of “stem-like” that might be used for differentiation, and expansion for the treatment of ovarian dysfunction also make them candidate for future clinical treatment because of their putative stem nature. In addition, the ovary can produce cells with the capacity to differentiate into separate somatic lineages that could be used to treat disease states in other organs but for future study. It can be suggested that marker genes (Foxl2, Fshr) for granulosa cells could be analyzed by qPCR to further substantiate the AMH results; it is necessary to evaluate the granulosa gene markers initially to compare the somatic gene profile of isolated cells at the beginning and after a long culture *in vitro*.

Taken together, our data suggest that multipotent stem cells with wide developmental plasticity are present in the ovary. Like most organs, the ovary has stem cells that can be manipulated *in vitro* to form multiple cells types. Their role *in vivo*, if one exists, remains to be determined.



**Figure 4.** AMH detection in media of ovary-derived Sca-1/CD15-positive cells. ELISA analysis of culture media conditioned for 2 wk revealed the presence of AMH in each treatment group. Only BMP-4 alone significantly increased AMH production. \*,  $p < 0.005$ .

**Author's contributions** MMZ conceived the study; GSE performed PCR and FACS analysis; FA and MA designed the overall experimental scheme; JJ participated in the design, coordination, and data interpretation and helped to draft the manuscript; EB did data analysis. All authors read and approved the final manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Ethics approval and consent to participate** All experimental procedures were approved by the ethics committee of Tehran University of Medical Sciences.

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