Characterization of ovine surface epithelium-derived stem cells and the modulatory role of phenol red in their proliferation and differentiation

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Abstract

Introduction: The ovarian surface epithelium (OSE) is postulated to harbor stem cells in adults, yet direct evidence remains limited. Phenol red, a pH indicator with weak estrogenic activity, may influence estrogen-sensitive stem cell differentiation, but its role is not well defined. This study examined the effect of phenol red on the isolation, characterization, and oocyte-like differentiation of stem cells from sheep OSE.

Materials & Methods: OSE-derived cells were cultured for three weeks in media with or without phenol red. Isolated small round cells (2–4 µm) were expanded and assessed for pluripotency markers (SSEA-1, SSEA-3) and germ cell/oocyte markers (VASA (DDX4), ZP3) via immunocytochemistry. Expression of germline genes (*c-Kit*, *ZP3*, *DDX4*) was analyzed by quantitative real-time PCR and statistically compared between groups.

Results: The isolated cells showed high proliferative and colony-forming capacity and expressed pluripotency and germ cell markers, confirming their stem and germline identity. These cells differentiated into oocyte-like structures *in vitro*. Cultures with phenol red showed higher ($P < 0.05$) expression of c-Kit (9.5-fold) and ZP3 (3.6-fold), along with increased colony number (15%) and cellular density (70%), compared to phenol red-free controls.

Conclusion: This study shows that ovine OSE contains putative stem cells capable of oocyte-like differentiation. Phenol red enhanced this process, indicating its role as a supportive, estrogen-mimicking factor in ovarian stem cell culture. This suggests that endogenous estrogenic signaling may regulate these cells *in vivo*. The findings contribute to the discussion on postnatal oogenesis and highlight the influence of culture medium composition on stem cell fate.

Keywords: germline stem cells; *in vitro* differentiation; oocyte-like cells; ovarian surface epithelium; phenol red; postnatal oogenesis.

1. Introduction

Gametes are the only mammalian cells that arise as products of meiotic division, a process that is highly conserved in sexual reproduction [1]. For more than a century, reproductive biology has been concerned with whether germ cells arise exclusively from a prenatally specified pool or can additionally be generated postnatally from somatic stem cells [2]. The long-standing consensus has been that females are endowed with their full supply of oocytes at birth, followed by a progressive decline in oocyte number until menopause [3]. However, accumulating evidence now challenges this dogma. It has been shown that female germ cells differentiate from the germinal epithelium during fetal ovarian development [2], and intriguingly this process may have a postnatal counterpart. Recent findings in rodents and humans suggest that the adult ovarian surface epithelium (OSE) may harbor stem cells capable of generating new germ cells under appropriate stimuli, offering a potential mechanism for postnatal oocyte-like differentiation [4,5]. This concept remains an active area of investigation and debate, but an increasing number of studies continue to provide evidence supporting the existence and functionality of ovarian stem cells.

A substantial body of research suggests that the OSE—a simple mesothelium lining the surface of the ovary—may serve as a potential source of germline stem cells in adult animals [6,7]. The *in vitro* study of OSE-derived cells is strongly influenced by culture conditions, particularly the composition of the culture medium. Phenol red, commonly used as a pH indicator in commercial tissue culture media, has been described as a weak estrogen agonist [8]. It can bind to estrogen receptors and stimulate the growth and activation of estrogen-responsive cells [8]. Its use as a differentiating factor in reproductive studies was first described by Bukovsky et al. [9], who generated human oocyte-like cells from scraped adult OSE cells and demonstrated that OSE cells are bipotential progenitors capable of differentiating into both granulosa-like and oocyte-like cells [6]. These findings highlight that relatively subtle components of the culture medium can influence stem cell behavior and emphasize the importance of carefully defining *in vitro* conditions when studying OSE-derived progenitors.

OSE-derived stem cells and their differentiation toward the female germ line are commonly characterized using a combination of pluripotency-associated and germ-specific markers. Stage-specific embryonic antigens SSEA-1 and SSEA-3 are well-established markers of undifferentiated pluripotent stem cells and have been reported in very small embryonic-like stem cells (VSELs) residing in adult mammalian ovaries [10–12]. In contrast, VASA (DDX4) is a highly conserved RNA helicase expressed exclusively in primordial and postnatal germ cells and is widely recognized as a definitive marker of germline specification [13]. Zona pellucida glycoprotein 3 (ZP3) is synthesized during the growth phase of the oocyte and represents a key indicator of oocyte-specific differentiation [14]. The combined evaluation of these markers therefore enables identification of undifferentiated stem cells as well as cells undergoing germ cell and oocyte-like differentiation.

Through the development of an ovine model for studying OSE-derived stem cells, we aim to bridge fundamental reproductive biology with applications in livestock reproductive biotechnology [28]. In this context, the present study had three main objectives: (1) to isolate and characterize stem cells from the ovine OSE; (2) to determine whether these cells can spontaneously differentiate into oocyte-like structures during a three-week *in vitro* culture period; and (3) to evaluate the effect of phenol red, an estrogenic component of the culture medium, on this differentiation process. By clarifying how phenol red influences the proliferation and oocyte-like differentiation of ovine OSE-derived stem cells, this work addresses an important gap in our understanding of postnatal ovarian stem cell biology in a large-animal model and may contribute to future strategies aimed at improving reproductive efficiency and fertility preservation in both humans and livestock.

2. Materials and Methods

2.1. Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (USA), Gibco (USA), and Abcam unless otherwise specified.

2.2. Ovarian Tissue Collection and Transport

Ovine ovaries were collected randomly from a local slaughterhouse. The ovaries were transported to the laboratory within 1 hour in Dulbecco's phosphate-buffered saline (PBS) supplemented with 1% penicillin-streptomycin at ambient temperature.

2.3. Isolation and Culture of Ovarian Surface Epithelial (OSE) Cells

Ovarian surface epithelial cell isolation was performed as previously described [9], with slight modifications. Briefly, upon arrival, ovaries were gently washed three times in sterile PBS containing antibiotics to minimize blood contamination. Each ovary was then placed in a sterile 60 mm Petri dish (FALCON®, Corning) containing 3 mL of pre-warmed (37°C) culture medium. A sterile surgical blade (Feather, Osaka, Japan) was used to gently scrape the ovarian surface, carefully avoiding penetration into the cortical stroma.

To generate a single-cell suspension, the scraped material was subjected to a two-step enzymatic digestion. First, the tissue was transferred to a digestion medium consisting of Collagenase Type IV (1 mg/mL; Sigma C5138), Dispase II (1 mg/mL; Sigma D4693), and Hyaluronidase Type I-S (1 mg/mL; Sigma H3506) in PBS and incubated in a shaking water bath at 37°C for 10 minutes. The resulting suspension was centrifuged at 100 × g for 2 minutes at room temperature. The pellet was then subjected to a second identical enzymatic digestion step. After the second digestion, the cell suspension was filtered through a sterile 40 µm nylon cell strainer (Falcon) to remove undigested tissue aggregates.

The isolated OSE cells were counted using a hemocytometer, and 1×10^6 viable cells were seeded per well in 3 cm tissue culture plates (TPP, Switzerland). Cells were expanded under two distinct culture conditions for 21 days in a humidified incubator (Memert, Germany) at 37°C with 5% CO_2. The culture medium for both groups was replaced every 72 hours. The first group was

cultured in Phenol Red-Free medium: High-glucose Dulbecco's Modified Eagle Medium (DMEM-HG; Gibco) supplemented with 13.5 g/L sodium bicarbonate, 1x non-essential amino acids (Gibco), penicillin (100 IU/mL), streptomycin (100 µg/mL), and gentamicin (40 µg/mL). The second group was cultured in Phenol Red-Containing medium: High-glucose Dulbecco's Modified Eagle Medium (DMEM-HG; Gibco) containing phenol red at the manufacturer's standard concentration (20 mg/L), supplemented identically to the phenol red–free medium. For colony assessment, a gridded coverslip was placed beneath culture plates in both groups, and colonies were manually counted using an inverted microscope at 10x magnification. To quantify cell density per colony, approximately 20 colonies were randomly selected from micrographs and the number of cells within each was enumerated.

2.4. Characterization of OSE Cells and Oocyte-Like Structures

Cells were characterized at two time points: immediately after isolation (Day 0) and after three weeks of culture (Day 21). For morphological and immunocytochemical analysis, cells were fixed in freshly prepared 4% paraformaldehyde (PFA, pH 7.4) for 10 minutes at room temperature. For molecular analysis, separate cell aliquots were snap-frozen in liquid nitrogen (-196°C) and stored at -80°C for subsequent RNA extraction. Characterization focused on OSE stem cells, spontaneously formed oocyte-like structures, and signs of EMT using specific markers via immunocytochemistry (ICC) and quantitative real-time PCR (qRT-PCR).

2.5. Immunocytochemistry (ICC)

Markers were selected to distinguish pluripotent stem cells (SSEA-1 and SSEA-3) from cells committed to the germline and oocyte lineage (VASA/DDX4 and ZP3), as established in previous studies of ovarian stem cells and *in vitro* oogenesis [4, 5, 11]. For ICC, cell clusters were fixed in 4% PFA for 20 minutes at room temperature. Following fixation, samples were washed twice with PBS containing 0.1% Tween-20 (PBS-T). Non-specific binding sites were blocked by incubating the samples with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS for 15 minutes at room temperature. Samples were then incubated overnight at 4°C with primary antibodies diluted in blocking solution. The primary antibodies used were: rat polyclonal anti-α6-integrin (1:100; Sigma-Aldrich, MAB1378), rat polyclonal anti-β1-integrin (1:100; Sigma-Aldrich, MAB1981), rat polyclonal anti-Oct-4 (1:100; Sigma-Aldrich, SAB4502567), and mouse polyclonal anti-Thy-1 (CD90) antibody (1:100; Santa Cruz Biotechnology, sc-53116).

The following day, samples were washed three times (5 minutes each) with PBS-T and incubated with appropriate secondary antibodies: FITC-conjugated goat anti-mouse IgG (1:200; Sigma-Aldrich, F0257) or FITC-conjugated goat anti-rat IgG (1:200; Sigma-Aldrich, F1763) for 45 minutes at room temperature in the dark. After three additional washes with PBS-T, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology) diluted 1:2000 in PBS for 10 seconds. Labeled cells were imaged using an fluorescence microscope (B-600 TiFL, Optika, Italy). Negative controls were processed identically but with the omission of the primary antibody.

2.6. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from freshly scraped OSE cells and from cultured cells after three weeks using the RNeasy Micro Kit (Qiagen, Cat. No. 74004) according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized from 500 ng of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Cat. No. 205311) under the following conditions: 42°C for 30 minutes, followed by enzyme inactivation at 95°C for 3 minutes.

Gene expression analysis was performed for the germ cell markers *DDX4 (VASA)*, *c-Kit*, and *ZP3*. *GAPDH* was used as the endogenous reference gene. Primer sequences were designed based on the sheep (*Ovis aries*) GenBank sequences using Primer-BLAST (NCBI) and are detailed in Table 1. qRT-PCR was carried out in 20 µL reactions using the QuantiFast SYBR Green PCR Kit (Qiagen, Cat. No. 204052) on a Rotor-Gene 6000 system (Corbett Research, Australia). Each reaction contained 1 µL of cDNA template, 10 µL of 2x SYBR Green Master Mix, 0.5 µL of each forward and reverse primer (10 µM), and 8 µL of nuclease-free water. The thermal cycling protocol was: initial denaturation at 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 40 seconds. A melting curve analysis was performed at the end of each run (ramping from 60°C to 95°C at 0.1°C/s) to confirm primer specificity and absence of primer-dimer artifacts. Amplification efficiency (E) for each primer pair was determined using a standard curve generated from a five-step, 10-fold serial dilution of pooled cDNA. Only primer sets with an efficiency between 95–105% and a correlation coefficient (R^2) ≥ 0.990 were used. Relative gene expression levels were calculated using the comparative 2^(-ΔΔCt) method [15].

Table 1. Primer sequences used in this study and their corresponding annealing temperatures.

Gene name	Direction	Prime sequence 5' to 3'	Accession (NCBI)	Product length (bp)
c-Kit	Forward	ATGACAGGCTGGTGAATGGC	XM_027970728	100
	Reverse	GAACACCTCTGCTCGGTTCC		
DDX4	Forward	ACTTCAGTAGCTGCCCGAGG	XM_042233781	111
	Reverse	ACGACCAGTACGCCCAATTC		
ZP3	Forward	CCTGAAGGTCACTCCGGTTG	XM_004020981	154
	Reverse	CATGGAACGGCCTGAAATGC		
GAPDH	Forward	ATGCTGGTGCTGAGTACGTG	XM_060411595	135

	Reverse	CCATCACAAACATGGGAGCG

2.7. Statistical analysis

All experiments were performed with a minimum of three biological replicates (ovaries from different animals), and each assay was repeated at least three times independently. Quantitative analysis of fluorescence intensity (if performed) was conducted using ImageJ software (NIH). Data were analysed using t-test in SAS version 9.4., and data are presented as mean ± standard error (SE). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization and Isolation of Ovarian Stem Cells

Initial isolation from adult sheep ovaries yielded a heterogeneous cell population. Histological analysis via Hematoxylin and Eosin (H&E) staining confirmed that the cells scraped from the ovarian surface possessed a characteristic epithelial morphology (Figure. 1A). During the early days of *in vitro* culture, this heterogeneity persisted. Microscopic examination revealed the presence of adherent epithelial cell clusters alongside two morphologically distinct populations of small, round, non-adherent cells resembling putative stem cells (PSCs) (Figure. 1B). These PSCs were often observed interspersed or physically entrapped within the larger epithelial cell clusters (Figure. 1C).

3.2. Spontaneous Differentiation into Oocyte-Like Structures

Cultured PSCs exhibited a capacity for spontaneous differentiation over a 21-day period. These cells progressively increased in size and adopted morphological features resembling immature oocytes. By week three, distinct OLS had formed. These OLS had an average diameter of 130 ± 15 µm, which is comparable to immature ovine oocytes *in vivo*. Key oocyte-specific morphological features were observed, including a prominent germinal vesicle (nucleus), perinuclear organelle accumulation (Figure. 1D), and occasional polar body-like protrusions, without direct evidence of meiotic progression (Figure. 1D). Furthermore, some OLS appeared to be surrounded by a translucent extracellular matrix resembling a nascent zona pellucida (Figure. 1D).

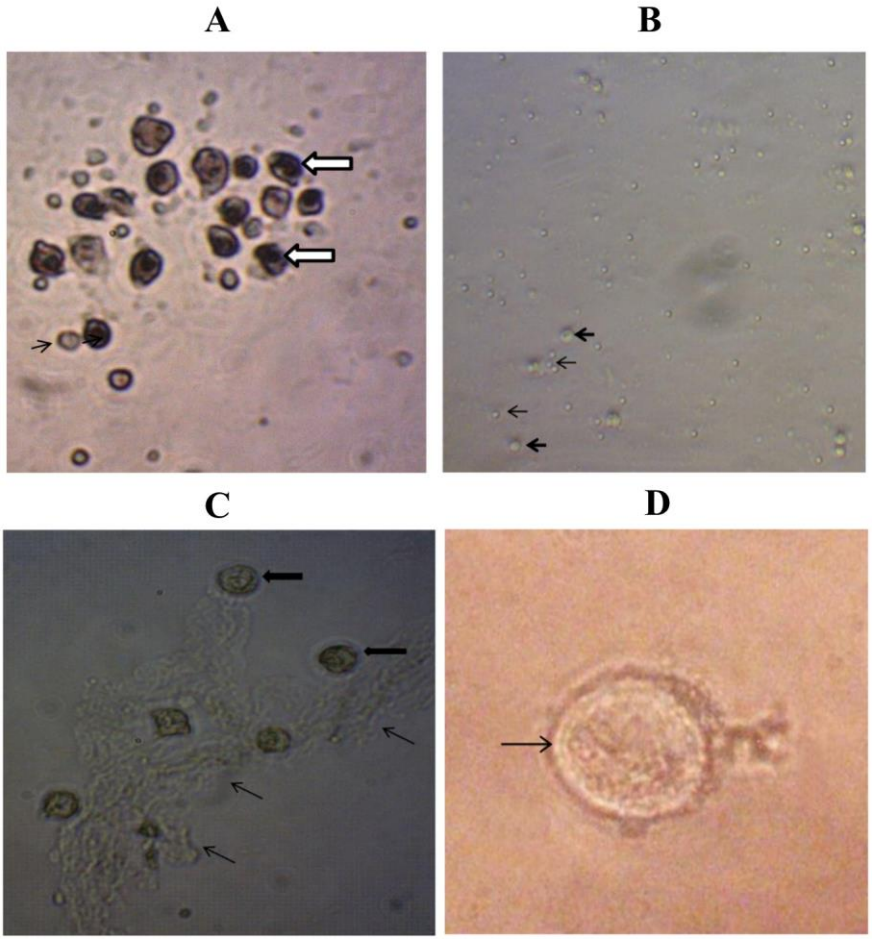

Figure 1. Morphological characterization of ovarian surface epithelial (OSE) cells and identification of putative ovarian stem cells (PSCs) following isolation from adult sheep ovaries. (A) Hematoxylin and eosin (H&E) staining showing epithelial morphology of cells obtained from the ovarian surface. Cells of the surface epithelium with abundant cytoplasm are indicated by a small arrow. Ovarian stem cells, indicated by a large arrow, exhibit a primary oocyte-like morphology with a large, dark nucleus. Figure (B) Early-culture phase revealing a heterogeneous population consisting of adherent epithelial cells and small, round, non-adherent PSC-like cells (2–4 µm). (C) PSCs observed interspersed within or entrapped among epithelial cell clusters, suggesting a potential niche association. (D) Formation of oocyte-like structures (OLS) after 21 days of culture, demonstrating enlargement and acquisition of oocyte-specific morphology. Mature OLS displaying a prominent germinal vesicle, perinuclear organelle accumulation, and zona pellucida-like extracellular material (showed by arrow), with occasional polar body-like protrusions.

3.3. Effect of Phenol Red on Ovarian Stem Cell Differentiation

3.3.1. Counting the Number of Cells and Colonies

The results of quantifying colony number and cell density in OSE-derived cell cultures in the laboratory environment show that, immediately four hours after the initial culture, the cells adhere to and settle on the bottom of the culture vessel. These cells remain quiescent in the culture medium for approximately two to four days, after which they begin dividing and proliferate actively. These

cells have colony-forming ability and produce colonies with regular or irregular shapes. Colonies appear by day seven and consist of single cells that are loosely associated with each other, and the cells remain distinguishable.

The colonies formed in both treatment groups were evaluated in terms of colony number and cell count per colony. The colony evaluation (Figure 2) showed that the mean colony number and the mean cell count per colony in the phenol red-free culture group were significantly lower than those in the phenol red-containing culture group ($P < 0.05$).

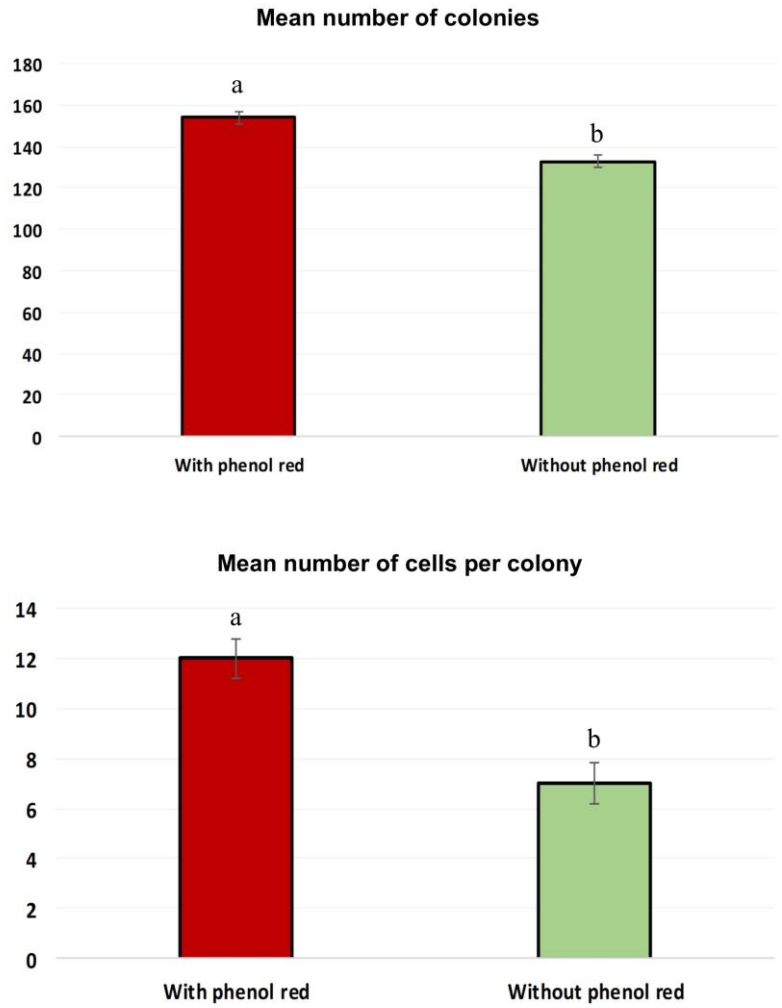

Figure 2. Phenol red increases colony formation and cell proliferation in ovine OSE-derived stem cell cultures. The bar graph shows the mean number of colonies and the mean cell count per colony after three weeks of culture. Both parameters were significantly higher in the phenol red-containing medium compared to the phenol red-free control. Data are presented as mean ± SE (n=3). Different letters indicate significant differences ($p < 0.05$).

3.3.2. Immunocytochemical (ICC) Analysis of Stem and Germ Cell Markers

To molecularly characterize the PSCs and their differentiated progeny, ICC was performed on 21-day cultures from both treatment groups. The expression of pluripotency-associated markers (SSEA-1 and SSEA-3) and germ cell/oocyte-specific markers (VASA, ZP3) was assessed. Cells within the colonies stained positively for all four markers (Figure 3). Specifically, strong immunoreactivity for the pluripotency markers SSEA-1 and SSEA-3 was detected on the cell surface. Concurrently, cytoplasmic staining for the germ cell marker VASA and the oocyte-specific zona pellucida protein ZP3 was evident, confirming commitment to a germ cell lineage. Staining was predominantly localized to the cytoplasm of the larger, DAPI-positive cells corresponding to the OLS, while smaller, putative stem cells showed distinct nuclear staining patterns for some markers (e.g., Oct-4, data not shown). Negative controls, processed without primary antibodies, showed no specific fluorescence (data not shown). The co-expression of pluripotency-associated markers with germ cell-specific proteins suggests the coexistence of undifferentiated stem cells and differentiating germline cells within the same cultures.

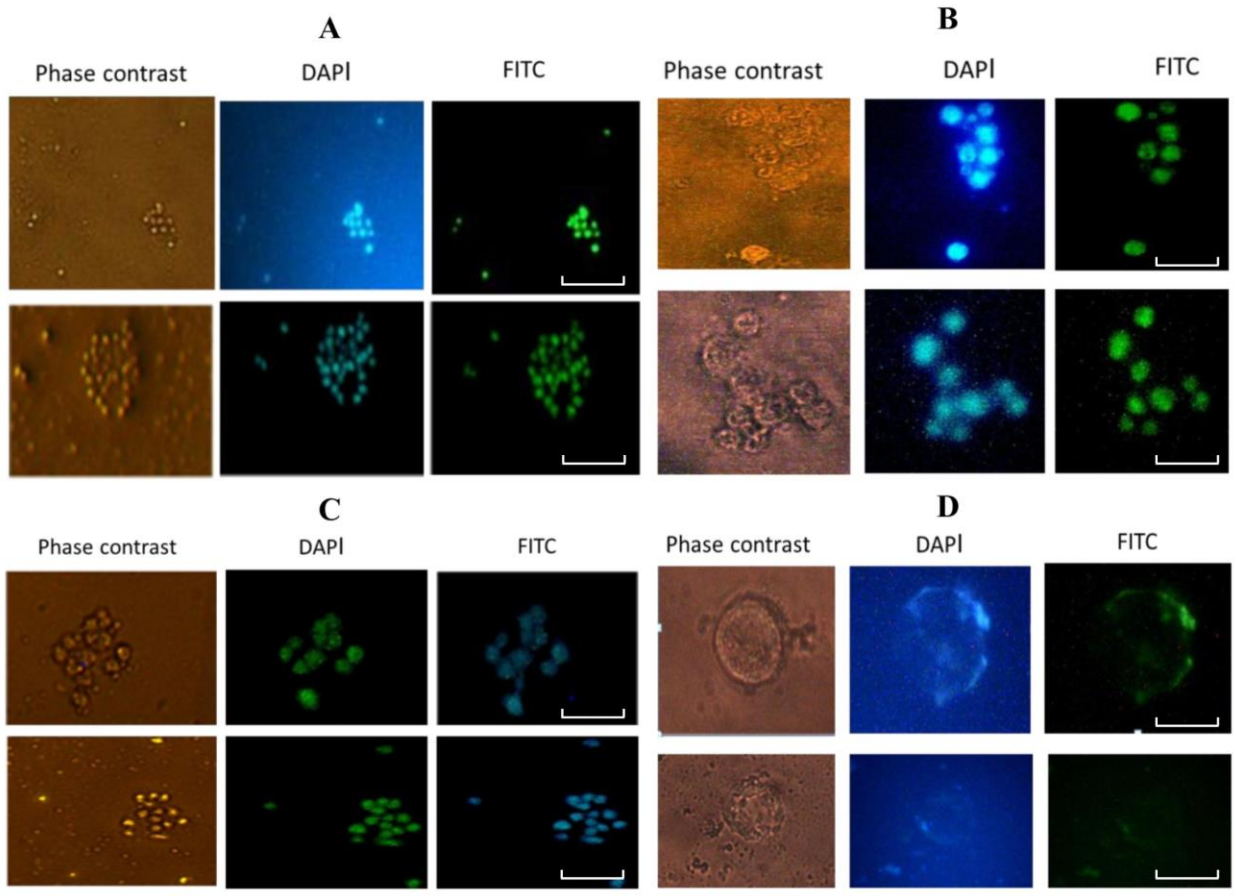

Figure 3. Immunocytochemical analysis of putative stem cell colonies after 21 days of *in vitro* culture, showing expression of pluripotency and germ cell markers. (A) SSEA-1 immunoreactivity (green) on the cell surface. (B) Cytoplasmic expression of the germ cell marker VASA (green). (C) SSEA-3 staining (green) indicating pluripotent characteristics. (D) Zona pellucida protein ZP3 (green) expressed in oocyte-like structures. Nuclei are counterstained with DAPI (blue). Scale bars: A, B and C captures = 200 μm; D capture = 50 μm.

3.3.3. Quantitative Gene Expression Analysis via RT-PCR

The expression of key germline genes (*DDX4 (VASA)*, *c-Kit*, and *ZP3*) was quantified using real-time PCR to provide a molecular correlate to the ICC findings and to assess the quantitative impact of phenol red. Transcripts for all three genes were detected in cultured cells from both the phenol red-free (Control) and phenol red-supplemented (Treatment) groups after three weeks, confirming the presence of cells with a germ cell molecular signature. The expression level of *DDX4* did not show a statistically significant difference between the Control and Treatment groups ($p > 0.05$). In contrast, the expression of both *c-Kit* and *ZP3* was significantly higher in cells cultured with phenol red compared to those cultured in its absence ($p < 0.01$ for both genes; data detailed in Figure 4).

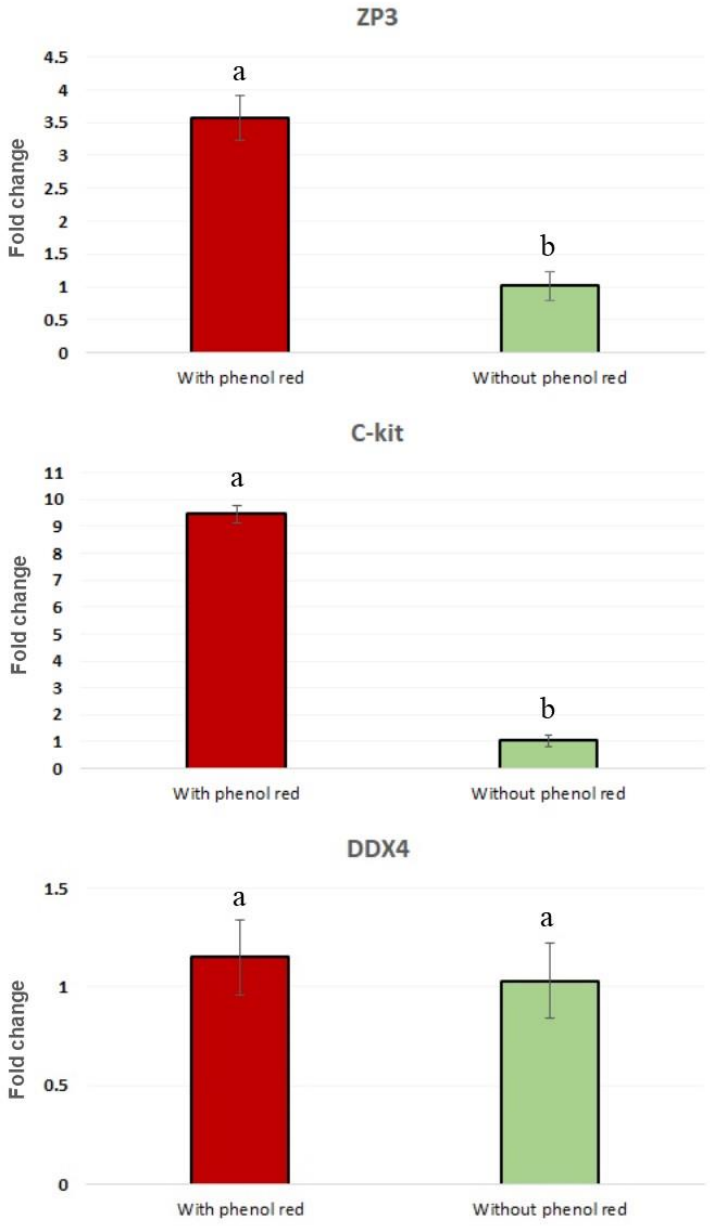

Figure 4. Comparison of mean numerical gene expression (±SE) between the two experimental groups. Expression level of *ZP3* and *c-Kit* was significantly higher in OSCs cultured in phenol red than control group. These values were not significant for *DDX4*. Values represent mean ± SE; n = 3 biological replicates. Different superscript letters indicate significant differences ($p < 0.05$).

4. Discussion

Our findings are compatible with the hypothesis that the OSE of adult sheep may provide a source of stem cells that can give rise to oocyte-like structures *in vitro* [6, 16, 17]. Moreover, our findings indicate that phenol red, a weak estrogen-like chemical in common cell culture medium, is associated with increased oocyte-like differentiation. These observations are consistent with and extend earlier studies on postnatal oogenesis. The concept of postnatal oogenesis in mammals has been controversial, yet there is increasing evidence that OSE contains two types of stem cells: very small embryonic stem cells (VSELs) and ovarian stem cells (OSCs); both are competent in germline differentiation as opposed to the traditional paradigm that an oocyte pool is fixed for lifetime [11, 12, 18].

The first description of our OSE-derived cell population indicated that it was a heterogeneous mixture, which was in agreement with previous report [11]. We observed small, round, non-adherent cells with high nuclear-to-cytoplasmic ratios, morphologically similar to the VSELs and slightly larger OSCs described in human and ovine ovaries [11, 12]. These PSCs were predominantly located in clusters of the larger epithelial cells, suggesting a potential niche or supportive interaction, as reported by Parte et al. [11]. These niches have been suggested to play a role in stem cell maintenance and differentiation, via similar mechanisms seen in other adult organs [12, 19], where the local epithelial microenvironment is involved directly or indirectly in providing signals that either promote stem cell quiescence or activation.

A significant observation is that the PSCs spontaneously differentiated into oocyte-like structures (OLS) when cultured in basic medium for 3 weeks. The quantitative differences observed in colony number and cell density per colony between phenol red–containing and phenol red–free cultures suggest that phenol red may enhance the proliferative and colony-forming behavior of ovine OSE-derived stem cells. Cultures supplemented with phenol red showed a significantly higher number of colonies as well as a greater number of cells per colony, indicating that this compound supports both stem cell activation and subsequent clonal expansion. Colony-forming efficiency is widely regarded as a surrogate measure of stem cell self-renewal and proliferative competence in adult stem cell populations, including ovarian stem cells [20]. Similar increases in colony formation and cell proliferation have been reported when ovarian stem cells are exposed to estrogenic or estrogen-mimicking stimuli, supporting the notion that estrogen signaling plays a permissive role in OSC activation and expansion. Given that phenol red has been shown to act as a weak estrogen capable of activating estrogen receptors, the enhanced colony formation observed in the present study may involve estrogen-responsive signaling pathways that influence survival, cell cycle entry, and differentiation of OSCs, although these mechanisms were not directly examined here [8]. These findings align with previous reports in sheep and human

ovaries demonstrating that estrogenic cues increase OSC proliferation and facilitate progression toward germline differentiation, reinforcing the importance of carefully controlling estrogenic components in *in vitro* ovarian stem cell culture systems [20, 21].

The OLS showed typical morphological characteristics, such as the diameter (~130µm) similar to an immature oocyte, a distinct germinal vesicle, and regular polar body-like extrusions and zona pellucida like formations. This capacity for spontaneous oocyte-like differentiation *in vitro* under specific culture condition from adult OSE was first reported in human cells over a decade ago [5, 18] and has since been replicated in other models [6, 20]. Our finding that this occurred in the absence of feeder layers or exogenous growth factors, within a simple serum-based medium, is consistent with the presence of intrinsic differentiation capacity among OSE-derived stem cells and may partially mimic aspects of a simplified *in vivo* environment [11]. This spontaneous differentiation has also been observed in other mammalian systems, suggesting that intrinsic determinants and cell–cell interactions within OSE cultures can suffice to drive germline trajectory *in vitro* [12, 19, 22]. Notably, a recent comparative study found that human OSE-derived stem cells exhibited a greater potential for *in vitro* oogenesis than murine cells [17], underscoring the importance of species-specific and culture condition optimization, such as the estrogenic influence of phenol red we report.

A key observation of this study is that phenol red appears to support OSC differentiation under our culture conditions. Phenol red is a well-documented weak estrogen able to induce the activation of estrogen receptors [8]. In our experiments, phenol red-containing cultures presented higher differentiation than that cultured in a phenol red-free medium. Immunocytochemistry assay indicated that cells in both groups expressed markers of pluripotency (SSEA-1, SSEA-3), and germ cell lineage (VASA, ZP3). While SSEA-1 and SSEA-3 expression reflects the persistence of an undifferentiated stem population, VASA and ZP3 expression indicates progression toward germ cell and oocyte-specific differentiation. These results indicate that while basic germ cell identity (as marked by DDX4) was maintained independently of phenol red, the expression of genes associated with oocyte differentiation and function (c-KIT and ZP3) was higher in the phenol red group. However, expression of these markers and associated morphological changes indicate acquisition of germ cell–associated characteristics and oocyte-like features but do not alone confirm functional oocyte identity [4, 12]. However, qRT-PCR indicated an notable difference: no significant difference was observed in DDX4 (VASA), a general germ cell marker between groups, while the expression of *c-Kit* and *ZP3* were higher in phenol red group. These findings suggest that even weak estrogenic signals can influence ovarian stem cell behavior and lineage commitment *in vitro*, reinforcing the idea that culture media components can inadvertently act as differentiation modulators [12, 18].

The specific upregulation of these two genes is highly informative. c-KIT, a tyrosine kinase receptor expressed in germ cells and various stem cells, is indispensable for the survival and proliferation of primordial germ cell and oocyte growth [23]. Its over-expression implies that phenol red supports the growth and/or survival of cells during oogenesis. Similarly, ZP3 is a major constituent of the zona pellucida and is produced only by growing oocytes [14]. Its significant elevation in the phenol red treatment strongly indicates advanced differentiation toward a

functional oocyte phenotype. This is consistent with that of Bukovsky et al. s work demonstrating that estrogenic action results in a differentiation of OSE cells into oocytes [24]. Indeed, reports have suggested that full oocyte maturation may depend on additional signals from mesenchymal or other somatic cells [6], which are not present in our monoculture systems. Future studies that include supportive somatic cell types or defined differentiation protocols will likely narrow the distance to full meiotic maturation and functional competence of OLS.

Our results are consistent with the model proposed by Bhartiya and colleagues, where pluripotent VSELs give rise to more committed OSCs, which can then form germ cell nests and differentiate [11, 12]. The estrogenic activity of phenol red likely acts on this cascade. Estrogen and FSH (which shares signaling pathways with estrogen) have been shown to activate ovarian stem cells, promoting their self-renewal and differentiation via asymmetric cell division [20, 25]. Phenol red may be mimicking this physiological estrogenic signal, thereby increasing colony-forming efficiency and driving a greater proportion of stem cells toward the oocyte lineage, as reflected in the higher *c-Kit* and *ZP3* expression. This mechanistic insight suggests that endogenous hormonal cues *in vivo* may similarly regulate OSC behavior, with potential implications for fertility regulation and *in vitro* gametogenesis.

A notable observation was the use of ovaries from adult sheep, which are past peak reproductive age. The successful isolation and differentiation of stem cells from such tissue affirm the results of Bukovsky et al. [6] who proposed that ovaries lacking an actively growing follicular population could conversely represent a stronger source of stem/progenitor cells, perhaps because there may be no inhibitory feedback from a cohort of larger follicles. This is also consistent with the data of VSELs residing in the aged ovary wherein follicular reserves are depleted, as reported in other mammalian models [12, 18].

5. Conclusion

This study demonstrates that the ovarian surface epithelium (OSE) of adult sheep contains putative stem cells capable of proliferation and spontaneous differentiation into oocyte-like structures *in vitro*. The isolated small OSE-derived cells exhibited strong colony-forming ability and expressed pluripotency and germ cell/oocyte markers, supporting their stem and germline identity. These findings reinforce the concept that the adult ovary may harbor stem/progenitor cells with the capacity to contribute to oocyte formation, even in animals beyond peak reproductive age. A major finding of this study is the significant influence of phenol red, a weak estrogen-like component commonly present in culture media, on the differentiation process. Cultures containing phenol red showed increased colony numbers, higher cellular density, and significantly elevated expression of the oogenic genes *c-Kit* and *ZP3* compared with phenol red-free conditions. These results indicate that phenol red can act as a supportive differentiation factor in ovarian stem cell cultures and highlight the importance of culture medium composition in regulating stem cell behavior and oocyte-like formation. Collectively, these findings provide further evidence supporting the

possibility of postnatal oogenesis in a livestock species and establish the ovine ovary as a valuable model for studying ovarian stem cell biology. Future studies should focus on optimizing culture conditions, elucidating the signaling pathways involved in OSE stem cell differentiation, achieving meiotic completion, and assessing the developmental competence of the resulting oocyte-like structures. Such advances may contribute to improved strategies in reproductive biotechnology and fertility preservation.

6. Limitations

Several limitations of this study should be noted. First, we did not include a separate estrogen or estrogenic analogue (e.g., estradiol) as a positive control, which limits our ability to definitively attribute the observed effects to classical estrogen receptor–mediated signaling rather than other, indirect culture-related mechanisms. Although phenol red was the only variable between culture conditions, its estrogenic effects may act through multiple indirect pathways, and the precise mechanisms driving enhanced oocyte-like differentiation were not explored. In addition, we evaluated only a single concentration of phenol red and did not perform a formal dose–response analysis, so we could not determine whether the effects on proliferation and oocyte-like differentiation are concentration-dependent or define an optimal effective range. Identification of stem cells and oocyte-like structures relied on morphology and marker expression (c-Kit, DDX4, ZP3), which alone cannot fully confirm germ cell identity or functional oocytes. Functional hallmarks of oogenesis, such as meiotic progression or fertilization competence, were not assessed, so the observed structures should be considered oocyte-like rather than fully mature. The small population of very small cells (2–4 µm) observed early in culture may represent stem cells, but their exact identity remains uncertain without further analysis. Finally, all experiments were performed *in vitro*, and the results may not fully reflect natural ovarian physiology.

Author contributions

M.A.C.: Conceptualization, Validation, Investigation, Data Curation, Formal analysis, Writing - Original Draft, Visualization. **M.Sh.**: Conceptualization, Supervision, Funding acquisition, Formal analysis, Writing - Review & Editing. **M.Zh.**: Conceptualization, Methodology, Resources, Formal analysis, Writing - Review & Editing. **M.M.**: Conceptualization, Formal analysis, Writing - Original Draft, Visualization.

Ethics Approval

Not Applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by the University of Tehran (grant number: 73130581.6.34).

Data Availability

All data and supplementary information would be available upon request from the corresponding author

Declaration of AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used GPT-4 to improve readability and refine language. The tool was applied selectively, and the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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511