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Retinoic acid and 17β-estradiol improve male germ cell differentiation from mouse-induced pluripotent stem cells

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Abstract

This research aimed to explore the impacts of retinoic acid (RA)/17β-estradiol (E) induction and embryoid body formation to enhance differentiation of mouse-induced pluripotent stem cells (miPSCs) into male germ cells in vitro. Flow cytometry and qPCR were conducted to describe miPSCs differentiation process. Various temporal expression profiles of germ cell-related genes were traced. *Stra8* gene expression increased in the RA group on the 4th day compared to other groups. The RA group experienced a more significant increase than E group. The expression of *Sycp3* increased in RA + E group on 4th day compared with other groups. Expression of *AKAP3* enhanced in the RA + E group than other groups on day 4. Moreover, miPSCs showed that this gene expression in the RA + E group was increased in comparison to RA and E groups on day 7. *AKAP3* gene expression on day 7 of miPSCs decreased in RA and E groups. Flow cytometry data indicated that 3%–8% of the cells in sub-G1 stage were haploid after RA and E induction compared to other groups on day 4. This study showed that miPSCs possess the power for differentiating into male germ cells in vitro via formation of embryoid body by RA with/or E induction.

KEYWORDS

17β-estradiol, differentiation, male germ cell, mouse-induced pluripotent stem cells, retinoic acid

1 | INTRODUCTION

Infertility is a key concern related to human's health, which is stimulated by the absence or disruption of germ so that it accounts for 10% to 15% couples (Greil, Slauson-Blevins, & McQuillan, 2010; Rafatmanesh, Nikzad, Ebrahimi, Karimian, & Zamani, 2018). Germ cell development is a complex multi-step biological process (Zhao et al., 2018). It is possible that unproductiveness results from failures in growing germ cell (Li et al., 2014). Transplantation of stem cell through advanced germ cells or primordial germ cells (PGCs) is a potential helpful therapeutic option to bring back male infertility that has no genetic justification (Dong et al., 2017).

The stem cell is a special cell type with the self-renovating and differentiating abilities. There are embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells in the stem cells (Mahabadi et al., 2018; Yang et al., 2017).

iPSCs generation was started from somatic cells for reproducible differentiation into various kinds of functional tissues for human clinical uses (Jung, Bauer, Bauer, & Nolta, 2012). Reprogramming of somatic cells to iPSCs is reprogrammed via ectopic expression

of four following transcription factor *OCT4*, *KLF4*, *MYC* and *SOX2*. There is similarity between mouse iPSCs (miPSCs) and human iPSCs (hiPSCs) with the human ESCs (hESCs) and mouse ESCs (mESCs) in terms of the capability of differentiation, morphology and expression of pluripotency marker (Fang, Li, Li, Zhao, Li, & Xiong, 2018). However, iPSCs are partially preferred over ESCs for reproductive drug use and generation of patient-specific spermatozoa due to a lot of sources and few ethical issues (Bock et al., 2011; Fang et al., 2018). However, there is no clear evidence on the in vivo regulators of human spermatogenesis process. Nevertheless, it was found that some regulators and inducers are crucial mediating agents of pluripotent stem cells (PSCs) differentiation towards germ cells of distinct maturation phases (Amini Mahabadi et al., 2018; Rombaut, Mertes, Mertes, Heindryckx, & Goossens, 2017).

Recently, it indicated the probability of achieving PGCs through miPSCs (Imamura et al., 2010; Vernet et al., 2006; Zhu et al., 2012). Nonetheless, there is no knowledge of whether miPSCs is able to generate late-stage male germ cells or not; therefore, it is hard to achieve spermatozoa (Zhu et al., 2012).

One of the derivatives of vitamin A is retinoic acid (RA), which has significant roles in cellular differentiation and embryogenesis and modulates meiotic initiation in mice (Dong et al., 2017; Zhu et al., 2012). Interestingly, RA could also improve spermatogenesis and induce downstream gene expression in male testes after birth (Bowles & Koopman, 2007) via activating major genes that begin meiosis (Rhinn & Dolle, 2012). One of these genes is *Stra8* that is triggered via RA. It displays the exact schedule of male-specific evolution in these episodes (Zhu et al., 2012).

Researches indicated that co-culture of neonatal testicular somatic cells and sequential exposure to morphogens and sex hormones could increase mESCs-derived PGCLCs for repeating the entire male gametogenesis in vitro (Zhou et al., 2016). Reports indicated that oestrogen contributes critically to regulate differentiation and survival of tissue-specific stem or progenitor cells or embryonic stem cells (Kitajima et al., 2015). Moreover, it is important in terms of signalling to the rapid growth of PGC in vitro (Eskandari et al., 2018).

Both male and female reproductive functions could be positively or negatively modulated by oestrogen and oestrogenic compounds. In fact, maturation and development of different reproductive gonads and tissues in mammals can be regulated by oestrogens (La Sala, Farini, & De Felici, 2010).

Diagnosing miPSCs differentiation into germ cells has been done mainly via analysing germ cell-specific gene and expressing protein in complicated stem cells cultures. Several research showed expressing genes such as *Ddx4 (Mvh)*, *Stra8*, *AKAP3* and *Sycp3*, which enhanced progressing nascent PGCs to begin meiosis and generate haploid cells specific to germ cell growth (Gholamitabar Tabari, Jorsaraei, Jorsaraei, Ghasemzadeh-Hasankolaei, Ahmadi, & Amirikia, 2018; Li et al., 2013; Silva et al., 2009).

The present study aimed to determine the effects of retinoic acid, 17β-estradiol and molecular mechanism to differentiate male germ cells from miPSCs.

2 | MATERIAL AND METHODS

2.1 | Ethical statement

Animal protection and experimental processes have been implemented precisely based on the ethical code rules of Kashan University of Medical Science, Kashan, Iran (IR.KAUMS.REC.1394.150).

2.2 | Development of mouse-induced pluripotent stem cells

Prof. Masoud Soleimani, who is an academic member in Stem Cells Technology Research Center, Tarbiat Modares University, Tehran, Iran, awarded male mice fibroblast iPSCs lines, which have been set up from male balb/c mice fibroblasts by retroviral transfers of transcription factors Oct4/Sox2/Klf4/c-Myc. The cells were cultured at 37°C with 5% CO2 and 95% humidity, and then maintained and expanded on a MEF feeder layer inactivated with mitomycin-C (Sigma, cat. no. M4287; Eskandari et al., 2018; West, Mumaw, Mumaw, Gallegos-Cardenas, Young, & Stice, 2010; Wongtrakoongate, Jones, Jones, Gokhale, & Andrews, 2013) in cell culture flask 25 cm^2 (Orange Scientific). Mouse iPSCs medium contained DMEM/F12 culture medium complemented with mmol/L L-glutamine (Gibco, cat. no. 25030-081), 0.1 mmol/L nonessential amino acids (NEAA; Gibco, cat. no. 11140-035), 20% (vol/vol) knockout serum replacement (KSR; Gibco, cat. no. 10828-028), 1,000 U/ml murine leukaemia inhibitory factor (LIF; ESGRO; Gibco, cat. no. ESG1107), penicillin/streptomycin (Gibco, cat. no. 15140-148), Insulin–Transferrin–Selenium (ITS; Gibco, cat. no. 41400-045), ROCK inhibitor Y-27632 (Sigma; cat. no. Y0503-1 MG; only once after thawing), and the medium was replaced every day. Mouse iPSC colonies passed enzymatically via 0.1% collagenase type IV (Gibco, cat. no. 17104-019) and moved to the inactivated MEF to be expanded (Figure 1) every 4–6 days.

2.3 | In vitro differentiation of miPSCs into PGCs

After remaining in the culture for 4–5 days, miPSCs cells were suspended into LIF-free medium for forming EBs (Figure 2). For EB formation, miPSCs were seeded onto 6-well floating culture-grade

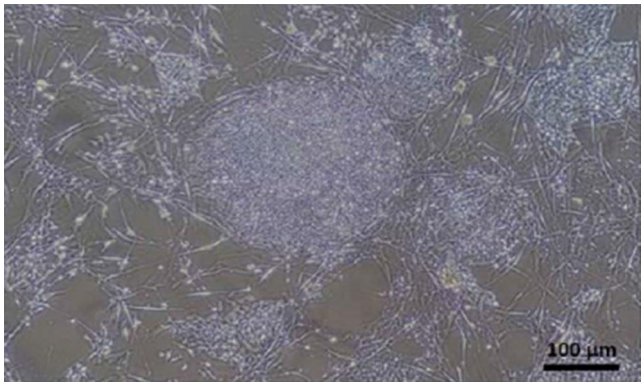

FIGURE 1 Morphology of miPSCs during culture on MEF feeder cells (the scale bar represents 100 µm)

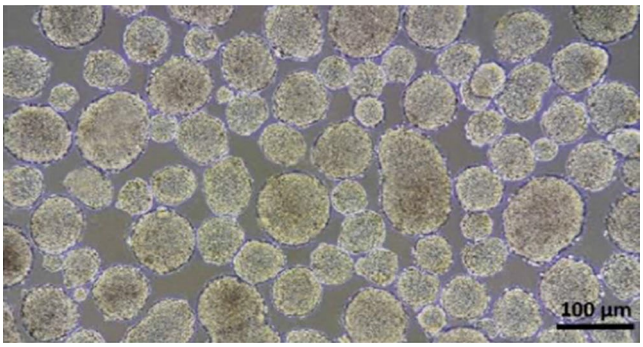

FIGURE 2 Embryonic bodies (EBs) formation through miPSCs in vitro. Morphology of EBs for 4 days after being cultured (the scale bar represents 100 µm)

dishes (Jet Biofil) at a density of 5×10^5 cells/ml in miPSCs culture medium with FBS 10% (Gibco, cat. no. 10270-106) and without LIF. The medium changed every 2 days. Then, EBs were transferred to 6-well plates coating with gelatin 0.1% (Sigma, cat. no. G9391-100G; Figure 3) and three wells for each group were considered. miPSCs-derived EBs were actuated to differentiate into primordial germ cells in vitro by treating cells with 10 nmol 17β-estradiol (Sigma, cat. no. E2758-1G; Eskandari et al., 2018; Hong, Zhang, Zhang, Sultana, Yu, & Wei, 2010) and 2 µmol retinoic acid (RA; Sigma, cat. no. R2625-50MG; Eguizabal, Shovlin, Shovlin, Durcova-Hills, Surani, & McLaren, 2009; Ghasemzadeh-Hasankolaei, Eslaminejad, Batavani, & Sedighi-Gilani, 2014; Gholamitabar Tabari et al., 2018; Huang et al., 2010; Li et al., 2013; Xuemei et al., 2013) at days 0, 4 and 7. miPSCs-derived EBs that had not treated with RA were used as a negative control (Figure 4). The protocol of differentiating miPSCs into PGCs in vitro via inducing RA has been performed based on the previous studies (Eguizabal et al., 2009; Li et al., 2013; Yang et al., 2017; Zhu et al., 2012).

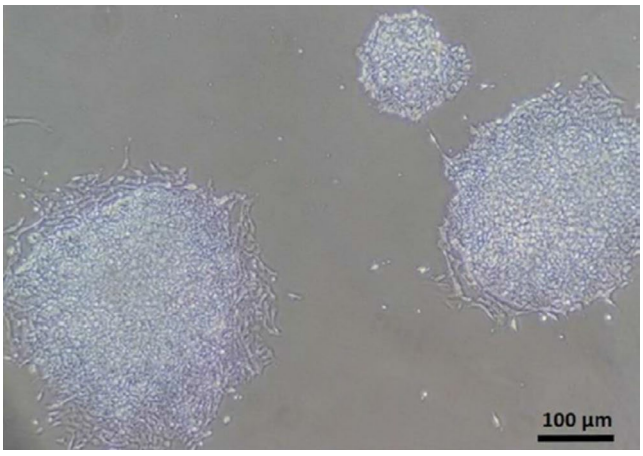

FIGURE 3 EBs had proper aggregation and spheroidal shape, and a proper adhesion was considered to provide the best conditions for differentiation. In order to culture and differentiate cell, EBs derived from miPSCs were plated on gelatin 0.1%-treated wells. As shown in the figure, the EBs attached well within 12 hr, and the EBs grew into monolayer surface, which might affect the differentiation of EB cells (the scale bar represents 100 µm)

2.4 | Extraction of RNA and quantitative real-time PCR

Total RNA was extracted from harvested cells by a commercial TRIzol Kit (Invitrogen, cat. no. 15596026) in accordance to the instructions of manufacturer. One microgram of RNA from each sample was reverse-transcribed to cDNA by PrimeScript™ RT reagent kit (TaKaRa BIO). qPCR was conducted for quantitative expression of the following genes: (a) *Stra8*, (b) *Sycp3*, (c) DDX4 and (d) *AKAP3*.

The entire sequences of the above genes were deduced from NCBI; specific primers were designed by Primer3 online software and were BLAST to find homology per cent. The primer sequences, annealing temperatures and reinforced product lengths for each primer sequences are given in Table 1.

The qRT-PCR mixture was composed of 10 µl SYBR Green PCR Master Mix Buffer (2×), 1 µl (100 ng) cDNA template and 0.5 µl each of forward and reverse primers in a total volume of 20 µl. *GAPDH* housekeeping gene was used as the internal controls for each sample. All qPCR reagents were ordered from CinnaGen Company. The qPCR programme includes initial denaturation at 95°C for 10 min, which was followed by 40 repetitive cycles (denaturation: 95°C for 30 s), annealing at a temperature which earlier mentioned for each gene for 30 s, and extension at 72°C for 30 s. A no-template control of nuclease-free water was involved in each run, and threshold cycle (Ct) values were computed for each reaction. All the qPCR reactions were conducted by triplicates in a LightCycler™ real-time PCR (Roche).

2.5 | Analysing flow cytometry of cell cycle distribution

The differentiated cells in this protocol were assessed at the termination of the differentiation process for the cell cycle assay. Initially, the EB colonies derived from miPSCs were collected by cell culture up and down. Then, these cells were centrifuged at 450 *g* for 10 min and washed two times with cold DPBS (Gibco, cat. no. 21600-010). Afterwards, cells were fixed with 1 ml of ice-cold 70% ethanol through adding dropwise while vortexing, stored at room temperature for 1 hr, and transferred at −20°C overnight to fix the cells. Next, the fixed cells were cleansed thrice with DPBS to eliminate final traces of alcohol. At the next step, cells were incubated with a staining solution 0.1% Triton X-100, 0.2 mg/ml of RNase A (Sigma, cat. no. R6513-10MG) and 0.02 mg/ml of propidium iodide (Sigma, cat. no. P4170-25MG) in DPBS at 37°C for 15 min. Ultimately, to analyse the cells, a flow cytometry analyser (Attune™ Acoustic Focusing Cytometer) was employed. PI intensity was used to analyse DNA content. Pulse processing, which uses pulse area versus pulse width, was employed to exclude the cell doublets, and forward scatter (FS) and side scatter (SS) were applied to identify the single cells. Of course, FlowJo® software was used to analyse cells in each sample.

2.6 | Flow cytometry analyses of Mvh-positive cells

There has been a relationship between the differentiated cells and 0.25% trypsin at days 0, 4 and 7. EB medium culture has been used to neutralise the cells. PBS has been applied for twofold washing the cells. Then, they have been centrifuged for 3 min at 115 *g*. Afterwards, the cells (at dark and RT) have been fixed with 4% paraformaldehyde for 30 min. Moreover, PBS/1%FBS/0.1% sodium azide has been applied for rinsing them two times. Next, the fixed cells have been permeabilised with 1% Triton X-100 in PBS 15 min. Then, PBS again has been used to wash the cells. Afterwards, 1% BSA in PBS has been applied at RT for 30 min for blocking the cells. Detection of the cells has been done for 1 hr at RT with FITC-conjugated anti-rabbit second antibody. When the cells have been again resuspended in PBS, FACS Calibur (BD Biosciences) has been used to analyse them and was washed two times. Normal miPSCs have been applied for controls.

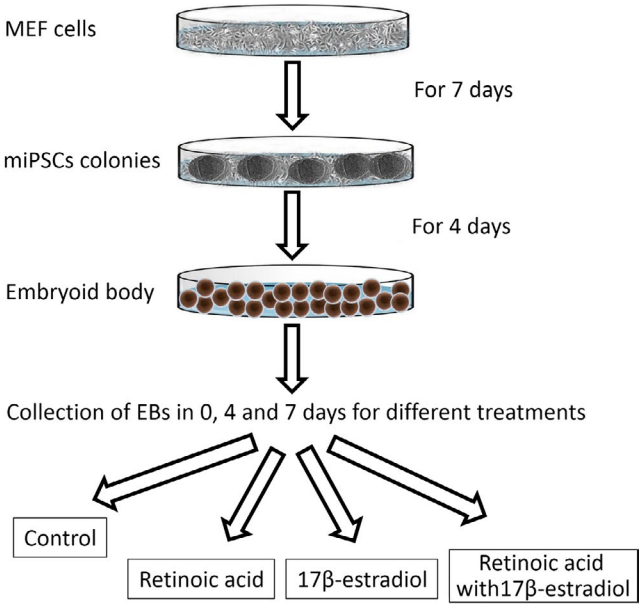

FIGURE 4 Schematic timelines and simplified protocols for miPSCs differentiation into male germ cells with retinoic acid and 17β-estradiol for 7 days

2.7 | Statistical analysis

One-way ANOVA followed by a Tukey post hoc test through a statistical software program (SPSS 19, IBM) was used to analyse qPCR data. Findings are expressed as mean ± *SEM*, and statistical significance was confirmed at $p < .05$. All tests were conducted separately for more than three times; unless otherwise expressed.

3 | RESULTS

3.1 | Gene expression assay

Figure 5 depicts the expression profile of *Stra8*, *Sycp3*, *Ddx4* and *AKAP3* genes in meiotic, mitotic and post-meiotic phases of spermatogenesis.

Relative expression profile of these genes during differentiating embryoid bodies of miPSCs on days 0, 4 and 7 was analysed

TABLE 1 Primers for analysis of qPCR of male germ cell markers

Gene	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Fragment size (bp)	Annealing temperature (°C)
Stra8	GAAGTGCCTGGAGACCTTTG	TGGAAGCAGCCTTTCTCAAT	150	59.7
Sycp3	GGGGCCGGACTGTATTTACT	GGAGCCTTTTCATCAGCAAC	246	58
Ddx4	CTTCAGTAGCAGCACAAGAGG	GGAGGAAGAACAGAAGAACAGG	267	57.5
AKAP3	CGCAAAGACCTGGAGAAAAG	CCACTTCCTCCACAAACGAT	82	58
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	223	57.5

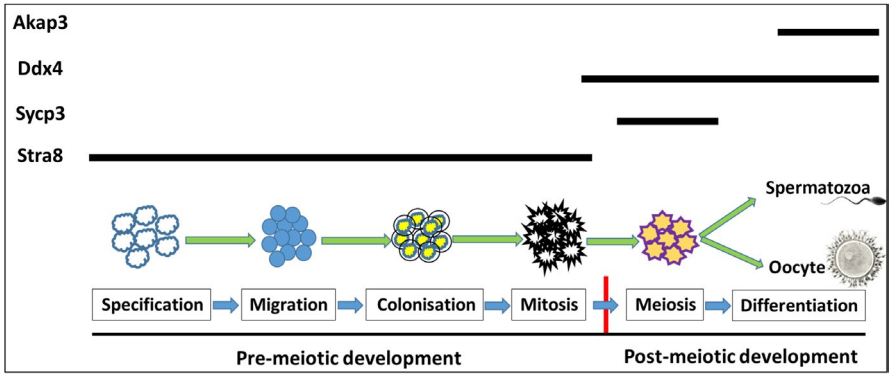

FIGURE 5 Scheme of representing transient gene expression during differentiation. Germ cells from the mitotic, meiotic and post-meiotic phases are portrayed, such as specification, migration, colonisation, mitosis, meiosis and differentiation. The temporal expression profiles of *Stra8*, *Sycp3* and *AKAP3* during differentiation are shown by the black bars

by real-time PCR. The capability of the cells' response to retinoic acid collected in the premeiotic germ cells is represented by *Stra8* (Li et al., 2013; West, Park, Park, Daley, & Geijsen, 2006). *Sycp3* (Synaptonemal complex protein 3) is a marker of meiosis, and sperm-specific *AKAP3* (A kinase anchor protein 3) is one of the dual-specificity anchoring proteins, which has an interaction with protein kinase and regulatory subunit via conserved N-terminal amphipathic peptides (Xu, Yang, Yang, Zhao, Wu, & Qi, 2014). Moreover, expressing the cells is just observed in the haploid male germ cells representing the condition of post-meiotic phase of the cells.

The expression of *Stra8* gene did not change on 0 and 7 days, but it increased in the RA group on the 4th day compared to other groups ($p < .01$). Moreover, a relative increase was observed in RA + E group compared to the control group, but it was not significant. Our results showed that there was a significant increase in the RA group compared to the E group ($p < .05$; Figure 6).

The meiosis marker of PGCs (*Sycp3*) was also analysed, and its expression level was measured at the different times during differentiation. Our qPCR results indicated no increase of *Sycp3* expression on 0 and 7 days. However, we observed the increased expression of this gene in RA + E group on day 4 compared with other groups ($p < .01$; Figure 6).

Real-time PCR has been used to analyse the expression of *Ddx4* gene. Similar to the above-mentioned genes, *Ddx4* express level was enhanced on day 4 and RA + E group compared to the controls ($p < .001$). Also, RA + E groups and RA and P groups had a significant difference on day 4 ($p < .01$). However, other groups did not differ on 0 and 7 days.

The expression of *AKAP3* gene during day 0 has not been detected. In contrast, expression of this gene significantly enhanced in the RA + E group compared to other groups on day 4 of EB formation ($p < .0001$). miPSCs indicated this gene expression in the RA + E group compared to RA ($p < .01$) and E groups ($p < .001$) on day 7. However, we did not observe a significant difference between RA + E and control groups on this day. The gene expression increased in RA + E group compared to the RA group ($p < .001$). Expression of *AKAP3* gene on day 7 of miPSCs decreased in RA ($p < .05$) and E ($p < .01$) groups compared to control group, whereas the expression of this gene was unchanged in RA + E group compared to the control group and E group compared to RA group respectively (Figure 6).

3.2 | DNA content analyses by flow cytometric

In addition, flow cytometry data showed that 3%–8% of the cells in sub-G1 stage were haploid after RA and 17β-estradiol (RA + E group) induction in comparison to other groups on day 4 (Figure 7), while a significant difference was not observed between groups in sub-G1 stage on 0 and 7 days. In general, findings showed that miPSCs could differentiate into haploid male germ cells with RA or oestrogen stimulation combined with EB formation.

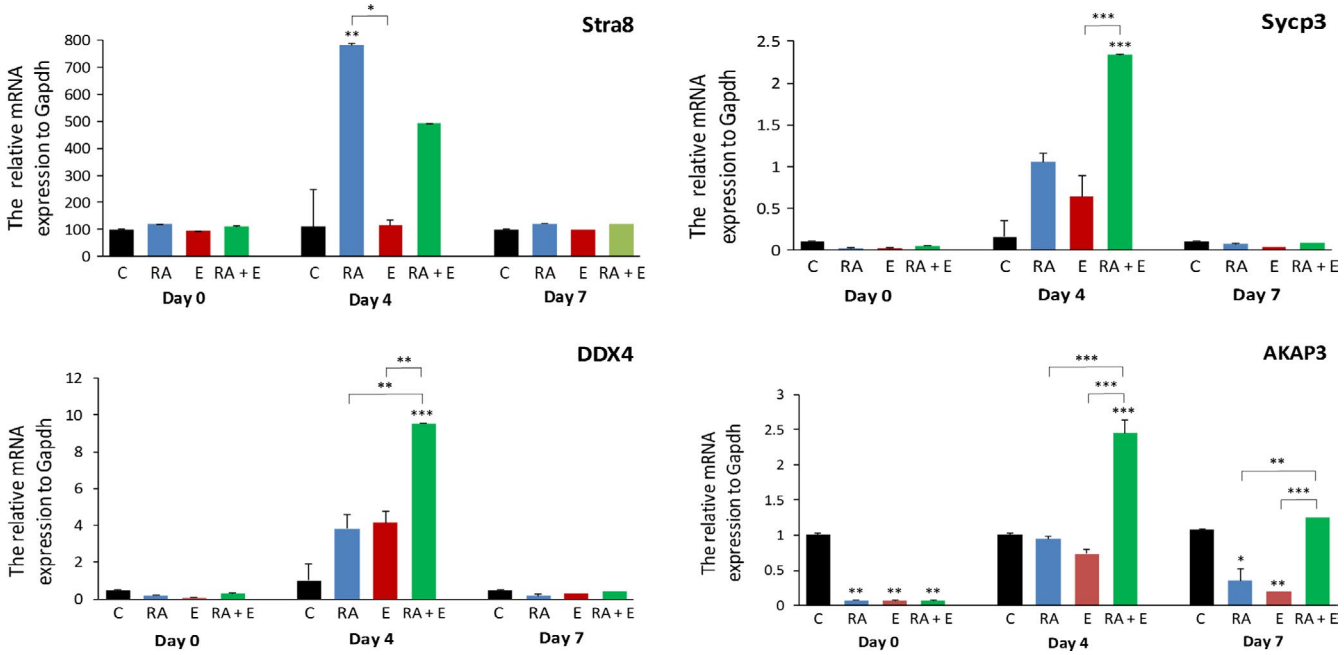

FIGURE 6 The expression profiles of germ cell-related genes in miPSCs treated with RA or 17β-estradiol in vitro. Real-time PCR was conducted by cDNA from EB cultures maintained for 0, 4 and 7 days in the absence or presence of RA and 17β-estradiol for detecting relative mRNA expression of *Stra8*, *Sycp3*, *Ddx4* and *AKAP3*. The mean normalised expression of each gene corresponding to that of GAPDH was depicted along the *y*-axis. C: the control group with no retinoic acid or induction of 17β-estradiol. E: the 17β-estradiol induction group. RA: the retinoic acid induction group. RA + E: the retinoic acid and 17β-estradiol induction group. * ($p < .05$), ** ($p < .01$) and *** ($p < .001$) indicated statistically significant differences in expression of mRNA between the RA- or/with 17β-estradiol-treated groups and the control

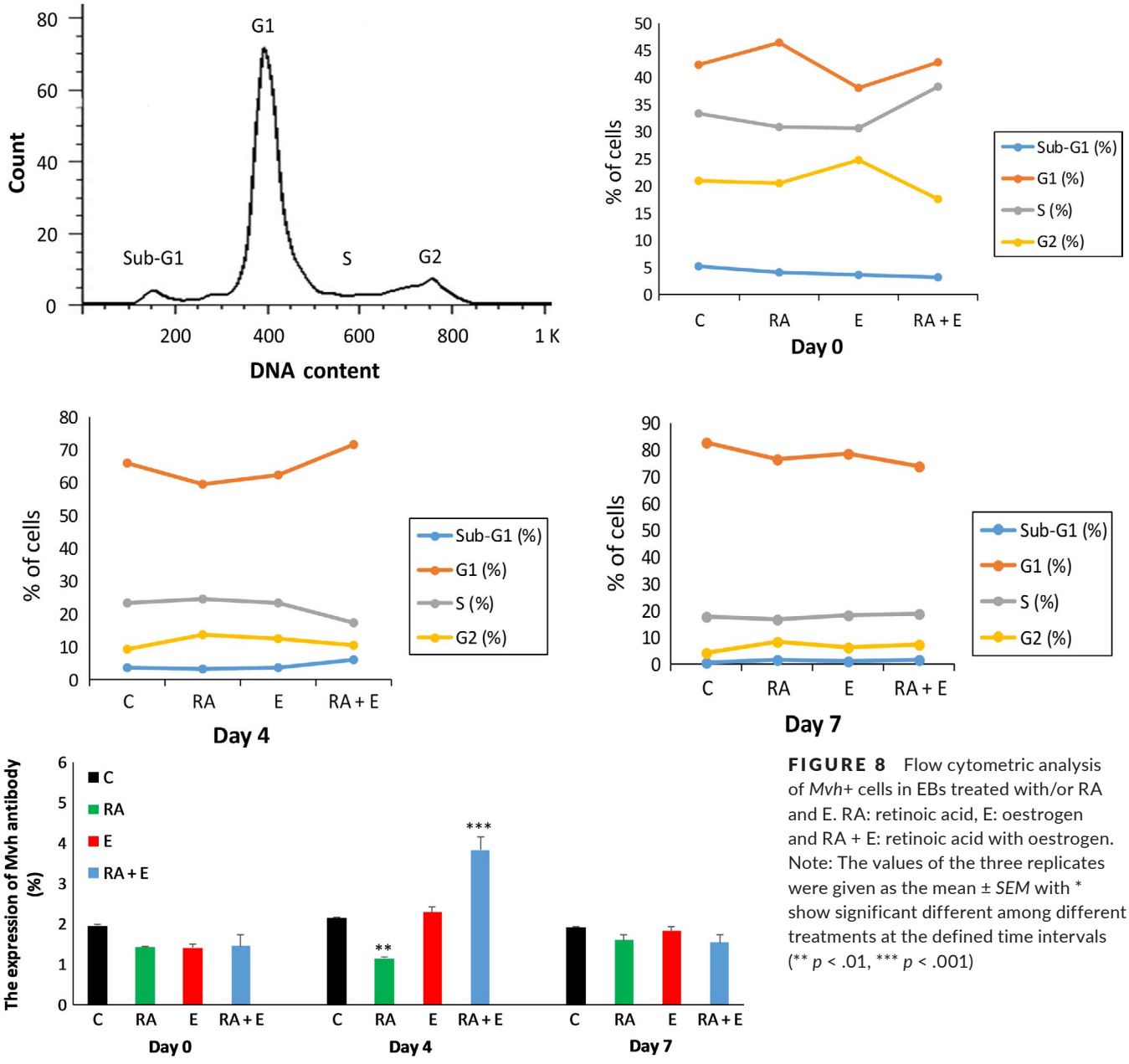

FIGURE 8 Flow cytometric analysis of *Mvh*+ cells in EBs treated with/or RA and E. RA: retinoic acid, E: oestrogen and RA + E: retinoic acid with oestrogen. Note: The values of the three replicates were given as the mean ± *SEM* with * show significant different among different treatments at the defined time intervals (** $p < .01$, *** $p < .001$)

FIGURE 7 Analysis of DNA content by flow cytometry in stages of G1, S and G2/M of the cell cycle

3.3 | Measuring the expression of Mvh protein via flow cytometry

For differentiation of spermatogenic lineage, this study dealt with the analysis of *Mvh* protein expressions, which is specific to differentiate male germ cells and a marker for PGCs gathered from culture from the final emigration stage to the post-meiotic stage of EBs. Detecting the mice *Mvh* protein expression has been performed on days 0, 4 and 7. Higher expression of the protein has been observed in male germ cells in the presence of RA with or oestrogen. Expressing the protein suggested a considerable enhancement in RA + E groups compared to the controls on day 4 ($p < .001$; Figure 8). Moreover, expressing *Mvh* declined in the induced groups with RA and controls on day 4 of induction compared to the controls ($p < .01$; Figure 8). Nonetheless, other groups did not differ on days 0 and 7.

4 | DISCUSSION

In this study, germ cell differentiation was conducted from miPSCs upon the process of EB formation and RA with oestrogen induction. miPSCs form cells aggregate called EBs, which can spontaneously differentiate into cells of all three germ layers such as primordial and more mature germ cells (Geijsen et al., 2004; Makoolati, Movahedin, Movahedin, & Forouzandeh-Moghadam, 2016). Moreover, many researchers stated that hiPSCs can

differentiate into PGCs in vitro (Panula et al., 2011; Park et al., 2009; Yang et al., 2012). Nevertheless, many efforts have been made to trigger iPSCs to be germ cells in vitro. However, there is no information of whether they can differentiate into germ cells in vivo (Yang et al., 2012). There are three main phases during the differentiation of stem cells to gametes in vitro including (a) stem cells differentiation into PGCs, (b) PGCs meiotic entry and (c) gamete maturation and formation of haploid cells (Tan et al., 2016). In addition, the expression profile of many genes related to male germ cell proliferation and/or differentiation (Li et al., 2013; Shah, Singla, Singla, Palta, Manik, & Chauhan, 2017; Silva et al., 2009; Xuemei et al., 2013).

Our real-time PCR data confirmed *Stra8* upregulation of premeiotic germ cell marker in the RA group and on day 4 when miPSCs are differentiated. This finding corresponds to the results of Li et al. (2013). They demonstrated that a significant increase of *Stra8* expression was observed by adding RA. As *Stra8* is the target gene of RA, this gene expression is inhibited in the absence of RA. It should be noted that the successful stimulation of *Stra8* in this study was observed by adding RA (Li et al., 2013). Results of real-time PCR in Dong et al. (2017) study revealed a considerable increase of *Stra8* expression in spermatogonial stem cells (SSC) conditions with RA on day 3. Decreased expression of *Stra8* expression in SSC conditions with RA was also observed on day 5. Dong et al. found that the common impacts of SSC and RA conditions could enhance the expression of *Stra8* for facilitating the differentiation initiation (Dong et al., 2017). In addition, Silva et al. (2009) determined that Stra8 expression in the treated cells with RA for 7 days experienced a significant increase compared to day 7 of untreated control cultures, which is inconsistent with our observations, because these studies worked on mESCs cultures treated with RA and testosterone (Silva et al., 2009).

The expression level of *Sycp3* (which is needed for meiosis synapsis phase) at different times was measured during differentiation by real-time PCR. According to the data deduced from this study, the gene expression of *Sycp3* significantly increased in RA + E group compared to the control group and E group respectively ($p < .01$). Dong et al. (2017) found that this gene expression remarkably increased at later time points. They demonstrated that RA combined with SSC conditions considerably increased the efficiency of mouse ESCs differentiation by enhancing spermatogenic genes expression (Dong et al., 2017). The results from the current study are in conflict with Dong et al.'s (2017) results because they worked on ESCs and RA with spermatogonial stem cell. Li et al. (2013) found that this gene expression increased from day 4 to day 7 of EB formation. Moreover, these researchers concluded that this gene expression decreased in miPSCs in responding to RA or testosterone alone (Li et al., 2013). However, the data from our study are compatible with Li et al.'s finding. We found that RA and oestrogen combination could induce miPSCs into germ cells on day 4.

Detection of *Ddx4* (*Mvh* or *Vasa*) gene expression, which is one of the genes in a form of PGCs in the differentiated EBs, has been observed. Findings revealed that expression of *Ddx4* enhanced in RA + E group compared to the controls on day 4. Eguizabal et al. (2009) generated primordial germ cells from pluripotent stem cells. They revealed differentiation of the cell lines by forming EBs via co-expression of *Oct4* and *Mvh* for identifying the novel PGCs in vitro (Eguizabal et al., 2009). Gholamitabar Tabari et al. (2018) assessed the effects of retinoic acid on expressing the gene of mice-specific germ cells in ESCs-derived germ cells in vitro. They showed the increased expression level of *Mvh* in ESCs given treatment with RA in comparison to the ones of without RA (Gholamitabar Tabari et al., 2018). Thus, this study finding had consistency with the present research outputs. However, it differs in terms of the kind of cells and treatments. Makoolati et al. (2016) observed expressing *Mvh* gene in undifferentiated ESCs and increasing the expression when the EBs differentiation procedure reached 2-day-old EBs (Makoolati et al., 2016).

Expressing *Mvh* protein significantly increased in RA + E groups as compared to the controls on day 4. As reported by Park et al. (2009), *Mvh* (*Ddx4*) protein-positive has been seen in <1% of the population in undifferentiated hESCs. Nevertheless, it is possible to identify Vasa positive cells after 7 days of differentiation (Park et al., 2009). Easley et al. (2012) studied the way of differentiating human pluripotent stem cells into haploid spermatogenic cells. They found that *Vasa*+ cells significantly increased with 40% of HFF1 cells and 60% of H1 cells after a 10-day culture. Easley et al. observed that *Vasa* and deleted in Azoospermia like (*Dazl*) are 2 RNA-binding germline-specific proteins, which contribute crucially to normal cell growth and spermatogenesis (Easley et al., 2012). Li et al. (2014) demonstrated that combining BMP8b, SCF and BMP4 is accompanied by the maximum potential for inducing the induced PGC-like cells, which results in a considerably greater expression of *Mvh* (Li et al., 2014).

The current project also revealed that treating miPSCs-derived embryoid bodies with RA and 17β-estradiol largely modified the expression of *Akap3* gene related to the male germ cell lineage on 4 and 7 days in comparison to other groups ($p < .001$). Silva et al. (2009) represented a significant first step to designing a plausible directed ESCs differentiation protocol for male germ cells by RA, and testosterone could not identify this gene expression during the time course (Silva et al., 2009). They found that the expression of *Prm1* (as a male germ cell gene) experienced a significant increase in comparison to day 7 of untreated control cultures when the cells were treated with RA for 7 days. This observation is not in agreement with our findings, because they studied ESCs differentiation into male germ cell by RA with testosterone. Likewise, the expression of *Akap3* gene did not change on 7 and 14 days, while the expression of other genes is associated with male germ cell increased over time. Li et al. (2013) determined that expression of *Akap3* gene increased from day 4 to day 7 of EB formation using RA and testosterone. Their results suggested that miPSCs enjoy the power of differentiation into haploid male germ cells in vitro on day 7 after EB formation phenotypically (Li et al., 2013). Their data are consistent with our results, but they evaluated the effect of testosterone on the differentiation of these cells.

Moreover, this research reported the presence of haploid cell population (5.96%) by flow cytometry in RA + E group on day 4, which suggests completion of meiosis. However, any significant changes were not found on 0 and 7 days. Concerning our knowledge, this is the first research explaining the identification of haploid cell population upon RA with E induction of male germ lineage differentiation in any species. Some preceding researches presented the haploid cell population detection upon FACS analysis. For instance, Kee, Angeles, Flores, Nguyen, and Pera (2009) demonstrated identification of ~2% haploid cell population on day 14 after overexpressing BOULE, DAZ and DAZL genes in human ES cells (Kee et al., 2009). It should be noted that no haploid cell population was observed in the differentiated buffalo embryonic stem cell monolayer cultures, which used cumulus cell-conditioned medium and testicular cell-conditioned medium upon analysis of FACS (Shah et al., 2017). Li et al. (2013) revealed that 2%–8% of iPS cells could differentiate into haploid cells via retinoic acid or testosterone therapy. In general, their findings showed that miPSCs could differentiate into haploid male germ cells with RA or testosterone stimulation combined with EB formation (Li et al., 2013). Easley et al. (2012) initially indicated efficient and direct generation of haploid spermatogenic cells from human ESCs and iPSCs in spermatogonial stem cell (SSC) conditions, which introduces a hopeful procedure for direct access of spermatid-like cells without genetic manipulation (Easley et al., 2012).

In conclusion, the differentiation of germ cells from PSCs brings about a favourable model for reproductive geneticists and a possible procedure to treat couples with infertility caused by germ cell defects. With regard to our knowledge, this research is the first draft that reports RA-induced differentiation with 17β-estradiol of miPSCs into germ lineage cells. In short, these cells capacity for expressing genes characteristic of germ cells in vitro was observed in the research. It was also demonstrated that RA and 17β-estradiol affect this process by gene expression and percentage of haploid cells. Therefore, these findings show a critical first stage for planning a reasonably directed differentiation protocol for male germ cells, so that additional investigations may provide culture conditions that promote the amount of male germ cell gene expression in a pattern compatible with the developmental programme of spermatogenesis and oogenesis. Moreover, future studies should emphasise the genome-wide epigenetic landscape of the pluripotent cell population before differentiating germ cells, because this will be useful in the analysis and following applications of germ cells for study or cell-based treatment. We suggest that further investigations should utilise more genes and proteins and also more techniques. Likewise, it is better to use other ingredients to increase differentiation in future studies. Moreover, there is another main limitation for our study which should be mentioned. We have just analysed the limited gene markers for mitotic and meiosis process which could increase these markers in further studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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