The combination of retinoic acid and estrogen can increase germ cells genes expression in mouse embryonic stem cells derived primordial germ cells

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A B S T R A C T

Generation of germ cells from embryonic stem cells *in vitro* could have great application for treating infertility. The temporal expression profile of several genes was expressed at different stages of germ cell development and examined in differentiation the mouse embryonic stem cells. Cells were treated in three groups of control, with 10^{-8} M of all-trans retinoic acid and the combination of 10^{-9} M of 17β-Estradiol and retinoic acid for 7, 12, 17 or 22 days. Quantitative RT-PCR and Immunofluorescent were used to investigate the possible inductive effects of estrogen on mouse embryonic stem cell-derived primordial germ cells. mRNA expression of *Oct4* and *Dazl* were downregulated in embryonic stem cells by the retinoic acid group, whereas *Mvh* transcription was reduced by retinoic acid and estrogen group in these cells compared to the control group. But, retinoic acid with estrogen group-treated cells exhibited increased mRNA expression of *Stra8*, *Fragilis*, *Sycp3*, *GDF9,* and *Stella* compared to untreated controls. The expression of *Stella* and *Mvh* proteins were remarkably increased in cell colonies. This study shows that estrogen affects the expression of specific markers of primordial germ cells. Also, estrogen and retinoic acid speed up and increase the level of expression of specific markers.

1. Introduction

Infertility is a global public health issue and one percent of people are not able to produce gametes [1]. About 10–15% of couples suffer from subfertility or infertility due to an error at any stage of gameto-genesis process [2,3].

Nowadays, gamete donation presents a solution to the couples with low or no sperm and/or oocyte, to have a baby. However, the genetic relationship will be changed so new methods for male infertility are essential [4]. In recent years, many studies conducting to generate germ cells from stem cells [5,6] have created new hope in the treatment of infertile couples that cannot produce gamete [7]. It was demonstrated that human and mouse embryonic stem cells (mESCs) are capable of differentiating into cells very similar to oocytes and ovarian follicle-like cells [8,9]. Also, it is reported that *in vitro*-differentiated embryonic stem cells (ESCs) give rise to male gametes that can generate mice offspring [10]. *In vitro* differentiation of several stem cells into pri-mordial germ cells (PGC) has been studied for bone marrow [10], in-duced pluripotent stem cells [2] and ESCs [11,12].

ESCs are pluripotent stem cells that are derived from inner cell mass of the pre-implantation blastocyst and are able of maintaining pluripotency [13]. ESCs can be induced to several features of early embryonic development and can differentiate into all three germ cell layers depending on the situation culture employed *in vitro* and *in vivo* [14]. Previous results showed that ESCs are capable to change not only to PGCs but also oocyte-like [15], sperm-like cells [16], blastocyst-like structure [17] *in vitro*. However, yet there is a big challenge to differ-entiate stem cells into germ cells and this technique requires further studies [18,19]. Researchers using a variety of experimental approaches demonstrated that some growth factors and steroidal hormones can stimulate the differentiation of stem cells to germ cells *in vitro* [7]. Estrogen is a steroid hormone that plays an important role in the menstrual cycle and involved in many processes related to reproduction [20]. The estrogen class of hormones is included estradiol (E_2), estriol (E_3), and estrone (E_1) [21]. Many studies have indicated estrogen sig-naling importance in the proliferation of PGC *in vitro* [20,22,23]. Var-ious studies showed that 17β-Estradiol has an inducing role in the differentiation of mESCs to motor neurons [24,25]. Retinoic acid (RA) involved in the initiation of meiosis [26] and is identified as PGC dif-ferentiation factor [2,27,28]. However according to these studies, yet these methods are not able to generate efficient germ cells in the de-sired range of quality and quantity [28]. A number of studies are

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Table 1
Primers for RT-PCR analysis of germ cell markers.

Gene	Forward Sequence 5'→3'	Reverse Sequence 5'→3'	Annealing Temp.
Oct3/4	CCTTGCAGCTCAGCCTTAAG	GCGATGTGAGTGATCTGCTG	63.9
Fragilis	ATGTGGTCTGGTCCCTGTTC	TCAGGATGCTGAGGACCAAG	64.5
Stella	GACCCAATGAAGGACCCTGA	CAATGCGGTTCCGTAGACTG	63.8
Dazl	AAATGGCCCGCAAAAGAAGT	ACTGCCCGACTTCTTCTGAA	64.3
Mvh	TCAGACGCTCAACAGGATGT	ACTGGATTGGGAGCTTGTGA	64.3
Stra8	CCTGGTAGGGCTCTTCAACA	CCCATCTTGCAGGTTGAAGG	64
Sycp3/Scp3	GGGGCCGGACTGTATTTACT	GGCTTCCCAGATTTCCCAGA	64.5
GDF9	GTTCCCAAACCCAGCAGAAG	GGAGGAAGAGGCAGAGTTGT	64.2
Prm1	ACAGCCCACAAAATTCCACC	CTTATGGTGTATGAGCGGCG	63.7
HPRT	CAGTCCCAGCGTCGTGATTA	GGCCTCCCATCTCCTTCATG	64.8

looking for an efficient method to differentiate stem cells into germ cells.

Real-time PCR (qPCR) analysis measured quantitative changes in mRNA abundance of these germ cell genes while expression of selected proteins was confirmed by Immunofluorescent. Thus, the main purpose of the study was to examine the effects of a combination of estrogen and RA on genes expression of mESCs into PGCs.

2. Material and methods

2.1. Ethics approval and consent to participate

All procedures performed in studies involving animal participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments.

2.2. Derivation of mouse embryonic fibroblast (MEF) and feeder cell preparation

Head and liver of mice embryos at 13.5 days postcoitum (dpc) were excluded, the body was crushed into smaller pieces by passing through needle (gauge 18) and transferred to a T75 flask with high glucose DMEM/F12 (Invitrogen, San Diego, CA, USA), supplemented with 12% fetal bovine serum (FBS) (Invitrogen, San Diego, CA, USA) and 1% penicillin/streptomycin (Invitrogen, San Diego, CA, USA). Next day, the medium was changed to remove cell derbies. The MEF of passage 3 were used as feeder cell. Inactivated MEF cells were incubated 3 h with 10 µg/ml mitomycin (Sigma-Aldrich, USA) in MEF medium. Cells were washed 3 times with PBS and next day using as feeder cell.

2.3. Embryonic stem cells culture

In this experimental study, undifferentiated C57BL/6 mouse XY ESCs (Royanb1) [29] were purchased from Royan Institute and cultured on 0.1% gelatin-coated cell culture flask containing mitomycin C-treated mouse embryonic fibroblast (MEF). To maintain the un-differentiated status, mESCs were cultured and grown in ESCs culture medium; knockout DMEM (Invitrogen, San Diego, CA, USA), supplemented with 15% ES qualified fetal bovine serum (FBS) (Invitrogen, San Diego, CA, USA), 2 mM L-glutamine (Invitrogen, San Diego, CA, USA), 1x non-essential amino acid (Invitrogen, San Diego, CA, USA), penicillin/streptomycin (Invitrogen, San Diego, CA, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, USA), and murine leukemia inhibitory factor (LIF) 1000 U/ml (Millipore, Billerica, MA, USA) [30]. When cells reached 70% confluency, they were harvested with collagenase type IV solution (Gibco™ 17104019). The cell suspension was diluted 3-fold and cultured in 0.1% gelatin-coated flask containing MEF and ESCs medium with LIF. The culture medium was changed daily.

2.4. Spontaneous differentiation of mESCs by retinoic acid (RA) or estrogen induction and embryoid body (EB) cultures

For embryoid bodies (EBs) formation, nearly 5×10^6 mESCs were transferred to 10 cm bacterial plates (BD Biosciences) containing 10 cm of mESCs culture media without LIF. EBs were retained in suspension for 5 days and culture medium changes daily. After 5 days, adherent cultures of EBs were fixed on gelatin-treated tissue culture plates without MEFs and protected in ESCs medium without LIF. The adherent cultures of EBs were exposed in triplicate to daily treatments with RA (10^{-8} M all-trans retinoic acid), RA + E (10^{-8} M of RA and 10^{-9} M of 17β.-Estradiol) for 72 h in 7, 12, 17 or 22 days considering the first day of adherent culture as day 0. During treatment (72 h), the medium was changed with fresh medium containing the treatment agent every day. After 72 h of treatment, the medium was changed and the cells were cultured with EB culture medium for 22 days, but RA was added to all the groups except control group. At the end of the treatment, cells were washed with PBS and total RNA was isolated using TRIzol Reagent (Invitrogen).

2.5. RNA extraction, cDNA synthesis, RT-PCR

Total RNA was extracted from the adherent cultures of EBs using TRIzol Reagent(Invitrogen) and cDNA was synthesized using a kit (Takara Bio, Japan) according to the manufacturer's instruction. RT-PCR was performed using specific primers which designed by Primer 3 software for mouse *Oct4* (OMIM: 164177), *Fragilis* (OMIM: 614757), *Stella* (OMIM: 608408), *Dazl* (OMIM: 601486), *Mvh* (OMIM: 605281), *Stra8* (OMIM: 609987), *Sycp3* (OMIM: 604759), *GDF9* (OMIM: 601918) and *Prm1* (OMIM: 182880) (Table 1).

PCR was carried out in a total volume of 20 µl consisting of 10 µl of 2× Master-mix PCR, 0.35 µM each of the forward and reverse primers, and 2 µl cDNA (all of PCR reagents were purchased from Bioneer Co., Korea). The PCR program was as follows: 35–40 cycles of 94 °C denaturation for 45 s; 55 °C–61 °C annealing for 30 s; 72 °C elongation for 60 s, with a final incubation at 72 °C for 10 min.

2.6. Immunofluorescence

For immunofluorescent localization of *Stella* and *MVH*, adherent EBs were prepared as previously described [30]. Similar to the experimental design that was explained in before section; the adherent EBs were exposed in triplicate to daily treatments of RA and estrogen.

In summary, cells were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature and washed twice with ice-cold PBS. Cells were incubated for 10 min with PBS containing 0.5% Tween-20 then blocked with 1% BSA and 0.5% Tween-20 for 60 min at room temperature. Cells were incubated with the primary antibodies against *Stella* (1 µg/ml) and *Mvh* (1 µg/ml) (Abcam, USA) overnight at 4 °C, then washed with PBS and incubated with the goat anti-rabbit secondary antibody Alexafluor 568 (Life technologies, USA) in the dark for

N. Eskandari et al.
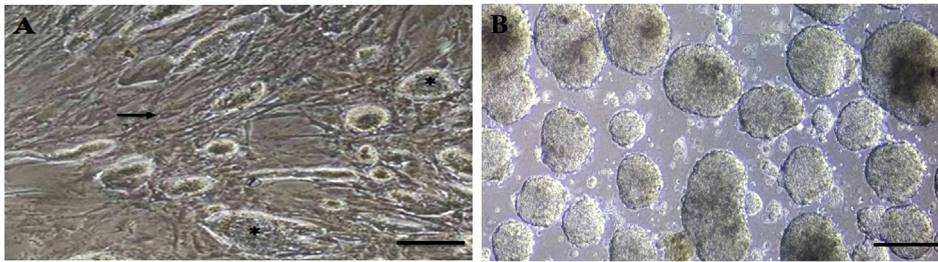
Fig. 1. Colony morphology of mESCs (Royan B1) in undifferentiated state and at the beginning of differentiation. A) Dome-shaped colony of undifferentiated mESCs (*) with smooth margin on iMEF (→). B) Morphology embryoid body formation of the mESCs. Scale bar = 100 μm.

A) Dome-shaped colony of undifferentiated mESCs (*) with smooth margin on iMEF ( ).
B) Morphology embryoid body formation of the mESCs. Scale bar = 100 μm.

60 min. Also, negative control cells were incubated with only secondary antibody. Nuclei were labeled by DAPI (Roche) staining and the images were recorded by an invert fluorescent microscope (Ti-E eclips, Nikon, Japan).

2.7. Statistical analysis

The results of three replicated experiments were presented as mean ± SD. Statistical analysis was performed using the ANOVA and Tukey posttest. The obtained data from RT-PCR were normalized with HPRT (as housekeeping gene). The *P* value was presented respectively as *P < 0.05, **P < 0.01 or ***P < 0.001 indicating as low, high or very highly significantly different. All of the data were analyzed with SPSS statics 19 version.

3. Results

Changes in cell morphology were monitored daily. Undifferentiated mESCs colonies were dome-shaped with smooth margins (Fig. 1A). Morphology of EBs was shown (Fig. 1B).

3.1. Temporal expression of germ cell-specific genes related with differentiation of EBs into PGC

The expressions of 9 genes were analyzed by RT-PCR. *Oct4* is a marker of ESC cells and PGCs [31,32], *Fragilis* is expressed in PGCs migrate to the genital ridge, *Stella* is known as a specific germ cell marker during early development, *Dazl* and *Mvh* are expressed in post migratory PGCs [33], *Stra8* (stimulated by retinoic acid gene 8) represents meiosis in both sex [34], *Sycp3* (Synaptonemal complex 3) is a meiosis specific protein that is expressed in PGC for meiosis [28], *GDF9* (Growth differentiation factor 9) and *Prm1* (protamin1) are as oocyte and sperm specific markers [35,36] respectively. Gene expression pattern is summarized in Fig. 2.

We found that RA reduced the expression of *Oct4*, while there was no significant change in the RA + E group (see Fig. 3). RA and RA + E treatment accelerated and increased the expression of *Fragilis*. Noticeably, on seventeenth day in RA + E group, the expression of this gene was dramatically increased compared to the other groups.

In RA + E group, the level of *Stella* expression was significantly increased on 7 and 17 days compared to other groups, while on other days the difference was not significant. On days 7 and 12, expression of *Dazl* was not observed in any groups than days 17 and 22 and retinoic acid reduced the expression of this gene. RA + E group was caused *Dazl* expression on seventeenth day and expression this gene increased in compared to RA group, but on 20-s day, the expression of this gene decreased in compared to RA and control groups.

RA + E treatment decreased the level of *Mvh* expression in all days, compared to other groups. In the control group, there was no expression of the *Stra8*, while in RA + E group it was accelerated and increased significantly. In the RA + E group, the level of *Sycp3* expression was increased on days 12 and 22 compared to the other groups. The expression of *GDF9* was only in the RA + E group increased on day 22 compared to other groups.

3.2. Immunofluorescence detection of proteins marker Stella and Mvh

Immunofluorescence results confirmed the expression of *Stella* and *Mvh* at the protein level in undifferentiated mESC genes in days 7, 12, 17 and 22 in RA and RA + E groups observed by PCR. The expression of *Stella* and *Mvh* were more intense in cell colonies edge than to the center. However, the outside of cell colonies did not show any expression of these genes. Immunofluorescence study showed that *Mvh* and *Stella* are expressed in all group (data not shown) but only on day 22, the RA + E group had a higher expression than other groups.

4. Discussion

During mESCs differentiation, the differential expression of specific genes in some ways imitate mouse embryogenesis *in vivo* [37]. Although many information is available about the process of embryonic stem cells differentiation into male germ line but still lots of people suffer from a lower quantity or quality of sperm [18]. In a precise gametogenesis process, many factors are needed to synchronize germ cell migration, proliferation, and meiosis initiation [26]. Spatial and temporal regulation of genes is responsible for appropriate germ cell proliferation and differentiation *in vivo*. These genes expression are regulated by inherent, interactive and exogenous factors [38], and hence are affected by a numerous of growth factors, differentiation factors, extracellular matrix proteins and signaling molecules. So, suitable culture conditions of germ cell will help to provide a pure population of gametes and necessary supporting somatic cell components from ESCs, which elicit a gene expression profile. This study was designed to examine the combination of retinoic acid and estrogen on the differentiation of mESCs into primordial germ cells [39].

We analyzed the expression of 9 genes by RT PCR and the results showed that they have distinctive expression profile at various stages of gametogenesis (i.e. mitotic, meiotic, and post-meiotic). In addition to, mRNA levels for some of the genes were partly increased in undifferentiated ES cells. The Expression profiles of these genes might be affected by of microenvironment of *in vitro* compared to *in vivo* experiment. Silva et al. (2009) showed that there is a group of founder cells within undifferentiated ESCs populations dedicated to differentiate into germ cell lineage [39]. Griswold et al. (2012) reported that retinoic acid functions as a meiosis-inducing substance but another study suggested that the germ cells do not need endogenous RA at the beginning of meiotic process [26]. However, Mi et al. (2014) for the first time showed the potential role of estrogen as a signaling molecule in germ cells differentiation with RA [40].

Our study showed that the combination of estrogen and RA can accelerate and increase the differentiation of mESCs into PGC. In this study, Royan B1 cell line was used and for the first time and it was shown that this cell line can be differentiation to PGC. In our study, we could see the expression of all the examined genes except *Prm1* that its expression has been shown in other studies [2].

Oct4 gene is expressed in all cell types along the differentiation path from PSCs to SSCs. Also, *Oct4* protein is known as a marker of PGC and ESC [41]; RA treatment reduced the expression of *Oct4* but the combination of RA and E (in RA + E group) was able to increase its
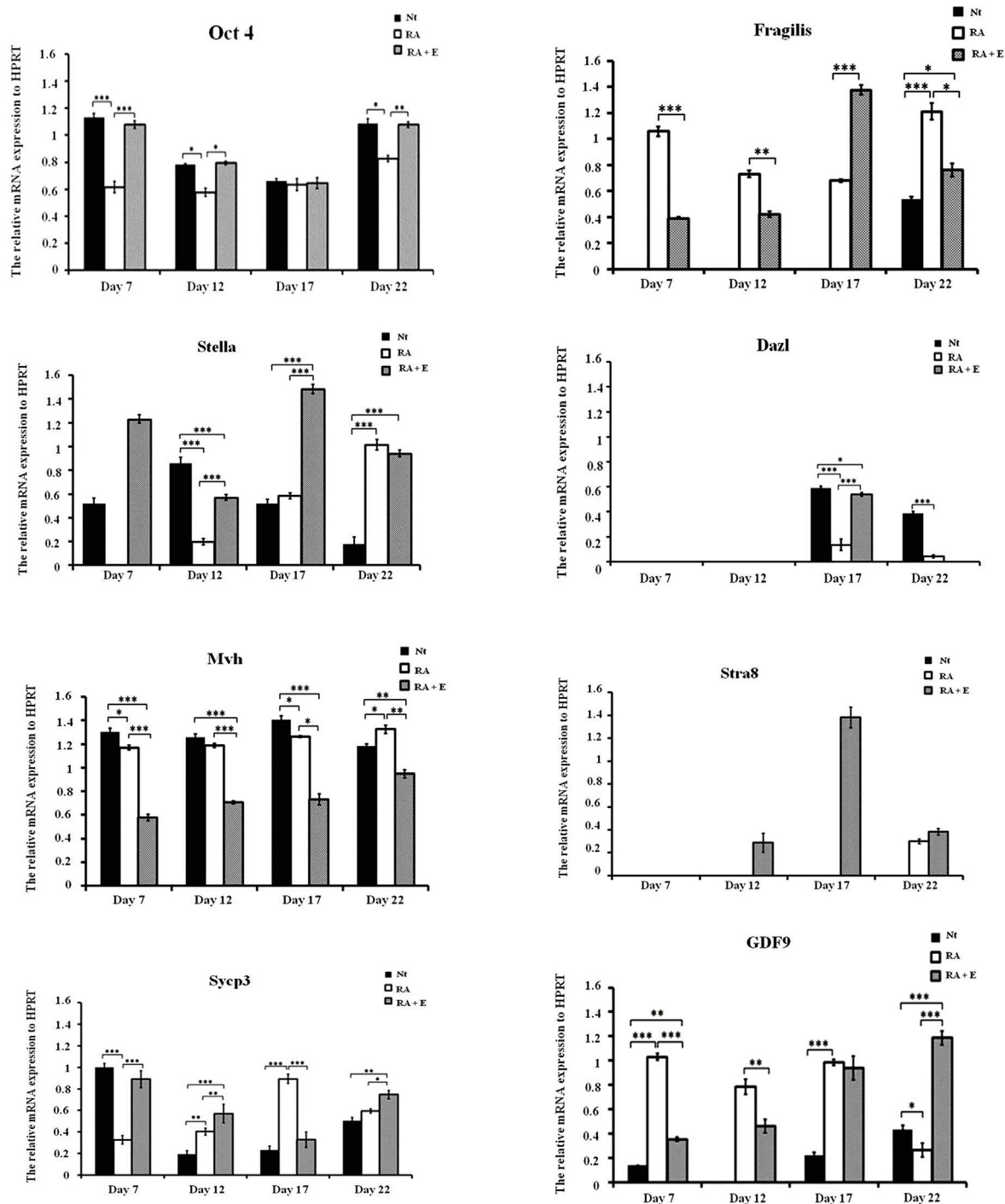
Fig. 2. The expression profiles of primordial germ cell-associated genes in mESCs treated with RA and combination of RA + estrogen (RA + E) *in vitro*. RT-PCR was carry out using the cDNA of mESCs in the presence or absence of RA and combination of RA + E groups. The normalized expression of each gene relative to that of *HPRT* as follows; Black bars: control, the group that was not treated with RA or RA + E. White bars: the group that was treated with RA. Gray bars: the group that was treated with RA + E. *P < 0.05, **P < 0.01 and ***P < 0.001.

expression. It seems that the RA + E help maintains ESCs pluripotency. *Fragilis*, as a putative interferon-inducible gene encodes a transmembrane protein, is apparently related to the acquisition of germ cell competence by epiblast cells [42]. In spontaneous differentiation, *Fragilis* expression is time-dependent by a cascade of molecular while the use of inducer factor (RA or RA + E) accelerated and increased expression. In our study, *Dazl* expression was entirely time-dependent and any of the treatment did not change the expression. In contrast to the Silva and et al. [28] study, RA treatment could increase *Dazl* expression.

In another study, co-culture of ESCs with granulosa cells decreased the level of *Mvh* expression [30] similar to RA + E treatment in our study that reduced the level of Mvh expression. These results show that this gene is not a reliable PGC differentiation factor.

Stella (*DPPA3*) is a novel gene specifically expressed in primordial germ cells, oocytes, preimplantation embryos, and pluripotent cells [43]. In our study, the *Stella* expression was increased by RA + E treatment. Wongtrakoongate et al. (2013) that worked on *Stella* facilitates differentiation of germ cell and endodermal lineages of human

N. Eskandari et al.
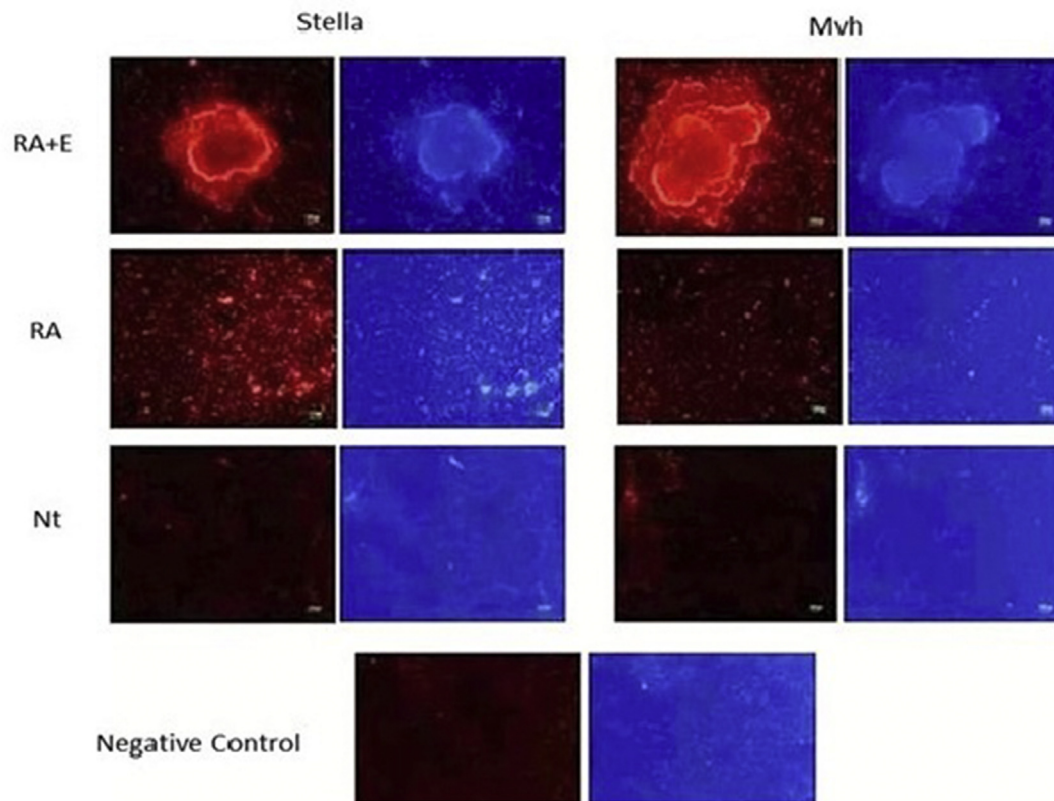
Fig. 3. Immunofluorescence localization of *Stella* and *Mvh* in adherent culture. The cultures were incubated with anti-*Stella* and anti-*Mvh* and the corresponding secondary antibodies. The control cells were only treated with secondary antibody. *Stella* and *Mvh* were visualized using confocal microscopy ($\times$ 250 magnification). White bars in each image represent 100 μm in length.

RA and Estrogen (RA + E).
Retinoic acid (RA).
Nt (control).

embryonic stem cells (hESCs), resulted *Stella* was expressed in hESCs and embryonic carcinoma (EC) cells, as well as in normal testis and ovary. These researchers found that the strongest expression was found in a testicular germ cell tumor, which shows characteristics of pluripotency [44]. *Stella* expression was detected in human oocyte and in EC cells, in which it was down-regulated after retinoic acid-induced differentiation. These findings strengthen the hypothesis that *Stella* might have a similar role in humans as in mice [45].

Our results showed that *Sycp3* and *GDF9* expression was increased with RA + E treatment. Stra8 was not expressed in the spontaneous differentiation and probably expression this gene needs to inducer factor that these findings are the same as Silva et al. [28] and contrary to studies Li et al. [2].

Although several studies reported the expression of *Prm1* [28,46] we did not detect any expression in our study. No expression this gene (*Prm1*) may be probably due to differences in culture and differentiation. The combination treatment of RA + E increased the expression of *Oct4, Fragilis, Stella, Dazl, Sycp3, Stra8* and *GDF9*. According to the most of increased gene expression levels and expression of oocyte-specific marker with not expression sperm-specific marker, it seems that the condition of this study was caused to create female PGC.

There are some limitations in this which should be mentioned. Firstly, we did not use Real time PCR for evaluation of gene expression. In addition, we used limited gene markers in the current study and further studies should consider more genetic markers.

5. Conclusions

In summary, our observations demonstrate the capacity of mESCs to

express the genes characteristic of germ cells *in vitro*. This study shows that estrogen induces the expression of specific markers of primordial germ cells. Estrogen and retinoic acid increased the level of expression of specific markers that were investigated. Thus, these data represent an important first step in designing a plausible directed differentiation protocol for germ cells such that further investigation may yield culture conditions that increase the level of germ cell gene expression in a pattern consistent with the developmental program of spermatogenesis.

Acknowledgements

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