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A Novel *in vitro* Co-culture Systems on Differentiation of Embryonic Stem Cells into Oocyte-like Cells in an *in vivo* Manner

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Abstract

Background: Differentiation of Embryonic Stem Cells into Oocyte-like cells *in vitro* is challenging. Successful derivation of oocyte from stem cells can provide an alternative source for curing ovogenesis problems. The current study aims to demonstrate a new protocol with two different types of media for differentiating embryonic stem cells (ESCs) into oocyte-like cells (OLCs).

Methods: After culturing mouse ESCs, embryoid bodies (EBs) were generated from ESCs by hanging drop (HD) method. To final differentiation of oocyte-like cells (OLCs), the EBs were cultured in two different types of media for 12 days (first 7 days EBs were cultured in *in vitro* maturation diluted in Granulose Cell-Conditioned Medium and Follicular Fluid [1:1:1] followed by 5 days of culture in in vitro maturation diluted in uterine condition medium [1:1]).

Results: According to the MTT test, the viability rate increased in the experimental group compared to the control EBs cultured alone. Expression of *Oct4*, as a pluripotency marker, decreased during the differentiation process of EBs in the experimental group. Co-culturing of EBs with our mentioned protocol increased germ cell markers (*Stella* and *Mvh*) and increased Oocyte-specific markers (ZP1, *Figα* and *GDF9*).

Conclusion: Our study introduces a promising *in vitro* protocol for achieving successful oogenesis through creating interactions of EBs with granulosa cells and uterine condition medium.

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Introduction

Embryonic stem cells (ESCs) are achieved from the inner cell mass of blastocyst stage of embryos before implantation (1). ESCs are pluripotent cells that can be differentiated into different cell types (2). In addition to differentiation of ESCs into the cells of three germ layers, ESCs can be differentiated into primordial germ cells (PGCs), sperm and oocyte-like cells (3). The differentiation ability of ESCs into both somatic cells and germ cells has been confirmed in *in-vivo* and *in-vitro* studies (4-6). At first, PGCs must be generated from ESCs, and then, PGCs are differentiated into oocyte and sperm through an important process which is called specification.

Providing oocyte from ESCs can improve the therapeutic strategies in the field of oogenesis issues (3, 7). The accurate procedure for inducing differentiation of ESCs into oocyte- like cells has not been fully elucidated, yet (8). In the body, complete ovogenesis occurs by interacting of granulosa cells, as somatic cells and oocyte, as germ cells (9). Also, this interaction should be provided in culturing oocytes conditions. ESCs start differentiation by generating a round structure which is called embryoid body **(EB).** EB stage contains both differentiated and undifferentiated cells that can finally differentiate into the different cell types (10, 11).

Several studies have confirmed the essential role of granulosa cells on differentiation of PGCs into oocyte (9, 12).

But, the role of granulosa cells improved with uterine condition medium on differentiation of ESCs into oocyte-like cells has not been evaluated in an *in-vitro* study. The aim of the current study was to evaluate the effects of co-culturing of granulosa cells improved with uterine condition medium on differentiation of ESCs into oocyte-like cells.

Materials and Methods

ESC Culture and Embryoid Bodies

In this experimental study, mouse ESCs, CGR8 were cultured on gelatin (0.1% Sigma) –coated flasks (Falcon) in ESC medium, containing DMEM (Gibco) with 15% fetal calf serum (Gibco), LIF (1,000 IU/ml; Chemicon), 1% w/w non-essential amino acids (Gibco), 0.1 mM β- mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco) and 1% w/w penicillin/streptomycin (Gibco) at 37°C and 5% CO_2 (13). The medium was exchanged every day.

For creation of embryoid bodies (EBs) from ESCs by hanging drop (HD) protocol with EB medium (ESC medium without LIF), a concentration of 2000 cells per 20 µl for 2 days was utilized (14-17). Then, EBs were seeded in individual wells of a low-attachment plate with induction medium (IM) and differentiation strategy was employed.

Differentiation Induction Protocol

To final differentiation of mESCs into oocyte-like cells (OLCs), the EBs were cultured in two different types of media for 12 days. For the first seven days EBs were cultured in *In Vitro* Maturation (IVM medium) diluted in Granulose Cell-Conditioned Medium (GCCM) and Follicular Fluid (FF) [1:1:1] followed by five days of culturing in *in Vitro* Maturation (IVM medium) diluted in uterine condition medium (UCM) [1:1] (uIVM).

Granulose Cell- Conditioned Medium

The granulosa cells of newborn mouse ovaries were cultured according to the method by Qing T (12). The granulose cells were grown to 80% confluence of the culture flasks. The cells were treated with mitomycin C (Sigma) for 2 hours and then, conditioned medium were collected every two days, filtered and sorted in – 20ºC until use.

Follicular Fluid

Adult mouse ovaries were obtained at the animal house of Mazandaran University of Medical Sciences and follicular fluid was collected by aspiration after sedimentation of cumulus oocyte complexes (COCs) and filtered and sorted in –20ºC until use.

IVM medium

IVM medium was composed of MEMα medium (Invetrogen) supplemented with 1U/ml pregnant mares` serum gonadotropin (PMSG), 0.23 mM Sodium pyruvate and 1mg/ml BSA.

Pseudopregnancy Induction

NMRI male mice, aged 6-8 weeks, were vasectomized and after recovery were used for induction of pseudopregnancy. NMRI female mice, aged 6-8 weeks, were kept under 12 hour light/12 hour dark condition. Pseudopregnancy was induced in a natural cycle. Estrus cycle was determined by daily vaginal smears. Immediately after that, female mice were caged individually with a vasectomized male of proven sterility overnight. The following morning was considered as the first day of pseudopregnancy if a copulatory plug was observed.

Preparation of Uterine Cell Cultures

On day 4 of pseudopregnancy, the mice were killed by cervical dislocation and the uterus with its horns was removed under sterile conditions for culture. The tissue was cut into sections and was placed in 0.05% protease (Protease IV: Sigma Pharmaceuticals) in DMEM medium for 20 min at 37°C and 5% CO_2.

Then, the tissue suspension was passed through a 120-pm pore size wire sieve to separate digested tissues from undigested tissues. Then, the cells were suspended in 5 ml DMEM medium plus 10% fetal calf serum (FCS, Gibco). Subsequently, the cells were centrifuged at 600g and the supernatant was disposed. The uterine cells were cultured on gelatin (0.1% Sigma) –coated 25-ml plastic flasks (Falcon, Becton Dickinson) in DMEM (Gibco) supplemented with 10% FCS and 1% w/w penicillin/ streptomycin (Gibco). The Cells were allowed to reach to 80% confluence of the culture flasks. For inactivation, the cells were treated with mitomycin C (Sigma) for 2.5 hours and then, the uterine conditioned medium (UCM) was collected every two days, filtered and sorted in –20ºC until use.

MTT Assay

Cell viability was evaluated based on mitochondrial function of living cells by reduction of tetrazolium salt (MTT, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide) at 4 different time points (2^{nd}, 4^{th}, 6^{th} and 8^{th} day) in 96-well microplates (Falcon). Briefly, 4.8×10^3 cells per 200 ml medium was seeded into each well of a 96-well micro liter plate for 24 hours at 37°C in 5% CO2. Then, the wells were washed with PBS, and 50 µl of 1 mg/ml MTT solution (Sigma-Aldrich) constituted in PBS was poured to each well and the cells were incubated for 4 hours at 37°C. After incubation, the culture medium was removed and 50 µl of 100 % Dimethyl sulfoxide (DMSO) was mixed and placed on a shaker for 10 min. The results were analyzed by spectrometric absorbance at 570 nm (PerSeptive Biosystems, Framingham, Massachusetts, USA).

Immunocytochemistry

The EB cells were washed with PBS and fixed in 4% PFA for 30 min and permeabilized in Tris buffered saline (TBS) with 0.1% Triton X-100 for 10 min. Blocking the nonspecific binding of the primary antibody was performed by incubation

of the EB cells in 0.5% Bovine Serum Albumin (BSA, Sigma) in TBST as a blocking solution (BS) for 30 min at RT. The EB cells were incubated with primary antibodies Ddx4/Mvh (Goat polyclonal IgG, ab566, Abcam system, diluted 1:1000 in BS) and GDF9 (Goat polyclonal IgG, Santacruze, SC- 12244, diluted 1:1000 in BS) overnight at 4°C. Further incubation with the appropriate secondary antibodies Phycoerythrin (PE) - conjugated Donkey polyclonal secondary antibody to Goat IgG (ab976, Abcam system, diluted 1:100 in BS) and FITC-conjugated Donkey polyclonal secondary antibody to Goat IgG (ab975, Invitrogen, diluted 1:100 in BS) were performed in 45 min at RT in darkness and the EB cells were washed 8 times, 30 min each in TBST. Nuclei were detected by DAPI (sigma) staining. Images were taken with an Olympus phase contrast microscope (BX51, Olympus, Tokyo, Japan) (18).

RNA extraction and RT- PCR

Total RNA extraction from cell cultures was performed by Qiazol lysis Reagent (Qiagen), and then 5µg RNA from each sample was transcribed into cDNA by using reverse transcriptase kit (Fermentas), according to the manufacturers' instructions. Primers were designed by AlleleID software version 7.6 (Primer Biosoft, Palo Alto, USA). Primer sequences are shown in table 1. The temperature profile in qRT-PCR amplification was (1) 95°C for 10, (2) 40 cycles of 95°C, 30 seconds; 60°C, 45 seconds; and 72°C, 45 seconds and (3) 72°C for 7 min for the final extension. The ΔΔCt method was used for rating the relative quantification of gene expression.

Statistical analysis

Statistical analysis was conducted by using one-way analysis of variance (ANOVA) test, followed by Tukey post-hoc test and through SPSS software for Windows, version 23. Differences between samples were considered statistically significant at $P<0.05$.

Table 1. The sequence of primers

Gene	Primer (forward/reverse)	Significance
Oct4	5'- CTCGAACCACATCCTTCTCT -3'	Pluripotency marker
	5'- GTTCTCTTTGGAAAGGTGTTC -3'	
Stella	5'- TGAAGAGGACGCTTTGGA -3'	Germ cell marker
	5'- CTTTCAGCACCGACA ACA -3'	
Mvh	5'- CGGAGAGGAACCTGAAGC -3'	Germ cell marker
	5'- CGCCAATATCTG ATGAAGC -3'	
ZP1	5'- CCTCTCACCCTCTGTGGAACAG -3'	Oocyte-specific marker
	5'- GAGCATGTATCAGACCCAGAGG -3'	
Figα	5'- CCGTTTCTACCACAGAGCAGG -3'	Oocyte-specific marker
	5'- TTCTTCAAGCCACTCGCACA -3'	
GDF9	5'- CCAGCAGAAGTCACCTCTACAA -3	Oocyte-specific marker
	5'- ACATGGCCTCCTTTACCACA -3	
GAPDH	5'- ACCACAGTCCATGCCATAC -3'	Internal Control
	5' - TCCACCACCCTGTTGCTGTA -3'	

Results

In this study, we used co-culture systems with two different types of media to mimic *in vivo* development of differentiating mouse ESCs into oocytes-like cells. In the first step, ESCs formed EBs to create germ cells. The CGR8 mouse ESC, was cultured and EBs were created at the end of passage 3 by hanging drop method (Figure 1).

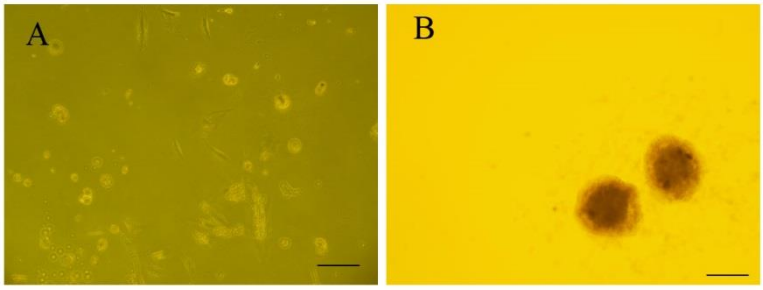

Figure 1. Cell morphology of primary cell culture of CGR8 (A) and morphology of EBs after the end of passage 3(B), Scale bars: 50 µm

In the experimental group, differentiation induction protocol was composed of the first seven days culturing of EBs in IVM medium diluted in Granulose Cell- Conditioned Medium followed by five days of culturing in IVM medium diluted in uterine conditioned medium. Cell viability was evaluated by using MTT assay in 4 different time points (2nd, 4th, 6th and 8th day). As shown in figure 2, cell viability increased in the experimental group compared to the control group at all 4 mentioned times. Except the 4th day, the increase in the rest of the days was significant.

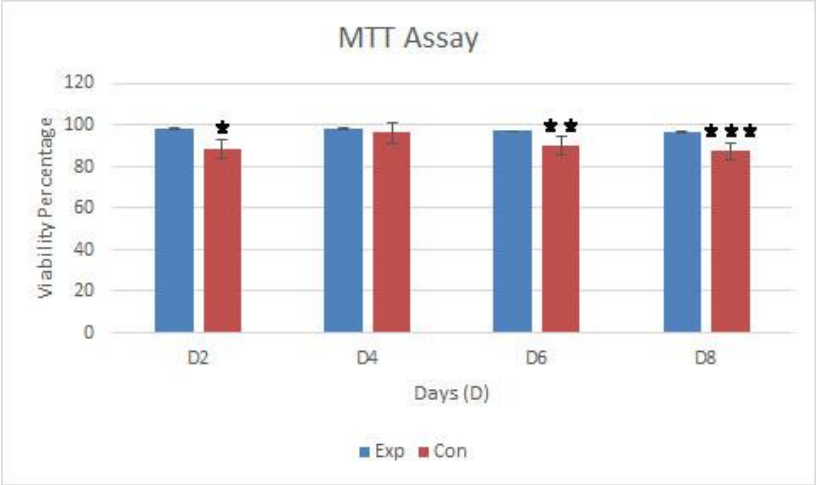

Figure 2. The viability rates in control (Con) and experimental (Exp) groups at days 2, 4, 6, 8 (P≤0.05), significance differences have been specified by star sign.

Expression of Ddx4/Mvh and GDF9 proteins were evaluated as described in EBs after differentiation induction strategy. Our Immunocytochemistry staining showed that Mvh positive cells were observed in almost all stages of induction protocol (Figure 3). Also, GDF9 was expressed only at the end stage of the induction protocol (D12) (Figure 4).

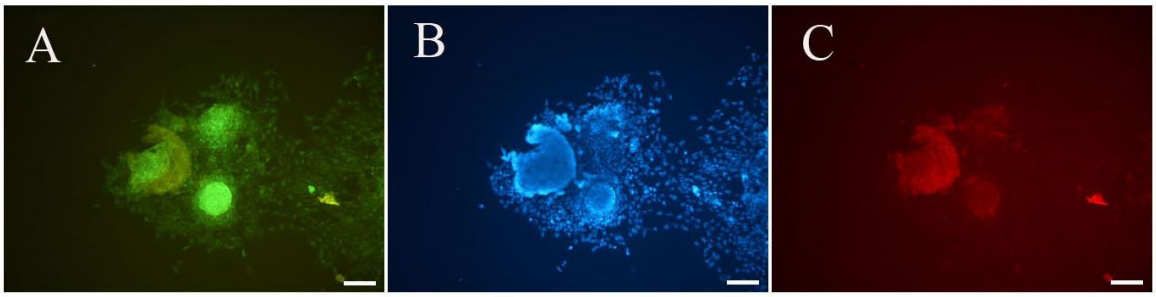

Figure 3. Immunocytochemistry analysis of co-culture cells to show Mvh positive cells

(A) Mvh positive cells, (B) DAPI and (C) Merge, scale bar approximately 50 µm

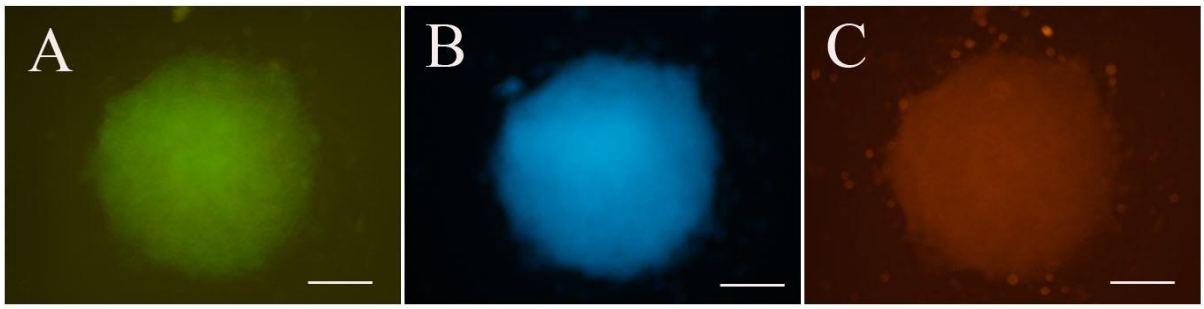

Figure 4. Expression of specific oocyte protein was analyzed by immunocytochemistry.

(A) GDF9, (B) DAPI and (C) Merge, scale bar approximately 20 µm

We performed RT-PCR analysis to evaluate the expression of pluripotency marker (*Oct4*), germ cell markers (*Stella* and *Mvh*) and oocyte-specific markers (ZP1, *Figα*, *and GDF9*) of the experimental and control groups (Figure 5). Our data showed that the expressions of *Oct4*, as a pluripotency marker, decreased during the differentiation process of EBs. Examination of the expression of *Stella* gene, as a germ cell marker, showed that in the experimental group the mean normalized expression of this gene was higher than that in the control group, at the whole time of the EBs differentiation progress. Expression of *Mvh* gene was observed just in the experimental group, with the highest amount at day 6th. Oocyte-specific markers ZP1, *Figα* and *GDF9* were expressed just in the experimental group, and they had no expression in the control group. The highest expression of *Figα* and *GDF9* happened at the end stage of the induction protocol (12th day) with the mean normalized expressions of 2.87 and 2.89 respectively. The highest expression of ZP1 happened at the mid-point of differentiation induction period (6th day) with the mean normalized expression of 3.21.

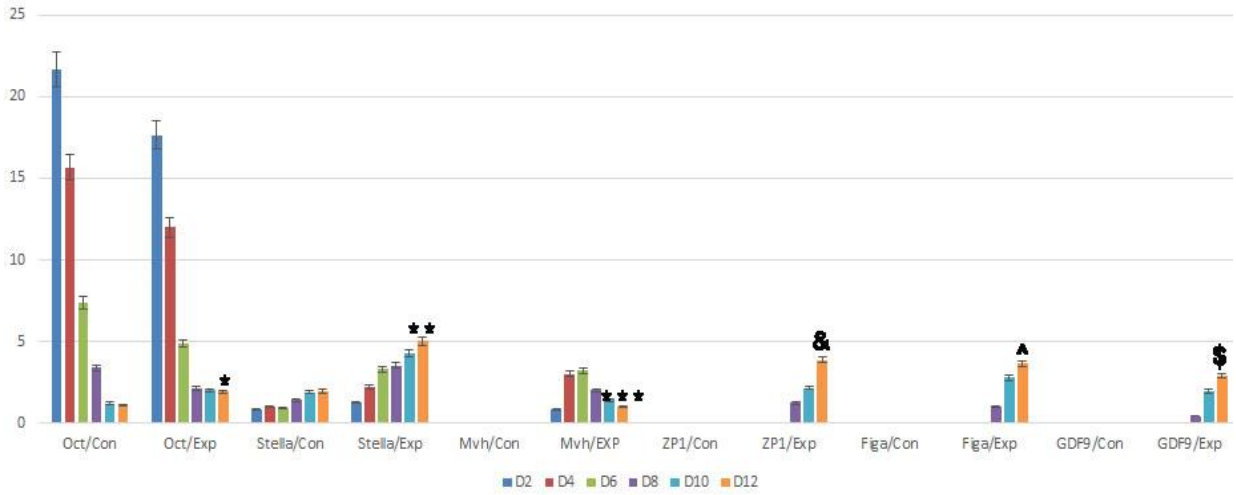

Figure 5. Mean normalized expressions of RT-PCR analysis for Oct4, Stella, Mvh, ZP1, Figα and GDF9 in the experimental (Exp) and control (Con) groups

The significance differences in D12 have been shown by different signs.

Discussion

Results of our study revealed a new promising approach for differentiating ESCs into oocyte-like cells that depends on culturing EBs in granulosa cell conditioned medium and uterine condition medium. After 12-day of induction protocol (first 7 days in IVM with granulosa cells and 5 following days in IVM with uterine conditioned medium), co-cultured cells expressed oocyte-specific markers (ZP1, *Figα* and *GDF9*), while in the control group that EBs were cultured alone no oocyte-specific marker was expressed. Our induction protocol was similar to the *in-vivo* condition because in both of these conditions, at first stage PCGs are generated from blastocyst and then PGCs are transformed into oocyte. We improved our induction protocol by either co-culturing following 5 days in uterine condition medium. In our study, viability rate in the experimental group was higher compared to the control group which can be caused by cell to cell contact and anti-apoptoic effects of granulosa cells.

Co-culturing of EBs with our mentioned protocol increased germ cell markers (*Stella* and *Mvh*) compared to the control group that EBs were cultured alone. *Mvh* expression was observed just in the experimental group while in the control group *Mvh* was not expressed. In our study, expression of *Oct4*, as a pluripotency marker, downregulated in both control and experimental groups. However, downregulation of *Oct4* in the experimental group was more than the control group, which indicated the higher differentiation in the experimental group following induction protocol. Our immunocytochemistry results confirmed the gene expression of the two markers, *GDF9* and *Mvh*. Expression of *Mvh* protein was observed in the experimental group, particularly at the beginning stage of induction protocol. While, expression of GDF9 protein was occurred at the end stage of induction protocol. *Mvh* positive cells are the indicator of germ cell colonies, while *GDF9* positive cells are the indicator of oocyte differentiation and

confirme the successful differentiation of EBs into oocyte-like cells.

Our study is compatible with previous studies that reported the valuable role of granulosa cells in germ cell development and oocyte differentiation (11, 19-22). The precious role of granulosa cell for oocyte differentiation is introduced by increasing cell to cell contact, providing essential ingredients, secreting paracrine signals and decreasing apoptosis (23-25). Qing *et al.* (2007) reported that after 10 days of co-culturing EBs with ovarian granulosa cells, germ cell markers (Mvh and SCP3) and oocyte-specific genes (Figa, GDF-9, and ZP1-3) were expressed in their experimental group and indicated that granulosa cells were effective in inducing the differentiation of ESCs into oocyte-like cells (12). Also, Parvari *et al.* (2016) demonstrated that co-culturing of ovarian stem cells with granulosa cells induced differentiation into oocyte-like structure (9). In another study, the roles of two regulatory proteins (DAZL and BOULE) which exit human embryonic stem cells from pluripotency and enter into meiosis and differentiate into ovarian follicle-like cells were introduced (26-30). Also, the positive role of Retinoic acid in enhancing germ cells differentiation from human embryonic stem cells has been reported (31-35). In another study, ESCs were pre-treated with BMP4 and then exposed to retinoic acid, which revealed upregulation in the expression of germ cell marker and oocyte maturation markers (5, 34-36).

We conclude that the use of 12-day induction protocol (first 7 days in IVM with granulosa cells and 5 following days in IVM with uterine conditioned medium) can support differentiation of mouse ESCs cells into oocyte-like cells. Viability rate of cells and gene expression of germ cell marker and oocyte-specific marker confirmed the efficiency of our induction protocol. But, still there is a need for further studies to access successful differentiation protocol for generating functional oocytes *in vitro*.

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