Iranian Journal of Basic Medical Sciences

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A vector-based system for the differentiation of mouse embryonic stem cells toward germ-line cells

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Sep 30,2013
Accepted: Dec 10, 2013

Keywords:
Differentiation
Germ-line cells
Mouse embryonic stem cell
Vector-based system

ABSTRACT

Objective(s): To culture the *in vitro* mouse embryonic stem cells (mESCs) and to direct their differentiation to germ-line cells; in present study we used a vector backbone containing the fusion construct Stra8-EGFP to select differentiated ES cells that entered meiosis. Retinoic acid was used to differentiate embryonic stem cells to germ cells.
Materials and Methods: A fragment of Stra8 gene promoter (-1400 to +7) was inserted in ScaI/HindIII multiple cloning site of pEGFP-1 vector. The electroporation was done on embryonic stem cells and positive colonies were selected as puromycin-resistant after three weeks of treatment with puromycin. All-trans retinoic acid (RA) was used for differentiation of mESCs at final concentration of 10^{-5}M. The expression of protamine 1 (Prm1) gene was checked as post meiotic marker in differentiated mESCs after 5, 10, 15, 21 and 30 days after RA induction.
Results: The PCR amplification by specific primers for Stra8-EGFP fusion gene was detected in DNA sample from mESCs after electroporation and puromycin treatment. GFP-positive mESC colonies were observed after 72 hr RA induction. The protamine 1 gene was expressed after 21 days of RA induction.
Conclusion: In this study, we demonstrated the *in vitro* generation of mouse embryonic stem cells to germ cells by using a backbone vector containing the fusion gene Stra8-EGFP. The Stra8 gene is a retinoic acid-responsive protein and is able to regulate meiotic initiation.

► *Please cite this paper as:*
Ebrahimzadeh-Vesal R, Shokrgozar MA, Nayernia K, Teimoori-Toolabi L, Miryounesi M, Nourashrafeddin S, Ranji N, Modarressi MH. A vector-based system for the differentiation of mouse embryonic stem cells toward germ-line cells. Iran J Basic Med Sci 2014; 17:566-570.

Introduction

Embryonic stem cells (ESCs) are pluripotent cells which have many characteristics such as self-renewal and ability to differentiate into all of the specific cell types, including germ line (1). These cells were isolated from the inner cell mass of preimplantation blastocyst (2-3). Injection of mouse embryonic stem cells to blastocyst of mouse embryo could generate chimeric mouse in which foreign ESCc are contributed to production of wide variety of somatic tissues (1).

Mouse embryonic stem cells are useful model to study stem cell fate and differentiation in laboratory. By using murine embryonic fibroblasts (MEF) as feeder layer and leukemia inhibitory factor (LIF) as supplementary factor in culture medium, mouse ES cells could be kept in undifferentiated state with high proliferation rate. (4).

Recent finding in stem cell researches have raised the possibility of *in vitro* germ cells production from a population of embryonic stem cells. *In vitro*-derived germ cells may be used as a choice of treatment of some cause of male infertility. Progression through the meiotic process is still a challenge in the *in vitro* generation of gametes. Germ cells produce gametes and are the only cells that can undergo meiosis as well as mitosis. Formation of the male gametes consists of sequential mitotic, meiotic, and post-meiotic cell divisions. Germ cells are critical cells for any species that multiplies through sexual reproduction. In the present study, the male mouse Embryonic Stem Cell line C57BL6/J with normal karyotype 46, XY, which was harboring a vector backbone containing the fusion construct Stra8-EGFP was cultured to select differentiated ES cells. Those were successfully imported to meiosis using RA.

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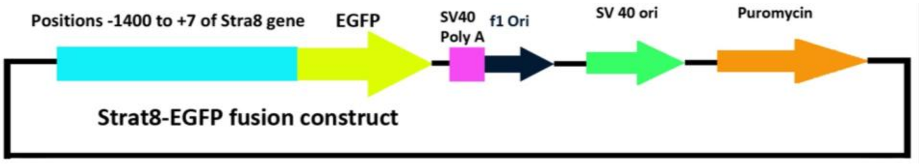

Figure 1. The schematic representation of the vector map and fusion construct used in this study. The pEGFP-1 is a promoterless EGFP vector which can be used to monitor transcription from different promoter sequences inserted into MCS located upstream of the EGFP coding sequence. A fragment including positions -1400 to +7 of Stra8 gene was inserted in multiple cloning site of vector backbone. This vector also contains puromycin resistance gene

Materials and Methods

The structure of the fusion gene constructs

We used a fusion construct that consists of the promoter sequence of Stra8 and coding regions of enhanced green fluorescent protein (EGFP). The Stra8 (stimulated by retinoic acid 8) gene, is a protein-coding gene that has been shown to be involved in the regulation of meiotic initiation in both spermatogenesis and oogenesis and expressed in spermatogonia (5). Previous findings demonstrated that 1.4 kb of the 5' flanking region of the Stra8 gene caused specific expression of GFP in premeiotic germ cells (6).

A fragment of promoter region of mouse Stra8 gene (-1400/+7) was inserted in the ScaI/HindIII multiple cloning site of pEGFP-1 vector (catalog 6086-1, Clontech, USA). This vector backbone also contains a puromycin resistance cassette to allow selection of transfected eukaryotic cells (Figure 1).

Electroporation into embryonic stem cells

After cloning and performing miniprep, the vector was linearized with NotI restriction enzyme and about 50 µg of this vector was used for electroporation. Briefly, mouse embryonic stem cells C57BL6/J were cultured in an undifferentiated state on mitomycin C-inactivated mouse embryonic fibroblast (MEF) as feeder layer as described previously (7). Approximately 8×10^6 ESCs was trypsinized and was prepared to undergo electroporation. Electroporation was performed with Bio-Rad Gene Pulser™ at conditions of 250 V and 500 µF (8). After 48 hr mESCs were treated with puromycin at final concentration of 1 µg/ml for three weeks. Puromycin-resistant colonies were selected. DNA from these colonies was extracted and tested for presence of Stra8/EGFP vector by PCR.

Differentiation of mouse embryonic stem cells and FACS

The mESC were cultured on 0.1% gelatin-coated culture dish (Sigma) in the absence of leukemia inhibitory factor (LIF). Differentiation was induced by retinoic acid (RA, Sigma-Aldrich). RA was added to the medium at a final concentration of 10^{-5} M for 72 hr. GFP-positive cells were selected using fluorescence-activated cell sorter (FACS). Cell sorting was carried out with about 6×10^6 cells of mouse embryonic stem cell by using FACS Aria flow cytometric cell sorter (FACSAria, BD Biosciences). GFP-positive cells were cultured on 0.1% gelatin-coated culture dish in the presence of RA in final concentration of 10^{-5} M for 30 days to continue differentiation.

Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted from differentiated mESC after 5, 15 and 30 days of RA induction by using TriPure RNA isolation reagent (Roche) according to the manufacturer's instructions. Then about 1µg of total RNA was subjected for cDNA synthesis using MMLV reverse transcriptase and random hexamers (RevertAid ™ First Strand cDNA Synthesis Kit, Fermentas). RT-PCR was performed using specific primers during different stages of meiotic differentiation. Also, phosphoglucomutase-1 (Pgm1) was used as the housekeeping gene to check the quality and the amplification reaction of cDNAs. The RT-PCR was performed in 95˚C for 3 min followed by 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 20 sec, and final extension at 72˚C for 10 min in a final reaction volume of 25 µl. The sequences of primers used in this study are listed in Table 1.

Table 1. List of primer sequences used in this study

Target gene	Primer	Sequence	Product size (bp)
Prm1	Forward	CTCACAGGTTGGCTGGCTCGAC	195
	Reverse	CGGCGACGGCAGCATCTTCG	
Pgm 1	Forward	GCTTCGATGCGAGAGCTCAC	190
	Reverse	TGCGACACGGTGTACGGCAC	
Stra8-EGFP	Forward	AGT TGAGCTCTGGAAACCCACAACGAAAGG	320
	Reverse	GGTGGTGCAGATGAACTTCAG	
Oct4	Forward	CTGAAGCAGAAGHAGGATCACC	180
	Reverse	TCGAACCACATCCTTCTCTAGCC	
Dazl	Forward	CAGGCATATCCTCCTTATCCAAG	263
	Reverse	TGTATGCTTCGGTCCACAGAC	
Sycp3	Forward	CCGGAGCCGCTGAGCAAACA	436
	Reverse	CCAGTTCCCACTGCTGCAACAC	

Prm1; Protamine 1, Pgm 1; Phosphoglucomutase-1, EGFP; Enhanced green fluorescent protein, Dazl; Deleted in azoospermia-like, Oct4; Octamer-binding protein 4, Sycp3; Synaptonemal complex protein 3

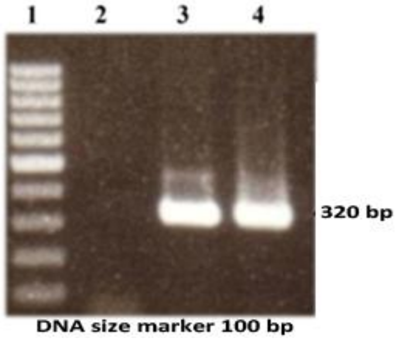

Figure 2. The PCR products for Stra8-EGFP fusion gene were electrophoresed on 2% agarose gel. The specific 320 bp product size was observed. (lane1) DNA size marker 100 bp, (lane 2) Negative control sample without template, (lane 3) DNA from vector as positive control sample, (lane 4) DNA from mouse embryonic stem cells

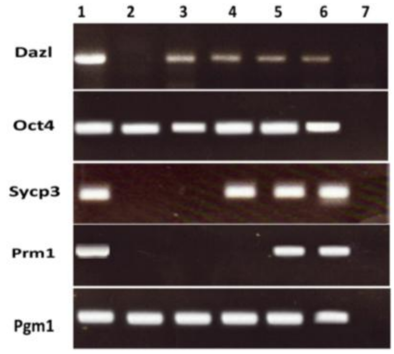

Figure 4. Expression marker genes in different stages of differentiation of mouse embryonic stem cell C57BL6/J. (lane1) Mature mouse testis tissue was used as positive control sample, (lane 2) Undifferentiated mESC C57BL6/J in absence of retinoic acid induction, (lane 3) Differentiated mESC after 5-days, (lane 4) after 15-days, (lane 5) after 21- days, (lane 6) and after 30-days of retinoic acid induction, (lane7) negative control sample without template. Expression of Pgm1 was used as housekeeping gene. Expression of Dazl and Oct-4 as pre-meiotic gene markers were seen in samples of RNA extracted at days 5, 15, 21 and 30. Also, the expression of Oct-4 was positive in undifferentiated mESCs. Expression of meiotic marker Sycp3 was positive in samples of RNA extracted at days 15, 21 and 30. Post-meiotic marker Prm1 expressed in RNA samples extracted at days 21 and 30

Results

The presence of the construct Stra8-EGFP was checked by PCR. The PCR reaction was done by specific forward and reverse primers for Stra8-EGFP fusion gene on extracted DNA from puromycin-resistant colonies after electroporation and three weeks of puromycin treatment. The amplified PCR products were electrophoresed on 2% agarose gel and the specific 320 bp product was visualized after ethidium bromide staining (Figure 2).

Mouse embryonic stem cells harboring the fusion construct Stra8-EGFP were cultured on 0.1% gelatin-coated culture dish under RA induction for 72 hr in concentration 10^{-5}M. The expression of GFP in cells was checked under a fluorescent microscope (Olympus IX53 USA) and GFP-positive colonies were observed. Then these cells were subjected to cell sorting and purified GFP-positive cells were obtained (Figure 3).

The purified GFP-positive mESC colonies after cell sorting were cultured on 0.1% gelatin-coated dish under RA induction for 30 days in concentration of 10^{-5}M. The expression of mouse Prm1 gene as the post-meiotic marker was checked in RNA samples sorted embryonic stem cells after 5 ,10, 21 and 30 days of RA induction by RT-PCR. The expression of Prm1 gene was observed in the samples of 21 and 30 days after RA induction (Figure 4).

Discussion

Based on these findings we used cloning and genetic manipulation of embryonic stem cells to generate germ cells. We used a vector backbone containing the fusion construct Stra8-EGFP to direct and select imported cells to meiosis and also retinoic acid to differentiate embryonic stem cells to germ line. *In vitro* generated germ cells may serve as a tool in elucidating the molecular mechanisms of spermatogenesis and expression analysis of germ cell-specific gene transcripts.

Embryonic stem cells are considered pluripotent which are derived from the inner cell mass of blastocyst with the ability for self-renewal and differentiation into endoderm, ectoderm and mesoderm and also specialized cells such as germ cells (2, 9-12). Some progress in isolation, culture and differentiation embryonic stem cells on mitomycin C-inactivated mouse embryonic fibroblast (MEF) or gelatin-coated culture dish, open new insight to stem cell research technology (13, 14).

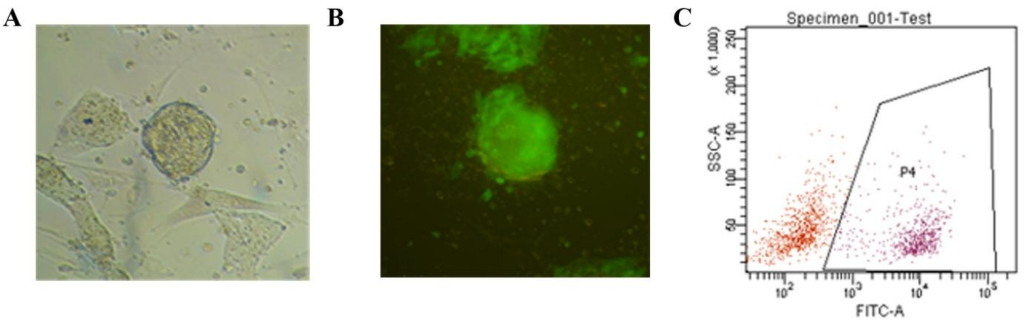

Figure 3. Mouse embryonic stem cell C57BL6/J after 72 hr RA treatment was checked under a fluorescent microscope and GFP-positive mESCs were seen. (A) Bright field image, (B) Fluorescent image 400 × magnifications, (C) GFP-positive embryonic stem cells were subjected to cell sorting by FACS Aria flow cytometric cell sorter (FACSAria, BD Biosciences)

Culturing of ESCs in undifferentiated state followed by its ability to differentiate into the germ cells simply by retinoic acid can be a powerful technique to investigate the molecular and cellular processes involved in spermatogenesis process. Progression through the meiotic process is still a challenge in the *in vitro* differentiation of gametes. Previous studies have shown the production of germ cells from mouse and human embryonic stem cells (11, 12, 15, 16).

All-trans retinoic acid is a vitamin A metabolite which is required for embryonic development. The rapid expression of germ-specific genes have been seen in RA treated ESCs (5, 6,14,15). That is why; RA is the most commonly used agent during *in vitro* germ cell generation from ESCs. All-trans retinoic acid provides instructive signals for the commitment of the germ cell lineage from ESCs (4). Specifically, the role of RA has been identified in the differentiation of embryonic stem cells to germ cells in culture medium and the induction of Stra8 expression in premeiotic germ cells (17). The precise molecular functioning mechanisms of RA on germ cell commitment is unknown but there is some evidence of the activity RA on bone morphogenetic Protein (18).

Several retinoic acid responsive genes in the mouse genome have been identified which are collectively called Stra genes (19). Stra8 gene knockout mice have deficiency in meiotic initiation and progression (20). This gene is specifically expressed in mammalian germ cells before transition of mitosis into meiosis cell division (21). In mouse, Stra8 is required for the transition into meiosis in both female and male germ cells and Stra-8 deficient mice are infertile (22).

Control of differentiation embryonic stem cells under appropriate culture conditions for generation of a broad spectrum of cell lineages can cause their possible use in future therapies and uncover molecular mechanisms controlling cell lineage determination. Successful *in vitro* differentiation of ESCs into functional sperm cells appears to have an extremely attractive potential for the treatment of human male infertility, particularly caused by spermatogenic arrest.

Conclusion

The ability to isolate and culture embryonic stem cell under *in vitro* conditions in this era is possible. The genetic manipulation and control of differentiation of embryonic stem cell to various cell lineages is a powerful tool to elucidate molecular mechanisms involved in differentiation and cell fate determination. The stem cell culture and differentiation can influence biomedical research and in the future open a route for development of new medical treatments for human diseases.

Acknowledgment

We would like to thank The Pasteur Institute of Iran especially National Cell Bank College and the department of flow cytometry at Royan Institute of Iran. This work was supported by a grant by Tehran University of Medical Science (TUMS), grant no. 20517. The results described in this paper were part of student thesis. The authors have no conflicts of interest.

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