Original Article

Evaluation of *in vitro* Spermatogenesis System Effectiveness to Study Genes Behavior: Monitoring the Expression of the Testis Specific 10 (Tsga10) Gene as a Model

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Abstract

Background: *In vitro* generation of germ cells introduces a novel approach to male infertility and provides an effective system in gene tracking studies, however many aspects of this process have remained unclear. We aimed to promote mouse embryonic stem cells (mESC) differentiation into germ cells and evaluate its effectiveness with tracking the expression of the *Tsga10* during this process.

Methods: mESCs were differentiated into germ cells in the presence of Retinoic Acid. Based on developmental schedule of the postnatal testis, samples were taken on the 7th, 12th, and 25th days of the culture and were subjected to expression analysis of a panel of germ cell specific genes. Expression of *Tsga10* in RNA and protein levels was then analyzed.

Results: Transition from mitosis to meiosis occurred between 7th and 12th days of mESC culture and post-meiotic gene expression did not occur until the 25th day of the culture. Results showed low level of *Tsga10*expression in undifferentiated stem cells. During transition from meiotic to post-meiotic phase, *Tsga10* expression increased in 6.6 folds. This finding is in concordance with *in vivo* changes during transition from pre-pubertal to pubertal stage. Localization of processed and unprocessed forms of the related protein was similar to those *in vivo* as well.

Conclusions: Expression pattern of *Tsga10*, as a gene with critical function in spermatogenesis, is similar during *in vitro* and *in vivo* germ cell generation. The results suggest that *in vitro* derived germ cells could be a trusted model to study genes behavior during spermatogenesis.

Keywords: *Tsga10*; embryonic stem cells; differentiation; *in vitro* germ cells generation

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Introduction

About 15% of couples have fertility problems and male factor infertility accounts for half of the cases.[1] Numerous pathologic conditions are involved in infertility problems.[2] To elucidate the causes of male infertility, it is important to know about the mechanisms of germ cell specification, development and differentiation. Due to lack of a robust *in vitro* culture system for differentiation of germ line progenitor cells into mature gametes, enough knowledge in this area is not available.

Recent studies have shown that embryonic stem cells (ESC) can give rise to primordial germ cells (PGCs) and sperm-like cells *in vitro*.[3] This ability provides an *in vitro* model to analyze the biology of germ cells development and new therapeutic approaches in reproductive medicine.[4]

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Consequently we tried to create an *in vitro* spermatogenesis system and monitor a testis specific gene, *Tsga10*, in the process of stem cells differentiation, and compare it with *in vivo* system. This gene has been studied in our team in several research projects and adequate information about its *in vivo* characteristics has been obtained.

The *Tsga10* is mainly expressed in the testis tissue. It has been shown that *Tsga10* has a critical role in spermatogenesis and its protein contributes to the fibrous sheath of sperm tail.[5] In addition *Tsga10* is expressed in actively dividing cells and fetal tissues.[6] This gene is a member of cancer testis (CT)[7,8] genes family. Its aberrant expression is reported in acute lymphoblastic leukemia and some primary cancers.[9] *Tsga10* mRNA is translated into a 65-kilodaltone protein. During spermatogenesis, it is processed to a 27-kilodalton N-terminal segment. This portion is localized to the fibrous sheath of mature spermatozoa.[10]

In this study, for the first time the alterations in *Tsga10* expression during *in vitro* differentiation of embryonic stem cells to germ cells was analyzed.

To confirm the different developmental stages of the differentiated cells, the expression panel of 6 stage specific genes, *Stra8*, *Sycp3, Dazl*, *Prm1*, *Spata19*, and *Plcz* in the development process was studied.

Retinoic Acid (RA) is now confirmed to be a key signaling molecule in meiosis induction.[11] RA induces a significant increase in the expression of *Stra8* (a pre-meiotic gene) and *Sycp3* (a meiotic

gene) in cultures of human fetal testis.[12] According to gene knockout studies, *Stra8* is believed to be the gatekeeper of the mitotic/meiotic switch.[13] *Dazl* is a testis-specific gene with a critical role in spermatogenesis specifically in meiosis initiation.[14,15] *Sycp3* is a meiotic specific gene responsible for the formation of the synaptonemal complex. This complex has an important role in synapse formation, recombination and segregation of chromosomes during meiosis.[16] *Sycp3* is positively regulated by Dazl through binding to 3′-UTR of its transcript. Protamine is coded by *Prm1* which is a post-meiotic gene with specific expression in haploid round spermatids. During development of mature spermatozoa histones are replaced by protamines.[17,18] *Spata19* is a testis specific gene that is expressed specifically in round spermatids.[19] Finally, *Plcz* is the physiological agent of oocyte activationand its protein causes Ca^{2+} osciliation in oocyte similar to those seen in fertilization. It was shown that *Plcz* mRNA is first detectable in spermatids.[20]

Materials and Methods

Cell culture

Mouse embryonic stem cell line C57BL6 with normal male (XY) karyotype (Invitrogen) was cultured in an undifferentiated state on a feeder layer of mitomycin-C inactivated mouse embryonic fibroblast.[21,22] Culture medium composed of Knockout Dulbecco's modified Eagle's medium (Knockout DMEM, GIBCO-BRL) supplemented with 12.5% (v/v) ES qualified FBS (GIBCO-BRL), 2mmol L-Glutamine (GIBCO-BRL), 1X nonessential amino acids (NEAA; GIBCO-BRL), 50 $\mu g ml^{-1}$ penicillin and streptomycin, as well as 50 µmol β-mercaptoethanol and 10^3 unit ml^{-1} LIF (Milipore).

Construction of germ cell specific reporter gene and recombinant mESCs

According to our previous published data, a segment of *Stra8* gene promoter (-1400/+7) was amplified from genomic DNA and inserted in the SacI/HindIII site of modified pEGFP-1 vector where neomycin-resistance cassette was replaced with puromycin-resistance gene (Figure 2d).[23] ES cells were trypsinized and approximately 7×10^6 cells were prepared for performing electroporation. Linearized vector (35 µg) was electroporated into ES cells. Electroporation was performed on Bio-Rad GenePulser device at 250 V and 500 µF. After electroporation, mESCs were transferred to a 6-well plate and cultured 72 hours without antibiotic. Then puromycin (final concentration 1 $\mu g\ ml^{-1}$) was added to medium and cells were cultured in the presence of puromycin for three weeks. Puromycin resistant colonies were selected. DNA from these colonies was extracted and tested for the presence of *Stra8*/EGFP vector by PCR and positive colonies were grown in complete medium with LIF for one month.[24]

Derivation of germ cells from mESCs

RA (Sigma) was added to the medium at a final concentration of 10^{-5} mol for 72 hours to induce differentiation. GFP-positive cells were selected using fluorescence-activated cell sorting (FACS). Cells were trypsinized and after pipetting up and down several times filtered through a 40 µm strainer (Falcon) to create single-cell suspensions. Sorting was carried out with 3×10^6 cells' using BD FACS Aria II flow cytometer and undifferentiated ESCs were used as a negative control.[23] These cells were cultured on a mouse embryonic fibroblasts (MEF) feeder under non-induced condition for two weeks. Resulting colonies were cultured for 25 days on gelatin with RA treatment (final concentration 10^{-8} mol).

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured with Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific). The extracted RNA (2 µg) was used for cDNA synthesis using M-MuLV reverse transcriptase (Fermentase) with random hexamer and oligo dT primer together. RT-PCR was performed using specific primer for *EGFP*, *Oct4, Stra8, Sycp3, Dazl, Prm1, Plcz*, and *Spata19* genes (Table 1).

Real time RT-PCR

Real time RT-PCR was carried out using specific primer for *Tsga10* gene with StepOne Real time PCR system (Applied Biosystems). Typically, a 20 µL reaction contained 10µL 2 × SYBR® Green PCR Master Mix (Applied Biosystems), 1 µL (final 500nM) forward and reverse primer, 1 µL of cDNA and 8 µL distilled water. Expression analysis of *Tsga10* gene and a housekeeping gene, *Tbp* (Tata Binding Protein) were performed using StepOne Software v2.1 (Applied Biosystems).

Western blot analysis

Equal amounts of cell lysate proteins of selected samples (adult testis and differentiated ES) were separated using 10% SDS-PAGE and transferred to a Polyvinylidene fluoride (PVDF) membrane (Milipore). The membrane free binding sites were blocked by treating the membrane with 5% (wt/ vol) skim milk for one hour at room temperature. After overnight incubation at 4°C with primary *Tsga10* goat polyclonal antibody (Santa Cruz), blots were washed 3 times with PBST. After incubation with the appropriate horseradish peroxidase-labeled secondary antibody (anti goat, Santa Cruz), blots were washed again as mentioned previously. Bands were detected using enhanced chemiluminescence (ECL).

Immunohistochemistry of TSGA10 in seminiferous tubules

Sections from testes (5-µm) fixed in Bouin's and embedded in paraffin, were deparaffinized in xylene and hydrated through a graded series of ethanol solutions. During hydration the sections were treated to abolish the endogenous peroxidase activity, neutralize residual picric acid and block free aldehyde groups. Once hydrated, the sections were blocked with 10% goat serum in TBS for 15 minutes, incubated with primary antibodies (polyclonal anti whole TSGA10 protein and polyclonal anti N-terminal) overnight at 4ºC, or at 21ºC for 2 hr, washed in TBS containing 0.1% Tween-20 (4 × 5 min.), blocked in 10% NGS in TBS and incubated with anti-goat IgG conjugated to peroxidase (Santa Cruz) in TBS (1:250) for 1 hr. After an extensive washing in TBS containing 0.1% Tween-20, peroxidase reactivity of the sections was tested by incubating the sections with 0.03% hydrogen peroxide and 0.05% diaminobenzidene tetrahydrochloride (DAB) in TBS containing 0.1 M imidazole, pH 7.6 for 10 min. The sections were then washed with double distilled water, counterstained with 0.1% filtered methylene blue, and immersed in tap water for 5 min. Following stain differentiation the sections were dehydrated, cleared in xylene and mounted with coverslips using Permount.[25]

Results

Establishment of spermatogonial stem cell (SSC)-like cells

Table 1. Primer sequence and expected size of PCR products of the *Oct4, Stra8, EGFP, Dazl, Sycp3, Spata19, prm1, Plcz*, and *Tsga10* genes.

Gene	Primer Sequence	Product size (bp)
Oct4	F: CTGAAGCAGAAGHAGGATCACC R: TCGAACCACATCCTTCTCTAGCC	345
Stra8	F : ACAACCTAAGGAAGGCAGTTTAC R: TGACCTCCTCTAAGCTGTTGG	174
EGFP	F: GCACCATCTTCTTCAAGGACGAC R: TCTTTGCTCAGGGCGGACTG	270
Dazl	F: CAGGCATATCCTCCTTATCCAAG R: TGTATGCTTCGGTCCACAGAC	263
Sycp3	F: CCGGAGCCGCTGAGCAAACA R: CCAGTTCCCACTGCTGCAACAC	430
Prm1	F: CTCACAGGTTGGCTGGCTCGAC R: CGGCGACGGCAGCATCTTCG	192
Spata19	F: CTGAAGATGATCATTACA R: TTAGCATTCTGAGCAAGAAGTC	475
Plcz	F: TGGCCTTATCTGATCTTGT R: GCCACCATCTGACAACCTA	256
Tsga10	F: AGACCTTCCTCCTTTATGAGCA R: AAGGCTACATCTCTCTCCGTC	141

Table 2. Expression pattern of stage specific genes during differentiation of mESCs to germ cells.

	Nanog	*Oct4*	*Stra8*	*Dazl*	*Sycp3*	*Prm1*	*Spata19*	*Plcz*
Un differentiated mESCs	+	+	–	–	–	–	–	–
After 72 h induction with RA	+	+	+	–	–	–	–	–
Day 7	+	+	+	+	–	–	–	–
Day 12	+	+	+	+	+	–	–	–
Day 25	+	+	+	+	+	+	+	+

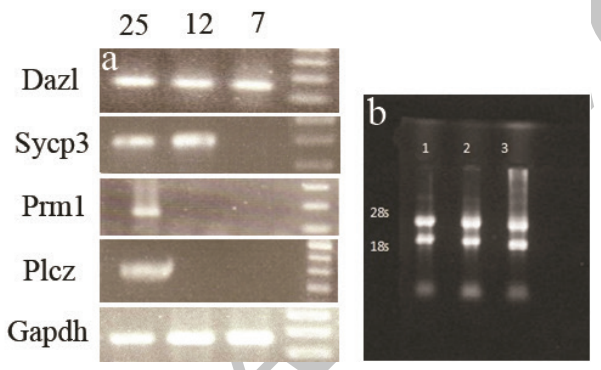

Figure 1. a) RT-PCR analysis of saples (7th ,12th, and 25th days) for meiotic (*Dazl* and *Sycp3*) and post-meiotic (*Prm1* and *Plcz*) genes. **b)** Extracted RNA from samples were analyzed by gel electrophoresis

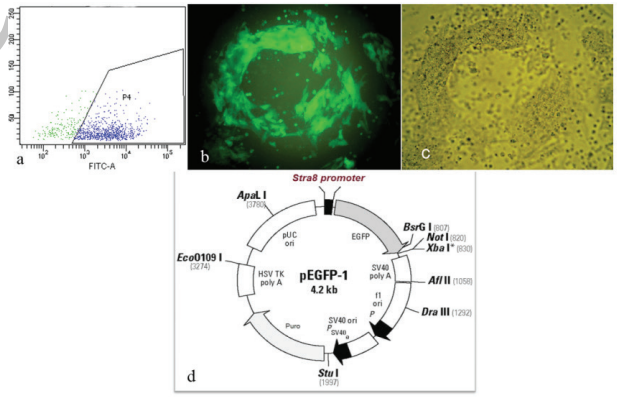

Figure 2. a) Diagram of GFP expressed mESCs sorted by FACS. **b and c)** After culture on MEF feeder, sorted cells showed high expression of GFP. **d)** Modified pEGFP1 vector which *Stra8* promoter was inserted in multiple cloning sites

In order to isolate differentiated stem cells, a specific reporter construct consisting of a germ line-specific segment of *Stra8* gene promoter and the coding region of enhanced green fluorescence protein (EGFP) were used. The use of this 1.4 Kb *Stra8* promoter resulted in the testis-specific expression of GFP. After selection with puromycin, ES colonies were picked up and PCR with GFP specific primers was performed to verify the presence of the construct in the genomic DNA of each colony. Induction with RA (final concentration of 10^{-5} mol) was performed for 72 hours. GFP expression was detected after RA treatment and colonies that were not induced with RA had no GFP shine. Approximately 60% of cells were shown to be GFP-positive by FACS (Figure 2a). These sorted cells were cultured under normal conditions (complete medium plus LIF) for two weeks to increase cell viability (Figures 2b and 2c). Differentiated cells were subjected to RNA extraction (Figure 1b), cDNA synthesis and analyzed for the expression of the six germ cell specific genes. RT-PCRs were performed for *Stra8* and *Oct4* as pre-meiotic genes, *Dazl* and *Sycp3* as meiotic genes and *Prm1* and *Spata19* as post-meiotic genes. *Oct4* was expressed in both induced and non-induced ES cells. *Stra8* showed high expression after 72 hours induction with RA. Expression of *Dazl*, *Sycp3*, *Prm1,* and *Spata19* was not detected in induced and

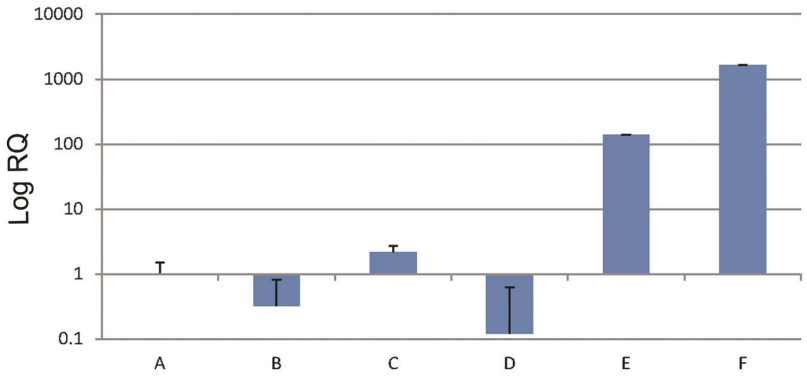

Figure 3. Real time RT-PCR results show elevation of *Tsga10* expression in post meiotic germ cells. **A)** undifferentiated mESCs, **B)** +12 day sample, **C)** +25 day sample, **D)** myocite drived from mESC, **E)** 15-day-old mouse testis, **F)** adult mouse testis

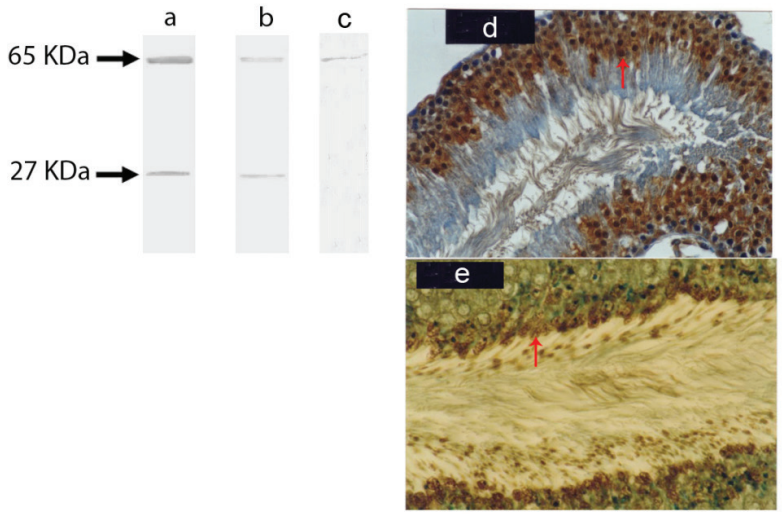

Figure 4. Analysis of TSGA10 protein, **a)** Western blot analysis showed a prominent 65-kilodalton and 27-kilodalton band in sample of adult Mouse testis. **b)** These bands in Post meiotic germ cells drived from mESC. **C)** 65-kilodalton band in undifferentiated mESC. **d)** Immunohistochemistry of Seminiferous epithelium (SE) in stage III-IV of the cycle of the SE showing intense immunostaining in the cytoplasm of round spermatids (arrow) and more moderate immunostaining in pachytene spermatocytes. Note the immunreactive tails of elongated spermatids in the lumen. **e)** SE in stage VII. Immunostaining is found in the cytoplasm of elongated spermatids (arrow) ready to be extruded into the lumen. Note the cytoplasmic droplet and principal piece of the tail are immunoreactive.

non-induced cells. *Stra8* is a molecular marker for spermatogonial stem cells[26] so it was revealed that induced cells were spermatogonial stem cell (SSC)-like cells.

Differentiation of SSC-like cells to germ cells

SSC-like cells were cultured in medium supplemented with RA (10^{-8} mol) and mRNA of these cells was extracted on 7th, 12th, and 25th days after initiation of treatment with RA. These samples were analyzed for pre-meiotic (*Oct4* and *Stra8*), meiotic (*Sycp3* and *Dazl*), and post-meiotic (*Prm1* and *Spata19*) genes expression. On the 7th day of cell culture *Oct4, Stra8*, and *Dazl* genes were expressed, but expression of *Sycp3* was initiated on the 12th day of culture. Expression of *Prm1, Plcz,* and *Spata19* did not start until the 25th day of culture (Figure 1a).

Tsga10 was up-regulated during production of germ cells

Expression analysis of *Tsga10* was performed by Real-Time RT-PCR. The melting-curves of *Tsga10* and *Tbp* products showed a single peak and agarose gel electrophoresis confirmed the results. Expression profile of *Tsga10* in selected samples on 12th and 25th days after initiation of RA treatment was investigated. Undifferentiated mESC showed low expression of *Tsga10* which was assigned to 1 for data analysis by StepOne Software. Results showed that during early phase of mESC differentiation to germ cells (12th day), relative *Tsga10* expression value dropped to 0.32. The relative expression of *Tsga10* was increased 2.2 fold in contrast to undifferentiated mESC after expression of post meiotic genes (25th day). As a control the expression of *Tsga10* decreased to 0.12 in the mESC differentiated into myocyte (details of the differentiation method not shown). *In vivo* analysis showed that the relative expression of *Tsga10* in 15-day-old mouse testis was 140. In adult mouse the relative expression of *Tsga10* was 1650 (Figure 3).

In western blot analysis, polyclonal TSGA10 antibody was used to identify the presence of TSGA10 protein. A prominent 65-kilodalton and a pale 27-kilodalton band for TSGA10 protein

were detected in the testis sample (Figure 4a). We detected the same bands in the post-meiotic germ cells derived from mESC (Figure 4b) and a pale 65-kilodalton band in undifferentiated mESC (Figure 4c). To examine localization of TSGA10 protein in adult mouse testis, we performed immunohistochemistry. Only seminiferous tubules were immunostained. The results showed that full length TSGA10 (Figure 4d) is intensely expressed in round spermatids (or early in spermiogenesis) and some cells appeared to be the early pachytene spermatocytes. The N-terminal portion of TSGA10 (Figure 4e) is strongly expressed in elongated spermatids, which are ready to detach from the seminiferous epithelium.

Discussion

In vitro differentiation of ESC provides a model for analyzing of developmental process. In the present study, we have developed a step-by-step differentiation model for analyzing stage specific gene expression.

During the transition of mammalian germ cells from mitosis to meiosis, *Stra8* gene is expressed.[25] A vector composed of promoter region of *Stra8* gene, and GFP coding sequence has been used in this study to select stable transfected ES cells that entered meiosis. We observed that if we did not separate GFP-positive cells, which entered the meiosis stage in the cell culture, differentiation process could be suppressed by neighboring undifferentiated cell populations. This finding has also been observed previously[17] and that is the reason for separation of cells using FACS.

In the mouse testis, the postnatal spermatogenesis development can be divided into three phases: proliferation of SSCs (days 1–7), initiation of meiosis and differentiation of SSCs into spermatocytes, and finally the haploid round spermatid generation (days 8–20), spermiogenesis and production of elongated spermatozoa (days 21–36).[28]

The start of *Sycp3* gene expression on the 12th day showed that after this period of time, SSC-like cells have been entered the meiosis. Expression of *Prm1* and *Spata19* genes in 25th day of culture confirmed that these cells are post-meiotic germ cells.

Previous studies showed strong correlation between *Tsga10* expression and spermatogenesis.[5] Full length TSGA10 protein is 65-kilodalton and its 27-kilodalton N-terminal fragment is a major component of fibrous sheath of the sperm tail. It has been shown that *Tsga10* has a chromosome segregation domain expressed in actively dividing cells, such as embryonic tissue and several cancers.[6] For the first time, the expression of *Tsga10* in embryonic stem cells was evaluated in this study. Our results showed that *Tsga10* was expressed in undifferentiated embryonic stem cells at low levels, which could support the role of this gene in a chromatin division during mitosis.

The results showed that expression of *Tsga10* in adult mouse testis was approximately 12 folds greater than in testis of 10- and 15-day-old mouse. This revealed that during the transition of germ cells from meiotic phase (15-day-old post-natal testis) to post-meiotic and mature spermatozoa (adult mouse testis), *Tsga10* expression is increased. In our experiment, expression of *Tsga10* in the 25th day of culture (after post-meiotic genes expression) was 6.6 folds greater than the 12th day of culture (meiotic phase). *In vitro* increase in *Tsga10* expression that occurred during the transition from meiotic phase to post-meiotic phase confirms the role of its protein in post meiotic events. Although both *in vitro* and *in vivo* studies showed up-regulation of *Tsga10* expression, the value of up-regulation was different in them, which may be due to differences in the efficiency of differentiation in testis *vs.* monolayer cell culture.

Reproductive biologists have limited available tools to determine the function of genes involve in germ cell development. These methods are laborious and time consuming.[29] Based on our finding we have suggested that *in vitro* spermatogenesis could be an alternative method for germ cell development studies.

One of the best methods for analyzing the effect of genes in development is generating knockout mice. Building knockout mice is an expensive and time consuming method. In addition, in the field of spermatogenesis, producing knockout mice is more difficult because these knockout animals are usually infertile. An alternative method for knockout animal technology is producing knockout ES cells, in which single or both copies of a testis specific gene is deleted, and differentiated these cells toward germ cells. According to the similarity of *in vitro* and *in vivo* spermatogenesis, the effect of the knockout gene in gametogenesis could be analyzed.

In conclusion, we suggest *in vitro* derived germ cells as appropriate models for analysis of germ cell specific genes expression.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Massart A, Lissens W, Tournaye H, Stouffs K. Genetic causes of spermatogenic failure. *Asian J Androl.* 2012; **14:** 40 – 48.
2. Van Golde RJ, Tuerlings JH, Kremer JA, Braat DD, Schoute F, Hoefsloot LH. DAZLA: an important candidate gene in male subfertility. *J Assist Reprod Genet.* 2001; **18:** 395 – 399.
3. Silva C, Wood JR, Salvador L, Zhang Z, Kostetskii I, Williams CJ, et al. Expression profile of male germ cell-associated genes in mouse embryonic stem cell cultures treated with all-trans retinoic acid and testosterone. *Mol Reprod Dev*. 2009; **76:** 11 – 21.
4. Kerr CL, Cheng L. The dazzle in germ cell differentiation. *J Mol Cell Biol*. 2010; **2:** 26 – 29.
5. Modarressi MH, CameronJ, Taylor KE, Wolfe J. Identification and characterization of a novel gene, TSGA10 expressed in testis. *Gene.* 2001; **26:** 249 – 255.
6. Behnam B, Modarressi MH, Conti V, Taylor KE, Puliti A, Wolfe J. Expression of *Tsga10* sperm tail protein in embryogenesis and neural development: From cilium to cell division. *BBRC.* 2006; **344:** 1102–1110.
7. Yazarloo F, Shirkoohi R, Mobasheri MB, Emami A, Modarressi MH. Expression analysis of four testis-specific genes AURKC, OIP5, PIWIL2 and TAF7L in acute myeloid leukemia: a gender-dependent expression pattern. *Med Oncol*. 2013; **30:** 368.
8. Dianatpour M, Mehdipour P, Nayernia K, Mobasheri MB, Ghafouri-Fard S, Savad S, et al. Expression of testis specific genes TSGA10, TEX101, and ODF3 in breast cancer. *Iran Red Crescent Med J*. 2012; **14:** 722 – 726.
9. Mobasheri MB, Modarressi MH, Shabani M, Asgarian H, Sharifian RA, Vossough P, et al. Expression of the testis-specific gene, TSGA10, in Iranian patients with acute lymphoblastic leukemia (ALL). *Leuk Res.* 2006; **30:** 883 – 889.
10. Modarressi MH, Behnam B, Cheng M, Taylor KE, Wolfe J, van der

Hoorn FA. *Tsga10* encodes a 65-kilodalton protein that is processed to the 27-kilodalton fibrous sheath protein. *Biol Reprod.* 2004; **70:** 608 – 615.
11. Griswold MD, Hogarth CA, Bowles J, Koopman P. Initiating meiosis: the case for retinoic acid. *Biol Reprod.* 2012; **86:** 35.
12. Chen W, Jia W, Wang K, Zhou Q, Leng Y, Duan T, et al. Retinoic acid regulates germ cell differentiation in mouse embryonic stem cells through a Smad-dependent pathway. *BBRC*. 2012; **418:** 571 – 577.
13. Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, et al. *Stra8* and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci USA*. 2008; **105:** 14976 – 14980.
14. Haston KM, Tung JY, Reijo Pera RA. *Dazl* functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells *in vivo* and *in vitro*. *PLoS One*. 2009; **4:** e5654.
15. Lin Y, Gill ME, Koubova J, Page DC. Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. *Science*. 2008; **322:** 1685 – 1687.
16. Mobasheri MB, Jahanzad I, Mohagheghi MA, Aarabi M, Farzan S, Modarressi MH. Expression of two testis-specific genes, TSGA10 and SYCP3, in different cancers regarding to their pathological features. *Cancer Detect Prev*. 2007; **31:** 296 – 302.
17. Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, et al. *In vitro*-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. *Dev Cell*. 2006; **11:** 125 – 132.
18. Peschon JJ, Behringert RR, Brinstert RL, Palmiter RD. Spermatid specific expression of protamine1 in transgenic mice. *Proc Natl Acad Sci USA*.1987; **84:** 5316 – 5319.
19. Ghafouri-Fard S, Ousati Ashtiani Z, Sabah Golian B, Hasheminasab SM, Modarressi MH. Expression of two testis-specific genes, SPATA19 and LEMD1, in prostate cancer. *Arch Med Res*. 2010; **41:** 195 – 200.
20. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update*. 2010; **16:** 690 – 703.
21. Guan K, Wolf F, Becker A, Engel W, Nayernia K, Hasenfuss G. Isolation and cultivation of stem cells from adult mouse testes. *Nat Protoc*. 2009; **4:** 143 – 154.
22. Nourashrafeddin S, Aarabi M, Miryounesi M, Ebrahimzadeh-Vesal R, Zarghami N, Modarressi MH, et al. Expression analysis of PAWP during mouse embryonic stem cell-based spermatogenesis *in vitro*. *In vitro Cell Dev Biol Anim*. 2014 doi10.1007/s11626-013-9722-1.
23. Nayernia K, Li M, Jaroszynski L, Khusainov R, Wulf G, Schwandt I, et al. Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells. *Hum Mol Genet*. 2004; **13:** 1451 – 1460.
24. Miryounesi M, Nayernia K, Dianatpour M, Mansouri F, Modarressi MH. Co-culture of Mouse Embryonic Stem Cells with Sertoli Cells Promote *in vitro* Generation of Germ Cells. *Iran J Basic Med Sci*. 2013; **16:** 27 – 38.
25. Oko RJ, Jando V, Wagner CL, Kistler WS, Hermo LS. Chromatin reorganization in rat spermatids during the disappearance of testis-specific histone, H1t, and the appearance of transition proteins TP1 and TP2. *Biol Reprod.* 1996; **54:** 1141 – 1157.
26. Lee JH, Engel W, Nayernia K. Stem cell protein Piwil2 modulates expression of murine spermatogonial stem cell expressed genes. *Mol Reprod Dev.* 2006; **73:** 173 – 179.
27. Hogarth CA, Mitchell D, Evanoff R, Small C, Griswold M. Identification and expression of potential regulators of the mammalian mitotic-to-meiotic transition. *Biol Reprod*. 2011; **84:** 34 – 42.
28. Malkov M, Fisher Y, Don J. Developmental schedule of the postnatal rat testis determined by flow cytometry. *Biol Reprod*. 1998; **59:** 84 – 92.
29. Childs AJ, Saunders PT, Anderson RA. Modelling germ cell development *in vitro*. *Mol Hum Reprod*. 2008; **14:** 501 – 511.