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Research Article

Ectopic expression of OCT4B1 Decreases Fertility Rate and Changes Sperm Parameters in Transgenic Mice

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Background: The octamer-binding transcription factor-4 (OCT4) is known as an established important regulator of pluripotency, as well as a genetic "master switch" in the self-renewal of embryonic stem and germ cells. *OCT4B1*, one of the three spliced variants of human OCT4, plays crucial roles in the regulation of pluripotency and stemness.

Objectives: The present study developed a transgenic mouse model containing an *OCT4B1*-expressing construct under the transcriptional direction of mouse mammary tumor virus promoter (pMMTV) to evaluate the role of OCT4B1 in the function of male germ cells in terms of fertility potential. Additionally, the effect of ectopic OCT4B1 overexpression on endogenous OCT4 expression was examined in mouse embryonic stem cells (mESCs).

Material and Methods: The *pMMTV-OCT4B1cDNA* construct was injected into the pronuclei of 0.5-day NMRI embryos. Transgenic mice were identified based on the PCR analysis of tail DNA. Further, Diff-Quik staining was applied to assess sperm morphology, while the other sperm parameters were analyzed through a conventional light microscopic evaluation according to World Health Organization (WHO) criteria. The fertility rate was scored by using in vitro frtilization (IVF) method. Furthermore, mESCs was electroporated with the *OCT4B1cDNA*-containing constructs, followed by analyzing through employing semi-quantitative RT-PCR and western blotting.

Results: The results demonstrated the changes in sperm morphology, as well as a statistically significant decrease in the other sperm parameters (count, viability, and motility) and fertility rate ($p<0.05$) in the transgenic mice compared with the control group. The assessment of the cause of the embryonic stem cell (ESC) death following transfection revealed a significant reduction in the endogenous OCT4 expression at both mRNA and protein levels in the transfected mESCs compared to the control ones.

Conclusion: In general, the *in vivo* results suggested a potential role of OCT4B1 in the spermatogenesis process. These results represented that the overexpression of OCT4B1 may induce its role in spermatogenesis and fertility rate by interfering endogenous OCT4 expression. However, further studies are required to clarify the mechanisms underlying OCT4B1 function.

Keywords: OCT4B1, Embryonic Stem Cells, Transgenic Mice, Sperm Parameters, Infertility

1. Background

The OCT4 (Octamer-binding transcription factor 4), a member of POU family, is considered as a key regulator of pluripotency and stemness state in embryonic stem/germ/carcinoma cells (ES, EG and EC, respectively) (1-6). The OCT4 is a genetic "master switch" in the totipotency-pluripotency regulatory network, and therefore it is vital for embryo development and maintaining pluripotency during the life cycle of mammals (7-9). OCT4 expression level is a crucial factor in regulating the balance between differentiation and self-renewal (10, 11). However, its expression is down regulated at gastrulation and finally becomes restricted to primordial germ cell (PGC) lineage, a small population of cells involved in spermatogenesis (12-14), and *OCT4* expression is the most important for survival of PGCs (15). In the male mammals, *OCT4* expression continues in proliferating gonocytes, pro-spermatogonia and in undifferentiated spermatogonia, while in female germ cells, after a repression at meiotic prophase I, it is re-expressed during the growth phase of the oocytes (14, 16-19). These gametes can be fertilized to develop the next generation.

The *OCT4* gene can potentially generate three spliced variants by alternative splicing, designated as *OCT4A*, *OCT4B* and *OCT4B1* (20, 21). The *OCT4B1* variant is highly expressed in human ES and EC cells and similar to *OCT4A*, is rapidly down-regulated upon differentiation. There is a potential relationship between the expression of *OCT4B1* and the pluripotent/undifferentiated state of ES/EC/Cancer Stem cells (21). Therefore, *OCT4B1* could be considered as a putative marker in the regulation of pluripotency and stemness (22).

Although the biological function of this variant is still largely unknown, some studies showed the expression ratio of *OCT4B1*/*OCT4B* transcripts and proteins was significantly elevated under the heat-stress condition (23). In addition, HSP40 family down regulation and enhancement of caspase-3 and caspase-7 activity following *OCT4B1* suppression indicated that *OCT4B1* might be involved in the cell stress response (24, 25). Furthermore, previous studies have indicated that expression of *OCT4B1* has been up-regulated and promoted the tumorigenic process in several cancers including colorectal (26, 27) gastric (28) and bladder cancers (29). In addition, *OCT4B1* as an oncogene, mediates the drug resistance, migration and invasion in colon cancer via decrease in the level of P-gp and ABCG2 and altering in EMT process *in vitro* (26). Moreover, the knockdown of OCT4B1 in three-dimensional spheroids from colorectal cancer decreased the self-renewal of cancer stem cells and reversed EMT through effect on miR-8064/PLK1 balance (30).

Therefore, this isoform may play some roles in tumor initiation and progression (28, 29, 31). On the other hand, the rate of apoptosis has been reported to increase in cancer cells treated with *OCT4B1*-siRNA and can down regulate several *BCL2* transcripts (28, 31, 32). In line withthese studies, *OCT4B1* was reported to extensively express in dental pulp tissues and cells (DPCs) in response to inflammation and suppressed apoptosis of DPCs (33). Therefore, due to its anti-apoptotic properties, it could affect the expression profile of stemness genes, such as *OCT4A*, *SOX2*, *NANOG*, and *KLF4* (32). Furthermore, in germ cell tumors (GCTs), a significant positive correlation between *OCT4A* and OCT4B1 has been shown (33). Although the relationship between OCT4B1 and OCT4A in germ cells, as well as their roles in infertility, remains unknown, accumulating evidence can point to the OCT4B1 function in germ cell development. Moreover, understanding the function of this variant in fertility and embryo development would provide a basis for proper manipulation of ESCs.

2. Objectives

According to the critical role of the OCT4 in spermatogenesis as well as the structural and functional similarities between OCT4 and OCT4B1, the main objective of the present study was to evaluate the possible roles of *OCT4B1* in fertility to resolve some ambiguities of OCT4B1 function. To this end, *OCT4B1* transgenic mice were generated and then fertility rate and sperm parameters were evaluated. Also, we analyzed presumably interference of *OCT4B1* in *OCT4* function in ESCs.

3. Materials and Methods

3.1. Animals and Care

All experiments were performed on NMRI mice, and animals were housed in a standard animal facility, with controlled temperature, photoperiod (12/12 hours of light/dark) and free access to food and water. All procedures and protocols were approved by the ethical committee of National Institute of Genetic Engineering and Biotechnology (NIGEB).

3.2. Construction of pCMV-OCT4B1cDNA-IRES-EGFP and pMMTV-OCT4B1cDNA-IRES-EGFP Vectors

The human OCT4B1-coding sequence (kindly provided by Prof. S.J. Mowla, Tarbiat Modares University, and Tehran, Iran) were amplified by B1-F and B1-R primers (**Table 1**). The PCR product and IRES-EGFP (from pIRES2-EGFP) were cloned into the *Bam*HI/*Not*I (Roche, Germany) sites of pcDNA3. The recombinant pCMV-OCT4B1cDNA-IRES-EGFP was verified by restriction using *Bam*HI/*Not*I (Roche, Germany) and OCT4B1 sequence analysis. For transgenic mice generation, CMV promoter was replaced by MMTV inducing Promoter at *Hinc*II/*Bam*HI sites of pCMV-OCT4B1cDNA-IRES-EGFP. Schematic pCMV-OCT4B1cDNA-IRES-EGFP and pMMTV-OCT4B1cDNA-IRES-EGFP maps are shown in **Figure 1A**.

3.3. Generation of Transgenic Mice

After digestion with suitable restriction enzymes to remove the bacterial elements of pMMTV-OCT4B1cDNA-IRES-EGFP, the construct was diluted to a concentration of 2.5 $\mu g.mL^{-1}$ in injection buffer (Millipore, MR-095-F) and microinjected into pronucleus of 0.5-day fertilized embryos NMRI mice (IM 300 microinjection; Narishige, Tokyo, Japan) and the surviving embryos implanted into pseudopregnant NMRI foster mothers. Then, genomic DNA was isolated from tail samples. Briefly, mouse tail samples (1-3 mm) were digested overnight at 55 °C with proteinase K (0.7 $\mu g.mL^{-1}$) in 200 µL buffer. DNA was extracted with phenol-chloroform and recovered by ethanol precipitation prior to PCR. The transgenic mice were genotyped by PCR using primers that span the junction between promoter and *OCT4B1cDNA* listed in **Table 1**.

3.4. Induction of OCT4B1 cDNA Expression in Transgenic Mice

MMTV promoter induction in F1 transgenic males (6-8 weeks old) was carried out by dexamethasone (DEX) intraperitoneal injection (1µg in 250µL per mouse) three times every other day. The *OCT4B1* mRNA expression was confirmed by RT-PCR in testis of induced transgenic animals. All primers used are listed in **Table 1**.

3.5. Sperm Analysis

For sperm analysis in transgenic mice, after dexamethasone induction of the OCT4B1 expression (three times every other day), both epididymides were removed from control and transgenic mice groups (four mice in each group) and were cleaned off from all fat and blood. The sperms were isolated from dissected epididymides and were allowed to swim into PBS to liquefy for 30 min at 36˚C prior to analysis. Sperm parameters were assessed by conventional light microscopic evaluation, according to World Health Organization (WHO) criteria (WHO, 2003) for sperm motility, morphology and counts. All sperm parameters were evaluated blindly by two independent expert laboratory staff. For motility observation and analysis with a light microscope, a drop of the sperm suspension was placed on a clean pre-warmed slide. A total of 200 sperms (motile and immotile sperms) were numbered under the 400× magnification light microscopy in each sample. Sperm movement was calculated by visual tracking based on WHO specific classification of sperm motility as type A;

Table 1. List of primer sequences used in PCR and RT-PCR

Name	(5' → 3')	PCR/RT-PCR Product Size
B1-F	GAAGGATCCGCCGCCACCATGCACTTCTACAGAC (*Bam*HI)	1045 bp (*OCT4B1cDNA*)
B1-R	GCCGAATTCTCAGTTTGAATGCATGGG	
TG-F	GCTCTGAGTGTTCTATTCTCC	300 bp
TG-R	CCACATCGGCCTGTGTATATC	
B2m-F	CTGACCGGCCTGTATGCTAT	150 bp
B2m-R	TTTCCCGTTCTTCAGCATTT	
mOCT4-F	GAGCCGTCTTTCCACCAGGC	436 bp
mOCT4-R	TGGGACTCCTCGGGAGTTGG	

sperms with rapid progressive motility, type B; motile sperms with slow progressive movement, and type C; Non-progressive motility sperms were considered as motile sperms and type D; dead sperms or sperms without any movement were considered as immotile. Then, the total movement of sperms from each mouse was calculated and reported as a percentage of total motile sperm. Epididymal sperm count was determined using a neubauer hemocytometer. The sperm suspension smear was stained with Diff-Quick for estimating the sperm's number with normal morphology by counting a total of 200 sperms under the light microscope under 400× magnification for abnormalities in their heads and tails. The viability of sperm was evaluated by using eosin–nigrosin in equal volume of sperm suspension and was counted a total of 100 sperms.

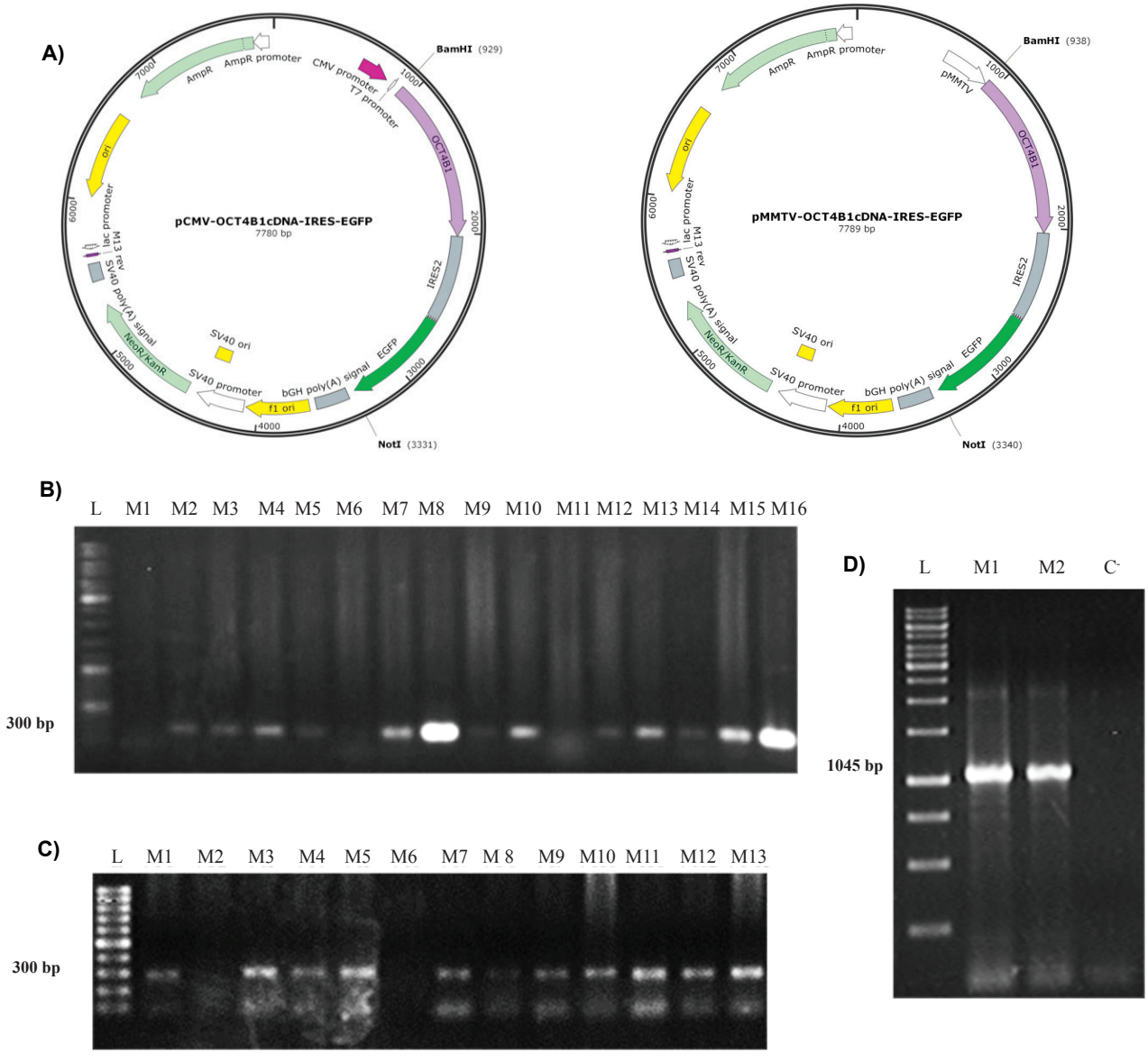

Figure 1. Screening of OCT4B1 transgenic mice using tail tips and tissue samples. Schematic pCMV-OCT4B1cDNA-IRES-EGFP and pMMTV-OCT4B1cDNA-IRES-EGFP maps that were used in this study **A)**. OCT4B1 transgenic mice were identified in F0 **B)** and F1 **C)** generations by PCR analysis of tail tip DNA using primers that span the junction between MMTV promoter and OCT4B1cDNA (The numbers of mice are indicated above the electrophoresis gel pictures, (L) DNA Ladder (GeneRuler DNA Ladder Mix, SM0331, Fermentas). OCT4B1 expression showed by RT-PCR analysis in two transgenic mice testis after MMTV promoter induction with DEX (M1-M3 from F1 generation), (L) DNA Ladder (GeneRuler 1 Kb DNA Ladder, SM1163, Fermentas), and (C^-) non- transgenic mice testis **D)**.

3.6. In Vitro Fertilization

Sperm analysis was performed in mice which were divided into two groups (transgenic and control mice), each consisting of four mice. Six female NMRI mice (six-eight weeks old) were superovulated by intraperitoneal injection of 5-10 IU PMSG, followed by 5-10 IU HCG 48 hours later. Approximately 12-14h after HCG injection, cumulus-oocyte complexes were isolated from oviducts in T6 medium supplemented with 5 $\mu g.mL^{-1}$ of BSA. Oocytes were inseminated with 0.15×10^6 spermatozoa in T6/BSA medium for 4 hours at 37 °C incubator. Eggs were then transferred to an embryo culture medium covered with paraffin oil. These eggs were evaluated in 2-cell and blastocyst stages.

3.7. Embryonic Stem Cell Culture and Transfection

The R1 mouse embryonic stem (mES) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented by 15% (v/v) fetal bovine serum (FBS, Gibco), glutamax (Gibco, 2 mM), MEM nonessential amino acids (Gibco, 0.1 mM), β-mercaptoethanol (Sigma, 0.1 mM), penicillin (50 $U.mL^{-1}$) /streptomycin (50 $\mu g.mL^{-1}$) (Gibco) and leukemia inhibitory factor (LIF, Sigma, 1000 $U.mL^{-1}$) at 37 ˚C, 5% CO_2 in a humidified incubator. Primary mouse embryonic fibroblast (MEFs) feeder cells were derived from NMRI mouse fetuses on 13.5 embryonic day (E13.5) and mitotically were inactivated by mitomycin C (Sigma). Transfection of mES cells with *pCMV-OCT4B1cDNA-IRES-EGFP* construct was performed using electroporation, with Neon™ Transfection System (Thermo Fisher Scientific, Life Technologies). After 24h incubation, transfection efficiency was evaluated using fluorescence microscopy.

3.8. RNA Extraction and RT PCR

Total RNAs were extracted from mES cells and mouse tissues using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. RNase-free DNase I (Fermentase, USA) treatment of total RNA was carried out to remove any genomic DNA contamination and cDNAs were synthesized from 1 µg of Total RNA by MMLV reverse transcriptase (Fermentase, USA). To detect the relative transcript levels of *OCT4B1* and *OCT4* in transfected mES cells compared with the control group, semi-quantitative reverse transcriptase PCR (RT-PCR) was performed by TaKaRa LA Taq™ (Takara, Japan). β2M (beta-2 macroglobulin) housekeeping gene was used as the control (standard) gene and all reactions were done in triplicate. Primer sequences are listed in **Table 1.**

3.9. Western Blot

OCT4 protein expression level in OCT4B1 transfected mES cells was determined by quantitative western blot. Total protein was isolated from transfected and non-transfected (control) mES cells using lysis buffer (M cell lysis Reagent, Sigma, USA) supplemented with 1X protease inhibitor (Roche). Total protein contents were determined by Bradford assay and equal amounts of proteins (60 µg) from transfected and control mES cells were separated on 12.5% SDS-polyacrylamid (PAGE) gel, then was transferred to a PVDF membrane. The blot was blocked with TBST (136mmol/L NaCl, $20mmoL.L^{-1}$ Tris-HCl, PH 7.6, and 0.1% Tween-20) containing 5% skim milk, then incubated with the anti-OCT4 antibody (sc-9081 Santa Cruz, USA, 1:500), a rabbit polyclonal antibody raised against amino acids 1-134 mapping at the N-terminus of Oct-3/4, which specifically recognizes OCT4A isoform and β-actin antibody (ab8226 Abcam, USA, 1: 5,000) at 4 ˚C overnight. Afterwards, the membrane was washed with TBST andthe membrane was incubated with a secondary peroxidase-conjugated antibody (sc-2004 Santa Cruz) for 1 h at room temperature. Luminescent signal was detected using the ECL advance western blotting detection Kit (Amersham, UK).

3.10. Statistical Analysis

Results were analyzed by descriptive statistics as mean ± standard deviation (µ±SD) for each group. For comparison between the groups, statistical analysis was done by t-test in SPSS and $p<0.05$ was considered statistically significant.

4. Results

4.1. Establishment of OCT4B1 Inducible Expression Transgenic Mice

The transgenic mice were generated using a construct containing *pMMTV-OCT4B1cDNA* and identified by PCR analysis in F0 and F1 generations (**Fig. 1B and C**). Thirteen OCT4B1 founder mice tested positive by PCR for transgene integration into the genome (5 males and 8 females). From those, the male founders were

infertile and one of the females died before the onset of puberty. Four lines were able to transmit the transgene to their offspring. After mating with non-transgenic mice, founder NO. 8 produced thirteen F1 mice from 2 litters with eleven OCT4B1 transgenic mice (5 males and 6 females). After three days of stimulation with DEX, *OCT4B1* expression was detected by RT-PCR analysis using OCT4B1 specific primers in two transgenic mice testis from F1 generation (**Fig. 1D**).

4.2. Decreasing Fertility Rate and Change in Sperm Parameters in OCT4B1 Transgenic Mice

The spermiogram results indicated that sperm parameters including count (concentration), viability, and motility decreased statistically significant ($p<0.05$) and morphology changed in in transgenic mice compare to that of the control group (**Table 2**). Prevalent sperm abnormalities in transgenic group including abnormal middle piece and mid-piece defect or bent neck are shown in **Figure 2** (Arrows, A-C).

To investigate fertility rate, transgenic mice sperms were used for IVF analysis with normal female mice oocytes and were compared to that of the control group. Fertility rates were scored at the two-cells and blastocyst stages of embryos. The fertility rate was calculated as two-cell rate by dividing the number of two-cells by total number of MII oocytes and blastocyst rate by dividing the number of blastocysts by total number of two-cells. The IVF results are summarized in **Table 3**. The results showed that transgenic mice had a significantly reduced fertility rate compared to that of the control group ($P < 0.05$).

4.3. OCT4B1 Overexpression Induces Cell Death in mESC Colonies and Decreases Endogenous OCT4 Expression

To identify the function of OCT4B1 variant *in vitro*, the effect of *OCT4B1* overexpression in R1 mouse embryonic stem cells was evaluated. These cells were transfected with the *pCMV-OCT4B1cDNA-IRES-EGFP*

Table 2. Evaluation of *OCT4B1* overexpression effect on sperm parameters including count, viability, morphology and motility in transgenic mice group compared with control group (M±SD)**.**

Group	Normal morphology %*	Motility %	Viability %	Sperm count*
Transgene	81.25±2.50	66.25±20.56	74.50±8.42	6.01±2.27
Control	90.25±6.34	83±7.39	91.25±14.19	12.98±3.15

The results of spermiogram revealed that sperm parameters were decreased in transgenic mice group in comparison with the control group. *: ($p<0.05$) (n=4mice/group).

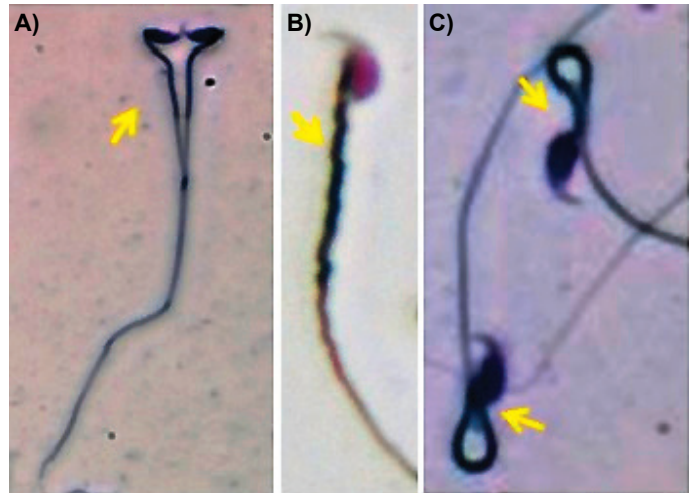

Figure 2. Morphology of epididymal sperms from OCT4B1 transgenic mice. Some of common sperm abnormalities in transgenic mice were head defect (doubled headed) **A)** abnormal middle piece **B)** and mid-piece defect or bent neck **C)**. Arrows show the defect piece of sperms.

construct (**Fig. 3A-C**). Transfected cells indicated cell death so that no ES colonies survived after 72 hours (**Fig. 3D-F**). The approximate starting time of cell death in ES colonies was 36 hours after transfection. The effect of electroporation pulses and IRES-EGFP vector alone on mES cells survival was investigated, however, no sign of death was observed in these cells (**Fig. 3G-J**).

Table 3. Evaluation of *OCT4B1* overexpression effect on IVF results in transgenic mice group compared with control group.

Group	No. MII oocytes	No. (%) 2cell*	No. (%) blastocysts*
Transgene	121	40 (38.64±17.10)	0 (0)
Control	102	90 (88.37±4.37)	15 (13.33±5.77)

The data were shown as No. (Mean ± SEM%). 2cell rate: No. 2cells /No. MII oocytes. Blastocyst rate: No. blastocysts/No. 2cells. *: ($p<0.05$) (n=4mice/group)

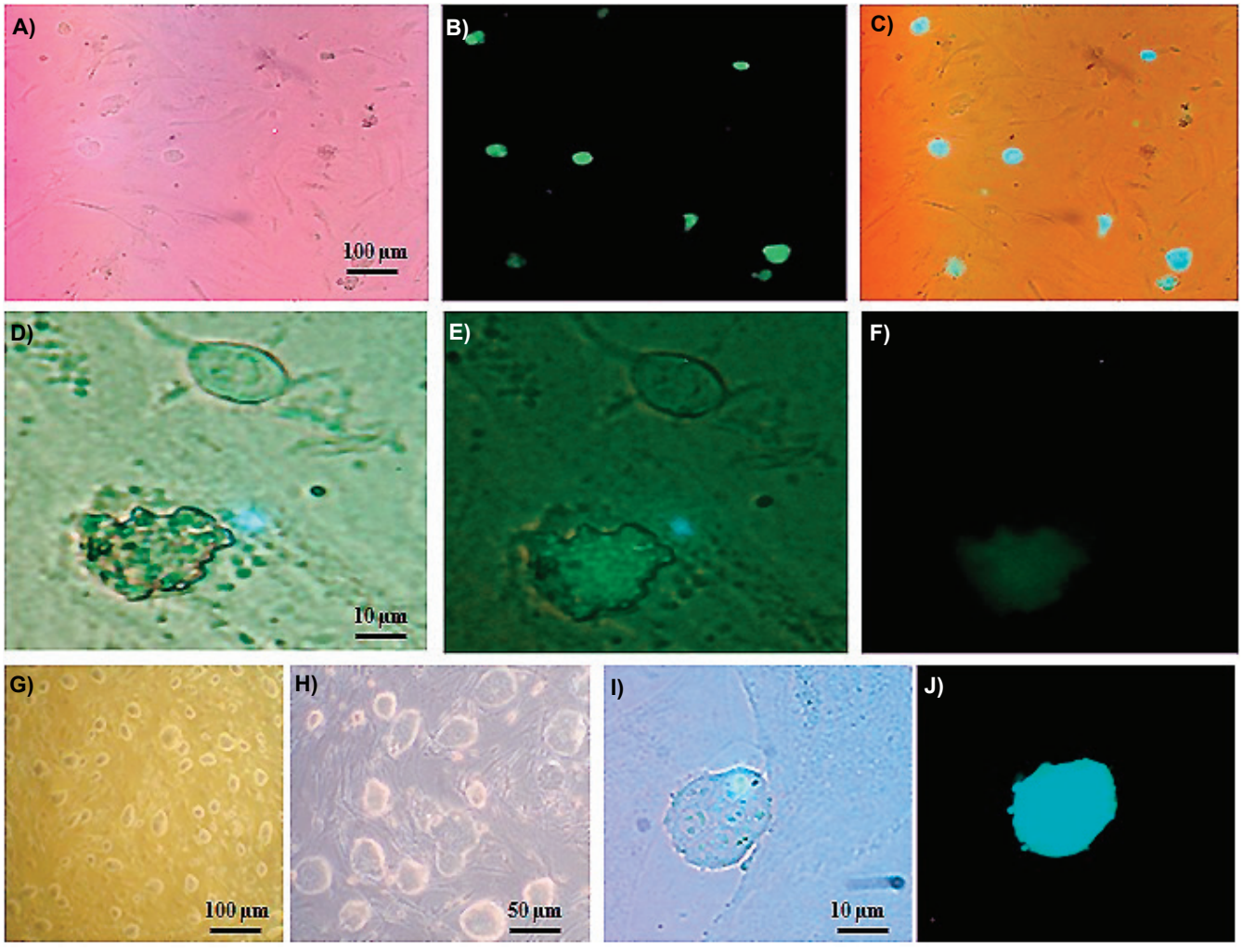

Figure 3. OCT4B1 overexpression in R1 mouse embryonic stem cells. ES cells were transfected by the *pCMV-OCT4B1cDNA-IRES-EGFP* construct and cultured on MEF feeder cells **(A-C)**; The signs of cell death were observed in these cells after transfection **(D-F)**. To evaluate the effect of electroporation pulses **(G** and **H)** and IRES-EGFP vector alone (**I** and **J**) on mES cells survival, cells were exposed to electroporation pulses without any plasmid construction and IRES-EGFP vector alone without OCT4B1, respectively. ES cells did not show any sign of death.

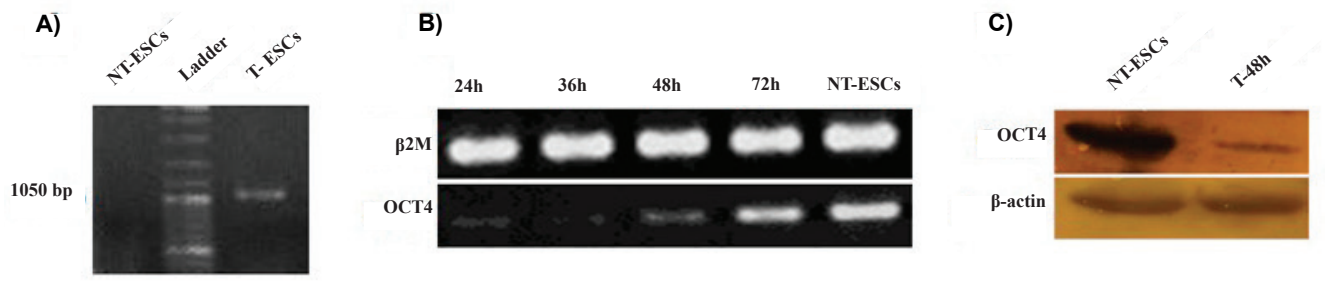

Figure 4. The inhibition effect of *OCT4B1* overexpression on endogenous OCT4 expression in *pCMV-OCT4B1cDNA* transfected ES cells compared with the control group. The expression of *OCT4B1* in transfected ES cells was confirmed by RT-PCR analysis, Non-Transfected ESCs (NT-ESCs), GeneRuler DNA Ladder Mix, SM0331, Fermentas, (L), Transfected ESCs (T-ESCs) **A)**; The expression of endogenous OCT4 was evaluated at 24, 36, 48 and 72 hours after transfection by semi-quantitative RT-PCR **B)** and western blot **C)**. (Significant decreasing in the OCT4 mRNA and protein was observed in transfected cells compare with the control ES cells, specifically at 36 hours after transfection).

PCR products from these transfected cells showed expected band size for OCT4B1 (**Fig.4A**). Our findings were further confirmed by PCR product sequencing.

Then, in order to investigate the reason for death in transfected ES colonies, the effect of *OCT4B1* overexpression on endogenous OCT4 expression was evaluated in these cells in the different time points. Cells were collected at 24, 36, 48 and 72 hours after transfection for subsequent analysis. Semi-quantitative RT-PCR analysis showed the expression level of *OCT4* mRNA was markedly reduced in transfected cells compared to the control group (**Fig. 4B**).

Western blot data also indicated significant down-regulation in the OCT4 protein expression in transfected cells compare with non-transfected ES cells as control that confirmed the results of qRT-PCR. (**Fig.4C**). Therefore, it was estimated that the cause of the colonies death could be sever decrease of the OCT4 expression in transfected embryonic stem cells.

5. Discussion

OCT4 transcription factor is an embryonic stem cell-specific and germline-specific marker- that some studies have reported different results for its functions. These controversies might be due to the presence of OCT4 pseudogenes (34-36) or non-discrimination between different OCT4 variants. For instance, discovery of a new variant, OCT4B1, that has a high expression in undifferentiated cells and is down-regulated in differentiating cells, has made some complications (21, 37, 38). The clear discrepancies between these variants in their gene expression pattern, protein localization and function, possibly due to post-transcriptional regulation, could be responsible for some contradictions in early reports. Due to the pitfalls of pseudogenes and isoforms, it was crucial to precise discriminating by further research on structure and function of these variants.

Some findings about OCT4B1, such as high expression in ES/EC cells, low or maybe undetectable expression in other cells and reduced expression after differentiation-inducing, suggest a potential relationship between OCT4B1 and pluripotency in human ES and EC cells (21). In fact, these findings show functional similarity between OCT4B1 and OCT4A. The results of international stem cells institute (ISCI) research suggested, it is likely that in the future, OCT4B1 would be a reliable marker of stemness instead of OCT4A (22), further strengthening the role of OCT4B1 variant. Given the importance of these issues, more investigations in the field of the OCT4B1 exact splicing pattern, function, and its role in tumorigenesis, cell stress, embryo development, and its other unknown aspects is needed.

In this study, for the first time, we developed the transgenic mice model to evaluate the OCT4B1 overexpression using MMTV inducible promoter. Our study in sperm and fertility rate analysis in OCT4B1 transgenic mice showed changes in sperm parameters and decrease in fertility rate. We also analyzed whether *OCT4B1* overexpression led to impairment in mES cells by transfection of *OCT4B1* construct. The *in vitro* results showed cell death in mES cells 72 hours after transfection and therefore, we did not achieve to any

stable mES cell line with OCT4B1 overexpression. It was interesting to determine whether *OCT4B1* overexpression led to disruption in the key regulatory pathway of *OCT4* expression in mES cells. In this regard, our semi-quantitative RT - PCR and western blot results analysis revealed a dramatic down-regulation in endogenous OCT4 expression following OCT4B1 overexpression in these cells, especially 36 hours after transfection that it was the approximate starting time of cell death in ES colonies. Since OCT4 is expressed in the precursors of spermatogonial stem cells (SSCs) and its expression has a crucial role in ESCs and PGCs survival and early stages of mammalian embryogenesis (8, 14, 15, 39, 40). It is likely that *OCT4* downregulation in germ cells, which is the result of *OCT4B1* overexpression, causes cell death and decrease in their population, as we observed in *OCT4B1* transfected ESCs. Therefore, it may have consequences such as sperm abnormality and decreasing fertility ability. This is in line with other studies where *OCT4* knockout led to embryonic germ cell death and embryonic lethal (15, 41). These findings from our *in vivo* and *in vitro* studies suggest the function of OCT4B1 during embryogenesis and thereby fertility. However, further studies are required to support this hypothesis.

Due to the different roles of OCT4 isoforms, it seems that *OCT4* variants have several regulation elements and can use different enhancers and promoters in their transcription. Also, since the OCT4 isoforms have similar sequences, it is likely to have close relationships in their biological functions as competitively or cooperatively in various levels of transcription. Therefore, according to the above-mentioned, it could be concluded that due to the presence of multiple alternative splicing isoforms of *OCT4* transcripts (A, B and B1) and proteins (A, B-164, B-256 and B-190) in various cell types -to overcome limited number of genes in genome and multiple biological functions- it is necessary to identify OCT4 isoforms, their expression patterns and exact function in stem cell biology (42, 43).

Further studies are required to elucidate how *OCT4B1* downregulates OCT4 expression. However, it could be considered that the *OCT4B1* may have a synergistic role for the different OCT4 splice variants or possibly function in concordance with OCT4A in driving pluripotency. Because of structural similarities between OCT4 and OCT4B1, it is presumably that ectopic *OCT4B1* expression could interfere with OCT4 function. *OCT4B1* might prevent OCT4A mRNA translation in stem cells by gene methylation in master gene or competitive miR binding, as has been suggested as a biological function of pseudogenes (44). Another possibility is interfering with OCT4A protein and inhibitory effect on its expression if the stable OCT4B1 protein is present. Due to the lack of known protein isoforms for OCT4B1and subsequent antibodies against it, study of OCT4B1 protein expression using western blot was not possible in our study.

Altogether, it is suggested further investigation on function of this variant especially as tissue-specific. Also, the role of OCT4B1 expression in spermatogenesis and fertility must be considered. If there is a correlation between OCT4B1 expression and fertility, we can proceed to treatment of related diseases with new strategies. Totally, more investigation on OCT4 and its variants could help to understand cellular and molecular mechanisms of stem cells pluripotency and to create new hopes for controlling these cells to use in regenerative medicine and tissue engineering.

6. Conclusion

In general, the results of the present study revealed an important and previously-unknown role of OCT4B1. The overexpression of OCT4B1 led to a decrease in fertility rate, evidenced by disruption in sperm parameters and IVF results. Therefore, OCT4B1 is closely related to fertility impairment through regulating OCT4 expression level, and may affect spermatogenesis. ESC death suggested the potential role of OCT4B1 in regulating stem cell self-renewal. These results further validated the general consensus that OCT4A expression level is a critical determinant of ESC fates, and a similar specificity can be considered for the OCT4B1 variant. However, further studies are required to gain more insights into the underlying mechanism in spermatogenesis and OCT4B1 role in embryo development. This study highlights the significance of differentiating between OCT4 variants, especially in expression pattern and biological functions. The results may help to decipher the molecular mechanisms involved in germ cell development, sperm abnormalities, and male infertility. Finally, the pMMTV-OCT4B1cDNA transgenic mice can be used as a model to evaluate the role of OCT4B1 in breast cancer due to mammary gland-specific expression.

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Declaration of Interest

All authors confirm that there is no conflict of interests associated with this paper.

Author's Contributions

M.N. and E.R.P. performed the experiments, analyzed the data, and wrote the manuscript. Me.S. and K.N helped to generate the transgenic mice model. M.M. and S.N. helped to perform the IVF experiments. M.K.S., Ma. S. and S.J.M. conceived the original idea and planned the experiments. Ma. S. helped supervise the project and designed the genetic constructs and the transgenic mice model. M.K.S. designed and supervised the study.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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