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Effect of Oct-4 Silencing on Proliferation and Differentiation of Mouse Undifferentiated Type A Spermatogonial Cells

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Abstract

Spermatogenesis is a complex process involving cell division and differentiation of Spermatogonial Stem Cells (SSCs), mediated by coordinated expression of a number of genes. The study of SSC biology was challenging due to the unavailability of proper SSC markers and their culture methods. In recent years, these hurdles have been somewhat overcome. A number of markers for undifferentiated spermatogonia are known to be involved in the process of SSC self-renewal. Oct-4 is one such marker expressed from Primordial Germ Cells (PGCs) of prenatal stages till undifferentiated spermatogonia of postnatal adult testes. Though, expressed by undifferentiated spermatogonia, its role in the process of their self-renewal, proliferation and differentiation is not clearly understood. In the current study, using shRNA mediated gene silencing approach, we show that Oct-4 silencing in undifferentiated type A spermatogonia *in vitro* leads to phenotypic differentiation of these cells to type A1-A4 spermatogonia, marked by the increased c-KIT expression, and decreased proliferation, marked by decreased PCNA expression. The study also demonstrates that Oct-4 silencing causes down-regulation of Plzf, Gfra-1, c-Ret, Bcl6b and Etv5 mRNA and protein. Therefore the current study suggests that Oct-4 does play an important role in deciding the fate of undifferentiated spermatogonia by controlling their proliferation and differentiation. In conclusion, the present study may provide useful insights into understanding the involvement of Oct-4 in proliferation and differentiation of undifferentiated spermatogonia in mice.

Keywords: Spermatogonial stem cells (SSCs); Oct-4; Self-renewal; shRNA

Introduction

Spermatogenesis is a complex process that involves continuous self-renewal of subset of type A spermatogonia called Spermatogonial Stem Cells (SSCs), which further undergo differentiation to give rise to spermatozoa thereby maintaining continual spermatogenesis from puberty till old age in males. SSCs help in transmitting genetic information from one generation to next generation [1,2]. Type A spermatogonia have several subtypes such as A-single (A_s), A-paired (A_{pr}) and A-aligned (A_{al}) cells [3,4]. Functional assays demonstrate that a subset of A_s, A_{pr} and A_{al} (undifferentiated spermatogonia) spermatogonia have stem cell potential [5].

The discovery of methods to maintain undifferentiated spermatogonia in culture has provided great promise for furthering the understanding of SSC biology [6]. Study by Kanatsu-Shinohara et al. [7] has shown that cultures of "Germline Stem" (GS) cells, derived from neonatal testes can be successfully expanded in culture for years while maintaining SSC colonization activity. These cells express markers of undifferentiated spermatogonia such as OCT4 and PLZF, as well as several cell surface markers, including RET, GFRa1, α6 and β1 integrins [6,8-10]. Recently it has been shown that SSCs from mouse and human testes can acquire pluripotency to become embryonic stem-like (ES-like) cells that can differentiate into three embryonic germ layers [11-13]. In other words, SSCs possess unique characteristics that distinguish them from other adult stem cells. Therefore, a detailed understanding of the molecular mechanisms of the genes, growth factors and signaling pathways regulating the fate of proliferation and differentiation of SSCs is important. This is essential for their possible use as a therapy for patients undergoing chemotherapy so as to preserve their fertility by maintaining the germline [14,15].

Several molecular markers such as Plzf, Oct-4, Gfra-1, Ret, and Sox-3 are known to be expressed in undifferentiated spermatogonia [16-21]. *Plzf*, a gene expressed in undifferentiated spermatogonia is known to play a key role in self-renewal process of SSCs. Spontaneous ("luxoid") or targeted mutation in the *Plzf (Zfp145)* locus, leads to male infertility due to progressive loss of germ cells. [16,17]. Earlier studies on *Gdnf, Gfra-1* and *Ret* have shown that any ablation in the Gdnf mediated Gfra-1 and Ret signaling pathways results in lack of SSC self-renewal and induces the progressive loss of spermatogenesis by germ cell depletion [20]. Another key gene that has been studied in SSCs is Oct-4. Like PLZF, OCT-4 is expressed in undifferentiated spermatogonia. However, OCT-4 expression is selective and expressed in the A_s cells [18]. Our earlier study demonstrated that Oct-4 is not only expressed in A_s cells of mouse adult testes but also in differentiated germ cells of 35days post partum (dpp) adult testes [22]. It is known that OCT-4 is involved in regulating Embryonic Stem (ES) cells pluripotency and its expression level is closely linked to the balance of self-renewal versus differentiation [23]. Oct-4 knockout mice have been shown to be embryonic lethal, due to indispensable role of Oct-4 in ES cells which further prevented analysis of the role of OCT-4 in SSCs using knock-out approach [24]. These studies were further supported by Kehler et al. [25], who revealed that tissue-specific knockout of Oct-4 in Primordial Germ Cells (PGCs) in the embryo led to embryonic

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germ cell death, preventing the analysis of OCT-4 function in SSCs, which are derived from PGCs.

Thus, although Oct-4 is playing a role in SSC self-renewal, its function in SSCs is largely unknown. Recently, Dann et al. [26] showed Oct-4 significance in self-renewal of SSCs but it was contradicted by Wu et al. [27] who demonstrated OCT-4 (POU5F1) expression is not essential for SSC maintenance *in vitro*. Our previous study revealed that Oct-4 expression at mRNA and protein level was highest between 5-10 dpp [22]. Therefore, we predicted that Oct-4 does play a crucial role in proliferation and in maintaining undifferentiated state of SSCs. The objective of the present study was to evaluate the possible role of Oct-4 in undifferentiated spermatogonia isolated from the testes of 5-6 dpp mice by shRNA gene silencing approach. The results demonstrated that Oct-4 silencing lead to the differentiation of type A spermatogonia from undifferentiated to matured differentiated germ cells type A1-A4 spermatogonia. Oct-4 silencing was found to cause decrease in the proliferation of undifferentiated type A spermatogonial cells and down regulation of genes involved in undifferentiated type A spermatogonial self-renewal process.

Materials and Methods

Experimental animals

For this study, adult CD1 male and female mice were bred to get the 5-6 dpp pups. All animals were housed at 25°C with 12L:12D photoperiod and were given water and a standard diet *ad libitum*. All animal experimental protocols were approved by the Institutional Animal care and Ethics committee (IAEC), National Institute for Research in Reproductive Health, Mumbai (IAEC # 08/09) in accordance with the guidelines of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) established by Govt. of India on animal care.

Germ cell isolation

Germ cells were isolated from 5-6 day old pups. Briefly, testes from the pups were dissected, detunicated, minced and subjected to two step enzymatic digestion method [28]. In the first step, the testes were put into digestion solution-I containing collagenase type IV (1.0 mg/ml) and DNase I (1 µg/ml) (Sigma-Aldrich, MO, USA) and incubated at 37°C for 10 min in shaking water bath set at 110 cycles/min. The interstitial cells were separated by sedimentation at unit gravity for 10 min and washed with DMEM. In the second step, the pellet was put into digestion solution-II containing collagenase (1.0 mg/ml), DNase I (1 µg/ml) and hyaluronidase (0.5 mg/ml) (Sigma-Aldrich) and the cell suspension was incubated at 37°C for 10 min. The dispensed cells were washed and filtered through 70 and 40 µ filters. The cells were pelleted down by centrifugation and put into 12 well culture plate containing DMEM+serum for 3 h in order to allow the sertoli cells to adhere. The remaining unattached spermatogonial cells were then subjected for laminin selection to obtain enriched population of undifferentiated type A spermatogonia.

Laminin selection to obtain enriched population of undifferentiated type A spermatogonia

The unattached spermatogonial cells from the previous step were further subjected to laminin selection. Approximately, $4X10^6$ germ cells were put on to the plates manually coated with laminin (20 µg/ml) (Merck Millipore-Chemicon, MA, USA) and were incubated for 15 min at 37°C. This was followed by 3 washes with PBS to remove the unbound cells. Attached cells were retrieved by trypsin (0.25%)-EDTA (1 mM) (Sigma-Aldrich) digestion for 5 min at 37°C. The cells were pelleted and used for culture. Our findings in this study and previous report suggest that the cells enriched by this method contain more than 90% undifferentiated type A spermatogonia [29,30].

Undifferentiated type A spermatogonial cell culture

Undifferentiated type A spermatogonial cells were put onto Mitomycin C (10 µg/ml) (Sigma-Aldrich) treated Sertoli cell feeder layer in a 12 well plate containing DMEM+10% FBS along with Non Essential Amino Acids (NEAA) (1X), Sodium Pyruvate (1X), Glutamine (2 mM), Penicillin-Streptomycin (100 U/ml and 100 µg/ml respectively) (Invitrogen, CA, USA) and growth factors such as GDNF (10 ng/ml), LIF (10 ng/ml) and bFGF (20 ng/ml) (R&D systems, MN, USA). The medium was changed once in every 3-4 days and cells were put onto fresh feeder layers.

RNA interference

The short hairpin RNA (shRNA) sequences cloned in pLKO.1 lentiviral vector targeting *Oct-4* mRNA were designed and commercially procured (Open Biosystems, CO, USA). The sequences of targeting Oct-4 shRNAs are listed in Table 1. In brief, colonies of undifferentiated type A spermatogonia were removed from the Sertoli cell feeder layer by gentle pipetting followed by digesting with 0.25% trypsin-EDTA. The digested single cell suspension was resuspended in antibiotic and serum free DMEM with GDNF, LIF, bFGF and plated at a concentration of $2X10^4$ cells/well in a 12 well culture plate without a feeder layer. The culture plate was kept in incubator at 37°C with 5% CO_2 (Day 0). These cells were then transfected next day (Day 1) with 1µg shRNA using Arrest-In transfection reagent (Open Biosystems). 48 h post transfection the cells were harvested for analysis. None Silencing Control Vector (NSCV), containing only backbone plasmid without shRNA and no shRNA (only cells) were used as controls.

RNA extraction and RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) from the freshly isolated and enriched undifferentiated type A spermatogonial cells as well as from the undifferentiated type A spermatogonial cells treated or untreated with Oct-4 shRNA plasmid. The extracted RNA was treated with DNase I (Sigma-Aldrich). First-strand cDNA was synthesized using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, CA, USA). Briefly, 5 X iScript reaction mix, iScript RTase, nuclease free water and 1 µg of RNA were mixed in a total volume of 20 µl. The protocol followed for cDNA synthesis was 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and final step of 4°C for ∞ (Bio-Rad). The RT-PCR was carried out to detect the presence of undifferentiated type A spermatogonia using markers such as *Oct-4, Plzf, Gfra-1, c-Ret, Bcl6b, Etv5*. The sequences of primers and annealing temperature are given in Table 2.

The cDNA (1 µl) was amplified using 0.1 µM of each primer, 1 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), PCR buffer with 1.5 mM $MgCl_2$, and 0.25 mM dNTPs in a 20 µl reaction volume in a PTC200 Thermal cycler (Bio-Rad). The amplification conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles comprising denaturation at 94°C for 30 sec, annealing at the optimized temperature for each set of primers for 30 sec, and extension at 72°C for 45 sec. The final extension was carried out for 7 min at 72°C. The products were analysed on 1.2%

Gene	Sequence of shRNA targeting Oct-4 (sense/*loop*antisense)
Oct-4 shRNA 1	CCGG**GCCGACAACAATGAGAACCTT***CTCGAG***AAGGTTCTCATTGTTGTCGGC**TTTTT
Oct-4 shRNA 2	CCGG**CCTACAGCAGATCACTCACAT***CTCGAG***ATGTGAGTGATCTGCTGTAGG**TTTTT
Oct-4 shRNA 3	CCGG**CGTTCTCTTTGGAAAGGTGTT***CTCGAG***AACACCTTTCCAAAGAGAACG**TTTTT

Table 1: Sequence of Oct-4 shRNAs targeting the different regions of Oct-4 mRNA.

Gene	Sequence	Product size	Annealing Temperature
Oct-4	**Forward 5'-cgggctgggtggatcctcga-3' Reverse 5'-ttcacggcattggggcggtc-3'**	**277bp**	**65°C**
Plzf	**Forward 5'-gcaggagccagcaaaggcga-3' Reverse 5'-gcagagaccccagggagggg-3'**	**196bp**	**65°C**
Gfra-1	**Forward 5'-ggctaggaggaggagatgct-3' Reverse 5'-ctggatgtgaccagggactt-3'**	**231bp**	**65°C**
c-Ret	**Forward 5'-tggcacacctctgctctatg-3' Reverse 5'-ctgttcccaggaactgtggt-3'**	**187bp**	**58°C**
Bcl6b	**Forward 5'-gcagcagtgaagaaggaacc-3' Reverse 5'-agccacagcctcacagttct-3'**	**206bp**	**61°C**
Etv5	**Forward 5'-gggagagacaaaaaccacca-3' Reverse 5'-atgggtgtgcagtttcttcc-3'**	**219bp**	**62°C**
Pcna	**Forward 5'-gaacaggagtacagctgtgta-3' Reverse 5'-caggctcattcatctctatgg-3'**	**220bp**	**60°C**
Pou3f1	**Forward 5'-cctggggtccttctaactcc-3' Reverse 5'- ggaggagaggggaagagaaa-3'**	**170bp**	**60°C**
c-kit	**Forward 5'-ttatcctttaggccgtgtgg-3' Reverse 5'-tgtggccccttaagtacctg-3'**	**230bp**	**58°C**
Scp3	**Forward 5'-ggggccggactgtatttact-3' Reverse 5'-aggctgatcaaccaaaggtg-3'**	**169bp**	**60°C**
Gapdh	**Forward 5'-aactttggcattgtggaagg-3'. Reverse 5'-acacattgggggtaggaaca-3'**	**223bp**	**64°C**

Table 2: Sequence of gene primers used in Real-time PCR studies.

(w/v) agarose gel stained with 0.5 mg/ml ethidium bromide (Sigma-Aldrich) and visualized under ultraviolet transilluminator. The product size was approximated using a 100-bp DNA ladder (Bangalore Genei, Bangalore, India). Negative control did not contain Reverse transcriptase (RT) enzyme in the reaction mixture. The gel pictures were recorded in Gel dock instrument (Bio-Rad).

Real-time quantitative PCR

Total RNA was isolated using TRIzol reagent from the undifferentiated type A spermatogonial cells 48 h post transfection treated with Oct-4 shRNA, NSCV and was treated with DNase I to remove the potential genomic DNA contamination. Total RNA (1µg) was reverse transcribed into the first-strand cDNA using iScript cDNA synthesis kit (Bio-Rad) as mentioned earlier. The effect of Oct-4 silencing was analysed using specific markers of spermatogonial development and differentiation using quantitative RT-PCR. The relative expression levels of *Oct-4, Plzf, Gfra-1, Ret, Bcl6b, Etv5* (markers for undifferentiated spermatogonia) mRNA and *c-kit, Scp3 and Pgk2* (differentiation specific markers) mRNA with respect to *Gapdh* housekeeping gene were estimated by CFX96 real-time PCR system (Bio-Rad) using SYBR Green chemistry (Bio-Rad). The amplification conditions were as follows: initial denaturation at 94°C for 2 min followed by 40 cycles comprising denaturation at 94°C for 30 sec, primer annealing at respective temperatures for different genes for 30 sec, and extension at 72°C for 45 sec. The final extension was carried out for 7 min at 72°C. The fluorescence emitted at each cycle was collected for the entire period of 30 sec during the extension step of each cycle. The homogeneity of the PCR amplicons was verified by running the products on 1.2% (w/v) agarose gels and also by studying the melt curve. Mean C_t values generated in each experiment using the CFX Manager software (Bio-Rad) were used to obtain fold change in the expression of different genes normalized to *Gapdh* in control and shRNA treated cells. The relative expression levels in terms of fold change were calculated by $2^{-\Delta\Delta Ct}$ method [31].

Western blot analysis

Undifferentiated type A spermatogonial cells treated with or without shRNA against Oct-4 were homogenized in RIPA lysis buffer with Protease-*Arrest* (G-Biosciences, MO, USA). The homogenates were centrifuged at 12,000 X g for 30 min, and the supernatants were collected. Aliquots of the preparation were stored at - 20°C till further use and the concentration of the total protein content was determined [32]. The samples were heated at 95°C for 5 min with Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% Glycerol, 2 mM dithiothreitol, 0.01% Bromophenol Blue) [33]. Electrophoresis was carried out on 10% (v/v) Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel under reducing conditions. Each lane was loaded with 40 µg of protein along with prestained molecular weight marker (Bio-Rad). The separated proteins were transferred on a Nitrocellulose membrane (Hybridization Nitrocellulose Filter, Millipore, MA, USA), followed by blocking with 5% (w/v) non-fat dry (NFD) milk powder (Bio-Rad) in PBS (0.01 M, pH 7.2) at RT for 2 h. The blots were incubated at 4°C for 18–20 h with anti-OCT-4 (ab19857; dilution 1:200; Abcam, Cambridge, UK), anti-BCL6B (ab87228; dilution 1:100; Abcam), anti-GFRA-1 (sc-10716; dilution 1:100; Santa Cruz Biotechnology, CA, USA) , anti-RET (ab51122; dilution 1:100; Abcam), anti-ETV5 (ab102010; dilution 1:100, Abcam), anti-PCNA (ab2426; dilution 1:200; Abcam), anti-c-KIT (sc13508; dilution 1:100; Santa Cruz Biotechnology), anti-

GAPDH (as a loading control) rabbit polyclonal (G9545; dilution 1:2000; Sigma-Aldrich) antibodies and anti-PLZF (ab104854; dilution 1:200; Abcam), anti-SCP3 (ab97672; dilution 1:100; Abcam) mouse monoclonal antibodies diluted in PBS. The blots were washed four times with PBST (0.1% v/v Tween20) for 10 min each and then incubated for 1 h at RT with horseradish peroxidase (HRP)–conjugated goat anti-rabbit secondary antibody (A9169; dilution 1:20,000; Sigma-Aldrich) for OCT-4, BCL6B, ETV5, GFRA-1, RET, PCNA, c-KIT, GAPDH and HRP–conjugated goat anti-mouse secondary antibody (A9917; dilution 1:10,000; Sigma-Aldrich) for PLZF and SCP3. The blots were then washed with PBST (0.1% v/v Tween 20) for 10 min each and detected using the ECL Plus chemiluminescence detection system (GE Healthcare, NJ, USA). Further, the relative expression levels of the amount of OCT-4, PLZF, GFRA-1, c-RET, BCL6B, ETV5, PCNA, c-KIT, and SCP3 were semi quantitatively determined by densitometry analysis.

Indirect Immunofluorescence (IIF) to analyze Oct-4 silencing in shRNA treated cells

Indirect immunofluorescence (IIF) study was carried out on the cells treated with Oct-4 shRNA to check whether Oct-4 was silenced in the type A spermatogonial cells. Briefly, 48 h post transfection cells treated with NSCV, no shRNA control cells, Oct-4 shRNA1 and 2 were harvested and cytospun on slides coated with poly-L-Lysine (Sigma-Aldrich). The smears made by cytospun were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich). The cells were permeabilized with 0.1% (v/v) Triton X-100 for 10 min. Blocking was done with 5% Normal Goat serum (NGS) for 1 h at room temperature (RT) in humid chamber. The slides were then incubated with OCT-4 rabbit polyclonal antibody (ab19857; dilution 1:100; Abcam) diluted in PBS at 4°C overnight. After washing with PBS thrice for 5 min each, the slides were incubated with FITC labelled goat anti-rabbit secondary antibody (1:200; Sigma-Aldrich) for 1 h at RT. The slides were mounted with Prolong gold anti fade reagent (Invitrogen) and the images were captured by Laser scanning confocal microscope (LSCM) (Zeiss, 510 meta, Germany) (X63) at NIRRH central facility.

Apoptosis assay

Determination of the percentage of apoptotic cells in cultured undifferentiated type A spermatogonial cells treated with or without Oct-4 shRNA was performed using ApoAlert DNA Fragmentation Assay kit according to the manufacturer's instructions (Clontech, CA USA) and analysed by confocal microscope. The percentage of apoptotic cells were then calculated in treated and in untreated cultures. The percentage was calculated by counting the number of apoptotic positive cells per field and ten such fields were counted. The experiment was repeated three times with three different biological replicates.

Statistical analysis

The data obtained on fold change expression was represented as mean ± SD. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA). For multiple comparisons, Bonferroni's multiple comparison post hoc test was used. All the statistical analysis was done using GraphPad Prism software version 5.0 (GraphPad software, CA, USA). The fold change in the expression was considered to be significant if the values obtained were less than 0.05 ($p<0.05$).

Results

Germ cell isolation, enrichment of undifferentiated type A spermatogonia and confirmation by RT-PCR using undifferentiated spermatogonial markers

To confirm the presence of SSCs in undifferentiated type A spermatogonial cells isolated and enriched from germ cells, cells were subjected to RNA extraction and RT-PCR analysis using primers for SSC specific markers. RT-PCR results revealed the presence of undifferentiated spermatogonia specific markers such as *Oct-4, Plzf, Gfra-1, c-Ret, Bcl6b and Etv5* (Figure 1). This confirmed that cells isolated and enriched by laminin method contain SSCs and can be further used for culturing and shRNA mediated silencing.

Effect of Oct-4 shRNAs on the expression of Oct-4 mRNA and protein

To analyze whether Oct-4 plays an essential role in the undifferentiated spermatogonial cell proliferation and differentiation, undifferentiated type A spermatogonia were subjected to Oct-4 silencing. In order to knock-down the expression of Oct-4 in undifferentiated type A spermatogonial cells, three shRNAs sequences were used for targeting different regions of Oct-4 mRNA. Of the three shRNAs tested, two were effective. These two targeting sequences were designated as Oct-4 shRNA1 and Oct-4 shRNA2.

Q-PCR results showed the expression of Oct-4 mRNA was markedly reduced in cultured undifferentiated type A spermatogonial cells treated with Oct-4 shRNA compared to NSCV and no shRNA control. Oct-4 mRNA expression was decreased by 73% and 78% in type A spermatogonial cultures treated with Oct-4 shRNA 1 and Oct-4 shRNA 2 respectively (Figure 2A- i and ii). Western blot data indicated the expression of OCT-4 protein was significantly down-regulated in cells treated with shRNA. The densitometric analysis demonstrated that OCT-4 protein decreased by 70% and 75% when treated with shRNA -1 and shRNA-2 respectively (Figure 2B- i and ii).

Furthermore, to rule out the possible off-target effect of shRNA, *Pou3f1* gene expression was analysed in Oct-4 shRNA silenced and non-silenced cells. It has been previously shown that *Pou3f1* and *Pou5f1* expression is not linked with each other in SSCs. Therefore, *Pou3f1* gene was a good candidate to rule out the possible off-target effect of Oct-4 shRNA on other genes. The results showed that there

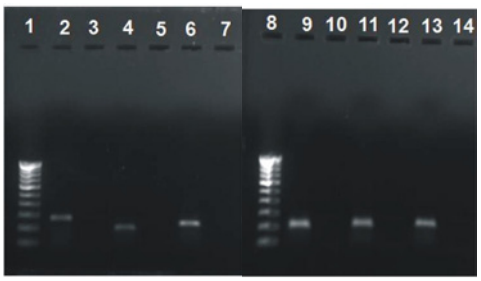

Figure 1: RT-PCR analysis for undifferentiated spermatogonial markers. Undifferentiated type A spermatogonia markers were expressed in isolated and enriched cells [1 and 8=100bp ladder, 2=Oct-4, 3=negative control for Oct-4, 4=Plzf, 5=negative control for Plzf, 6=Gfra-1, 7=negative control for Gfra-1, 9=Ret, 10=negative control for Ret, 11=Bcl6b, 12=negative control for Bcl6b, 13=Etv5, 14=negative control for Etv5].

was no change in the mRNA levels of *Pou3f1* in cells treated with Oct-4 shRNA as compared with untreated control cells (Figure 2C).

Indirect Immunofluorescence analysis of Oct-4 expression in undifferentiated type A spermatogonial cells silenced with Oct-4 shRNA

Indirect Immunofluorescence (IIF) analysis was carried out to assess the effect of shRNA mediated Oct-4 gene silencing on Oct-4 expression in cultured undifferentiated spermatogonia. The results revealed a significant knockdown of Oct-4 expression in cells transfected with Oct-4 shRNA-2 as compared to NSCV and only cells control (Figure 3).

Oct-4 silencing induces type A spermatogonial differentiation

To evaluate whether Oct-4 knock-down affects proliferation and induces phenotypic differentiation of type A spermatogonia into type A1-A4 or type B differentiated spermatognia or early spermatocytes or spermatids, real time PCR and Western blot analysis was performed using specific markers of spermatogonial proliferation (*Pcna*), differentiated spermatogonia (*c-kit*), early spermatocytes (*Scp3*) and spermatids (*Pgk2*).

Real-time PCR results revealed that *Pcna* mRNA levels were significantly decreased in type A spermatogonia treated with Oct-4 shRNA while that of *c-kit* and *Scp3* mRNA levels were significantly elevated ($p<0.05$). Western blot results showed that the expression of PCNA protein was reduced in shRNA treated type A spermatogonia, contrary to this KIT was up regulated in these cells thereby confirming the results of mRNA expression. The densitometric analysis revealed that *Pcna* mRNA levels decreased by 65% (Oct-4 shRNA1) and 67% (Oct-4 shRNA2) and PCNA protein levels decreased by 64 (Oct-4 shRNA1) and 66% (Oct-4 shRNA2) in shRNA treated type A spermatogonia (Figure 4A and 5A). Similarly, in shRNA treated type A spermatogonial cells, *c-kit* mRNA and C-KIT protein levels were increased by 2.80 (Oct-4 shRNA1) and 3.0 folds (Oct-4 shRNA2) at mRNA and 2.7 (Oct-4 shRNA1) and 2.9 (Oct-4 shRNA2) folds respectively at the protein level (Figure 4B and 5B). In contrast, *Scp3* mRNA showed 2.2 (Oct-4

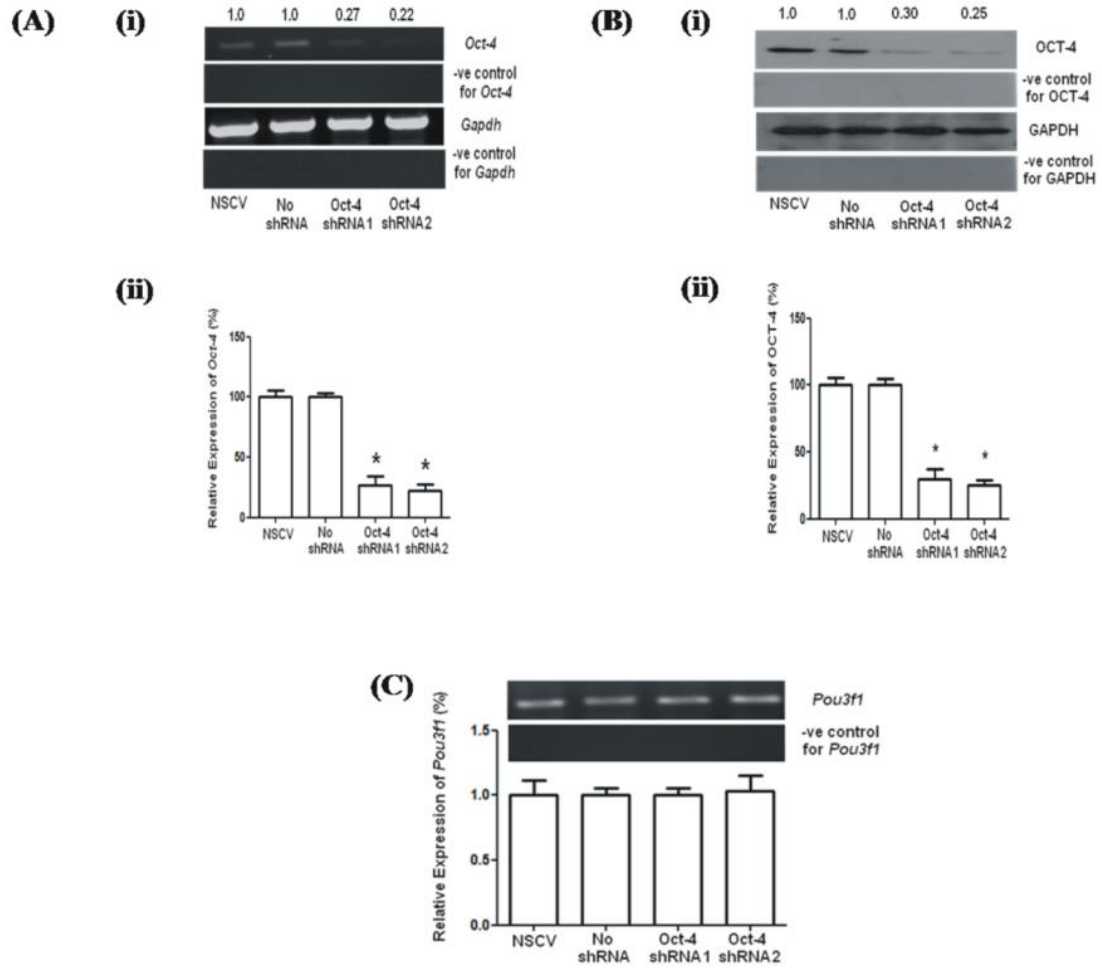

Figure 2: Effect of Oct-4 Knock-down on Oct-4 mRNA and protein. [A] (i) RT-PCR analysis of *Oct-4* using RNA isolated from NSCV, only cells and Oct-4 shRNA1 and 2 treated cells (ii) Relative expression of *Oct-4* mRNA quantified by Q-PCR in Oct-4 shRNA1 and 2 treated, NSCV and untreated cells. [B] (i) Western blot analysis of OCT-4 normalized against GAPDH (ii) Densitometry analysis of OCT-4 protein levels in Oct-4 shRNA1 and 2 treated, NSCV and untreated cells. [C] Relative expression of *Pou3f1* to rule out the possible off target effect of Oct-4 shRNAs. *Pou3f1* mRNA levels did not change in Oct-4 silenced cells. Negative control did not contain Reverse transcriptase (RT) enzyme in the reaction mixture for PCR. For Western blot, replacement of primary antibody with PBS was used as a negative control. Values are the mean ± SD. n=3 different biological replicates performed on three different occasions (*$p<0.05$).

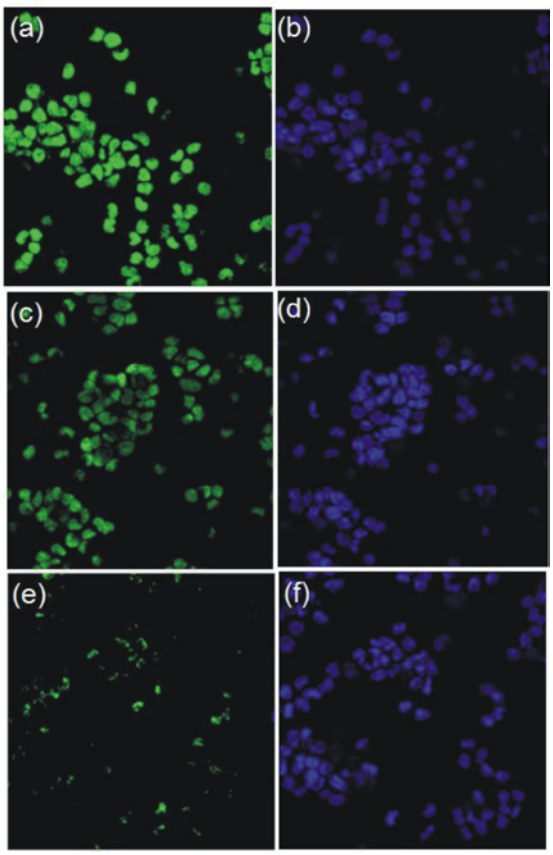

Figure 3: images of undifferentiated type A spermatogonia treated or untreated with Oct-4 shRNA captured on Laser scanning confocal microscope (LSCM). (a) Cells transfected with NSCV showing normal nuclear Oct-4 expression. (b) Undifferentiated type A spermatogonia transfected with NSCV shown by DAPI staining. (c) Undifferentiated type A spermatogonial cells without shRNA Transfection showing nuclear Oct-4 expression. (d) Undifferentiated type A Spermatogonial cells without shRNA transfection shown by DAPI staining. (e) **Undifferentiated type A spermatogonia 48 h post transfection with Oct-4 shRNA-2, showing significant knock-down of Oct-4 expression as compared to NSCV treated cells. (f) Undifferentiated type A spermatogonia 48 h post transfection with Oct-4 shRNA-2 shown by DAPI staining (X63). The figure shown is the representative pictures from three independent experiments.

shRNA1) and 2.3 (Oct-4 shRNA2) folds increase (Figure 4C) in Oct-4 shRNA treated cells but no SCP3 protein expression was observed in either treated cells or untreated cells, indicating differentiated cells did not display the phenotypes of spermatocytes at the protein level. Spermatid marker *Pgk2* was also checked in Oct-4 shRNA silenced type A spermatogonial culture. There was no Pgk2 mRNA and protein expression in the Oct-4 shRNA treated cells as compared to untreated cells.

Oct-4 silencing induces the down regulation of Plzf, Gfra-1, Ret, Bcl6b and Etv5

To check the effect of Oct-4 knock-down on the expression of undifferentiated type A spermatogonial markers, real-Time PCR and Western blot analysis was performed. Plzf, Gfra-1, Ret, Bcl6b and Etv5 were used as markers for undifferentiated type A spermatogonia.

Real-Time quantitative PCR results showed that there was 38%, 76%, 65%, 40% and 41% (Oct-4 shRNA2) (Figure 6A-E) decrease in the expression of *Plzf, Gfra-1, c-Ret, Bcl6b* and *Etv5* at mRNA level respectively. Western blot results showed the down regulation in protein expression of PLZF, GFRA-1, RET, BCL6B and ETV5 in shRNA treated type A spermatogonial cells further confirming the results of mRNA expression levels. The densitometric analysis showed 36%, 75%, 65%, 39% and 38% (Oct-4 shRNA2) (Figure 7A-E) reduction in the expression levels of PLZF, GFRA-1, RET, BCL6b and ETV5 in Oct-4 shRNA treated cultures as compared to untreated cultures.

Oct-4 silencing induces apoptosis in undifferentiated spermatogonial culture

Oct-4 shRNA-1 and Oct-4 shRNA 2 treated culture showed significant apoptosis ($p<0.05$) of type A spermatogonia. There was 3.67% apoptosis in NSCV treated cultures while in Oct-4 shRNA1 and Oct-4 shRNA 2 treated cells it was 10.33 and 13.66% respectively (Figure 8).

Discussion

SSCs form the basis of spermatogenesis and their self-renewal as well as differentiation is required for continuing male fertility. Due to the rarity of this cell population in the testis, studying SSC biology is a challenging task. In recent years, development of *in vitro* culture systems to maintain SSCs in the undifferentiated state has helped to study the pathways and molecules regulating SSC fate decisions and survival. Oct-4, a crucial transcription factor expressed in ES cells plays an important role in PGC survival [25]. It is a member of POU family transcription factors which play an important role in the development and in maintaining undifferentiated state of stem cells. Oct-4 is known to be expressed in the neonatal and adult testis [18].

Though Oct-4 is expressed in SSCs, its role in SSC self renewal, proliferation and differentiation is still not clear. Two independent studies by Dann et al. [26] and Wu et al. [27] tested the involvement of Oct-4 in SSC self-renewal and differentiation. However, these studies contradicted each other and failed to confirm the exact role of Oct-4 in SSC self-renewal and differentiation. The present study provides insight into the regulation of undifferentiated type A spermatogonial proliferation and differentiation governed by Oct-4. In this study, shRNA technology was used to reduce the expression of Oct-4 and its effect on the proliferation and differentiation of undifferentiated spermatogonia was studied. The enriched population of undifferentiated spermatogonia has a mixture of A_s, A_{pr} and A_{al} spermatogonia, it's difficult to separate A_s (SSCs) cells from A_{pr} and A_{al} cells. Therefore, the term undifferentiated type A spermatogonial culture has been used in this study for convenience instead of SSC culture. We exploited the ability of shRNA over siRNA to carry out stable Oct-4 knock-down *in vitro*. Lentiviral vector based shRNA expression is full proof and efficient in gene silencing [26].

The effect of Oct-4 knock-down was analysed on the proliferation and differentiation of undifferentiated type A spermatogonia by Real-Time, Western blotting and apoptosis assay methods. Using the shRNA approach we could achieve 78% Oct-4 knock-down *in vitro*. Our results showed that the suppression of Oct-4 expression led to decrease in the proliferation of undifferentiated spermatogonia in culture evidenced by the decrease in *Pcna* expression at mRNA and protein level. PCNA is a marker for identification of proliferating spermatogonia and early spermatocytes in the testes of rodents and primates [34]. We also analyzed whether Oct-4 silencing led to phenotypic differentiation by analyzing the expression of differentiation specific markers. Our

results showed that after the knock-down of Oct-4 the undifferentiated type A spermatogonial cells differentiated into more mature germ cells as evidenced by an elevated expression of *c-Kit* mRNA and its protein. KIT expression is considered as a hallmark for differentiating spermatogonia and type A1-A4 spermatogonial divisions [35-37].

It was interesting to determine whether Oct-4 silencing led to further differentiation of type A undifferentiated spermatogonia to spermatocytes and spermatid lineage. We used differentiation specific markers such as *Scp3* (early spermatocytes marker) and *Pgk2* (spermatid marker). In this study, *Scp3* mRNA expression was seen but *Pgk2* expression was not observed in the differentiating cells; subsequently western blot results did not show the expression of SCP3 protein suggesting Oct-4 silencing did not induce the differentiation till early spermatocytes or spermatid lineage.

Next, effect of Oct-4 silencing was checked on the expression of undifferentiated spermatogonia markers by analyzing Plzf, Gfra-1, Ret, Bcl6b and Etv5 expression. It was interesting to note that Oct-4 knock-down in undifferentiated type A spermatogonial cells,

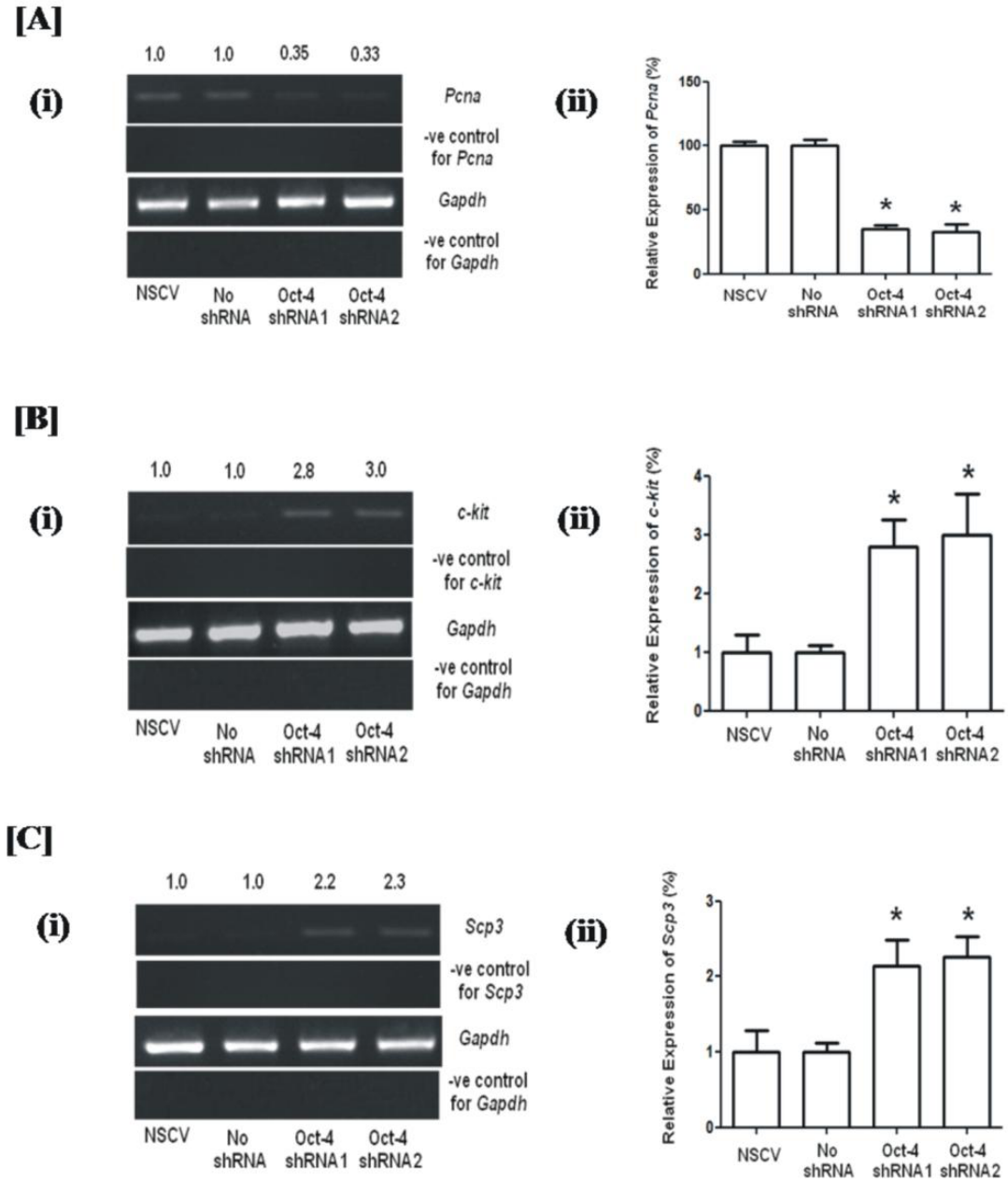

Figure 4: Effect of Oct-4 knock-down on type A spermatogonial cells in culture. [A] (i) RT-PCR analysis of *Pcna* and (ii) Relative expression of *Pcna* mRNA levels quantified by Q-PCR in Oct-4 shRNA treated and untreated cells. [B] (i) RT-PCR analysis of *c-kit* and (ii) Relative expression of *c-kit* mRNA levels determined by Q-PCR in Oct-4 shRNA treated and untreated cells. [C] (i) RT-PCR analysis of *Scp3* and (ii) Relative expression of *Scp3* mRNA levels quantified by Q-PCR in Oct-4 shRNA treated and untreated cells. Negative control did not contain RT enzyme in the reaction mixture. Values are the mean ± SD. n=3 different biological replicates performed on three different occasions (*$p<0.05$).

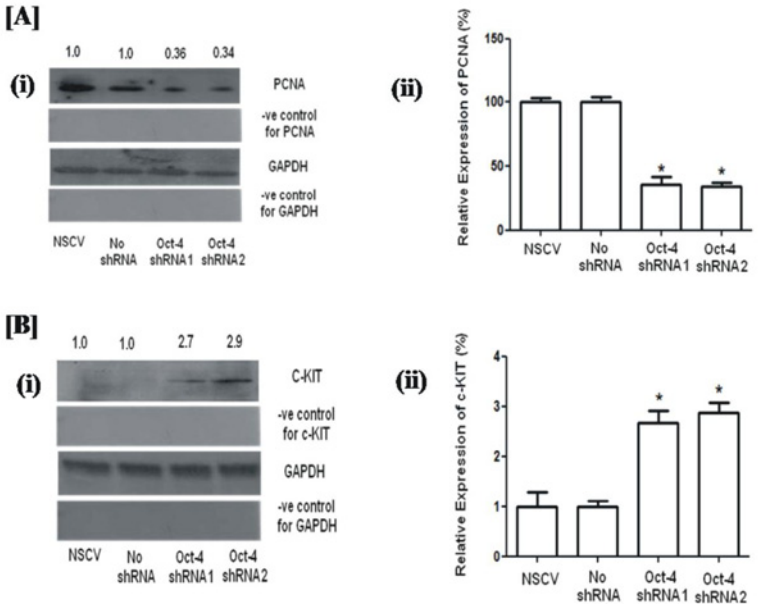

Figure 5: Effect of Oct-4 knock-down on type A spermatogonial cells in culture. [A] (i) Western blot analysis of PCNA and (ii) Densitometry analysis of PCNA normalized to GAPDH in Oct-4 shRNA treated and untreated cells. [B] (i) Western blot analysis of c-KIT and (ii) Densitometry analysis of c-KIT normalized to GAPDH in Oct-4 shRNA treated and untreated cells. The replacement of primary antibody with PBS was used as a negative control. Values are the mean ± SD. n=3 different biological replicates performed on three different occasions (*$p<0.05$).

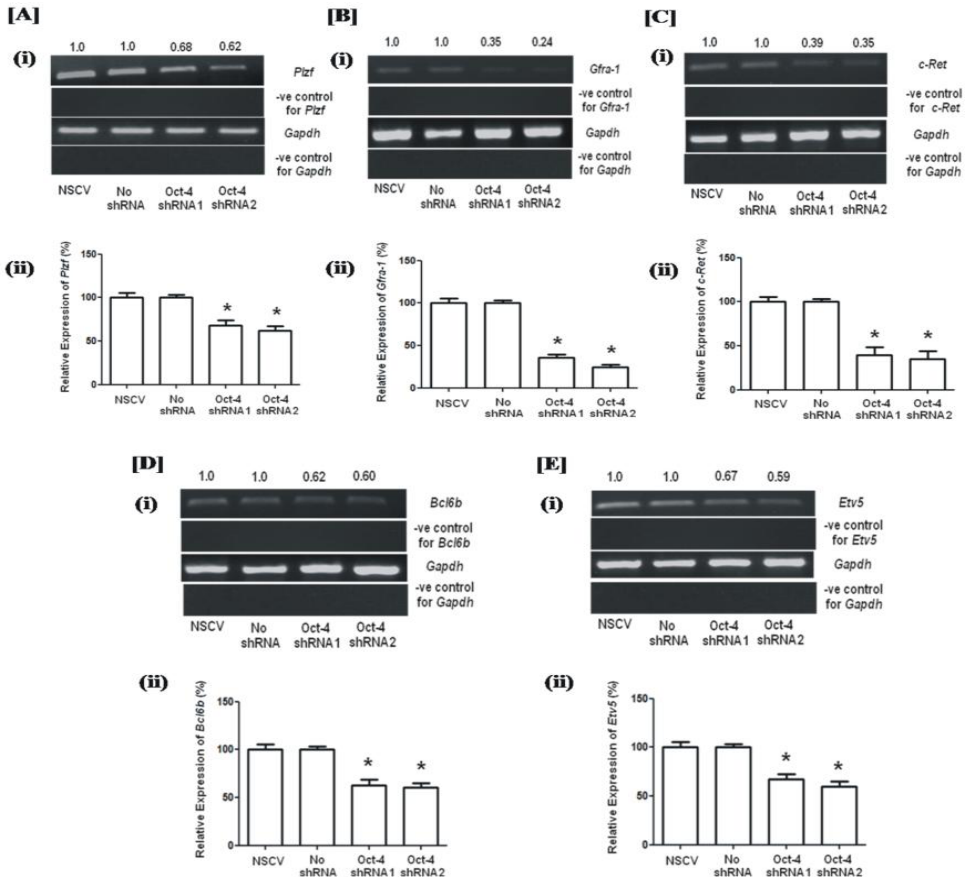

Figure 6: Effect of Oct-4 knock-down on the expression of markers for undifferentiated spermatogonia. RT-PCR of A(i) *Plzf*, B(i) *Gfra-1*, C(i) *c-Ret*, D(i) *Bcl6b*, E(i) *Etv5* and Relative expression of A (ii) *Plzf*, B (ii) *Gfra-1*, C (ii) *c-Ret*, D (ii) *Bcl6b*, E (ii) *Etv5* by Q-PCR in Oct-4 shRNA treated and untreated cells. Negative control did not contain RT enzyme in the reaction mixture. Values are the mean ± SD. n=3 different biological replicates performed on three different occasions (*$p<0.05$).

induced the down regulation of Plzf, Gfra-1, Ret, Bcl6b and Etv5 expression significantly ($p<0.05$) which explained the lower number of undifferentiated spermatogonia and greater number of differentiated type A1-A4 or type B spermatogonial cells in culture treated with Oct-4 shRNA.

The study by Dann et al. [26] demonstrated that Oct-4 does play an important role in the self-renewal of SSCs. The reduction of Oct-4 leads to impairment of SSC self-renewal *in vitro* and further treatment of GS cells with Retinoic acid (RA) leads to decrease in the number of GS cell colonies. This is due to RA mediated inhibition of Oct-4 promoter. In their study they used the GS cell culture conditions in which major portion of cultured GS cell population had c-KIT positive cells indicating the presence of differentiated spermatogonial cells. In our current study, undifferentiated type A spermatogonia were enriched using laminin selection method. It was found that there was almost undetectable c-KIT expression in cultured cells thereby the possibility of differentiated spermatogonia in culture was ruled out. Dann et al. [26] have established the GS cell culture using donor mice of age group between 9-10 dpp thereby the possibility of differentiated spermatogonial c-KIT positive cells are high in such culture and therefore use of such culture system as a model to study the role of Oct-4 in the process of self-renewal is a question. To rule out the possible contamination of differentiated spermatogonia in culture, in our study 5-6 dpp age group mice were selected for isolation of undifferentiated type A spermatogonial cells because at this point there are almost no differentiated germ cells existing in the testes.

The study by Wu et al. [27] showed that POU3F1 but not POU5F1 is an essential regulator of SSC fate decisions contradicting the findings of Dann's group. In the study conducted by Wu's group they used transient transfection using siRNA approach in SSC cultures *in vitro*. But this group did not study the effect of Pou5f1 knock-down on the proliferation and differentiation of SSCs. They did transplantation studies of the cells treated with Pou5f1 siRNA and found that there was no impairment in the maintenance of mouse SSCs. In our study, though we did not carry out transplantation experiments, the effect of Oct-4 knock-down on the expression of markers for undifferentiated spermatogonia as well as proliferation and differentiation potential of undifferentiated type A spermatogonial cells was studied. Based on the results of our study we see phenotypic differentiation of undifferentiated type A spermatogonial cells treated with Oct-4 shRNA into type A1-A4 spermatogonia marked by elevated expression of KIT protein in cultured cells. The current study also shows that Oct-4 knock-down causes decreased proliferation of undifferentiated spermatogonial cells marked by decreased expression of PCNA at the mRNA and protein level. We further demonstrate that Oct-4 knock-down in undifferentiated type A spermatogonial cells causes down regulation of markers of undifferentiated spermatogonia involved in self-renewal process. This implies that Oct-4 plays a crucial role in proliferation and differentiation of undifferentiated type A spermatogonia. This was

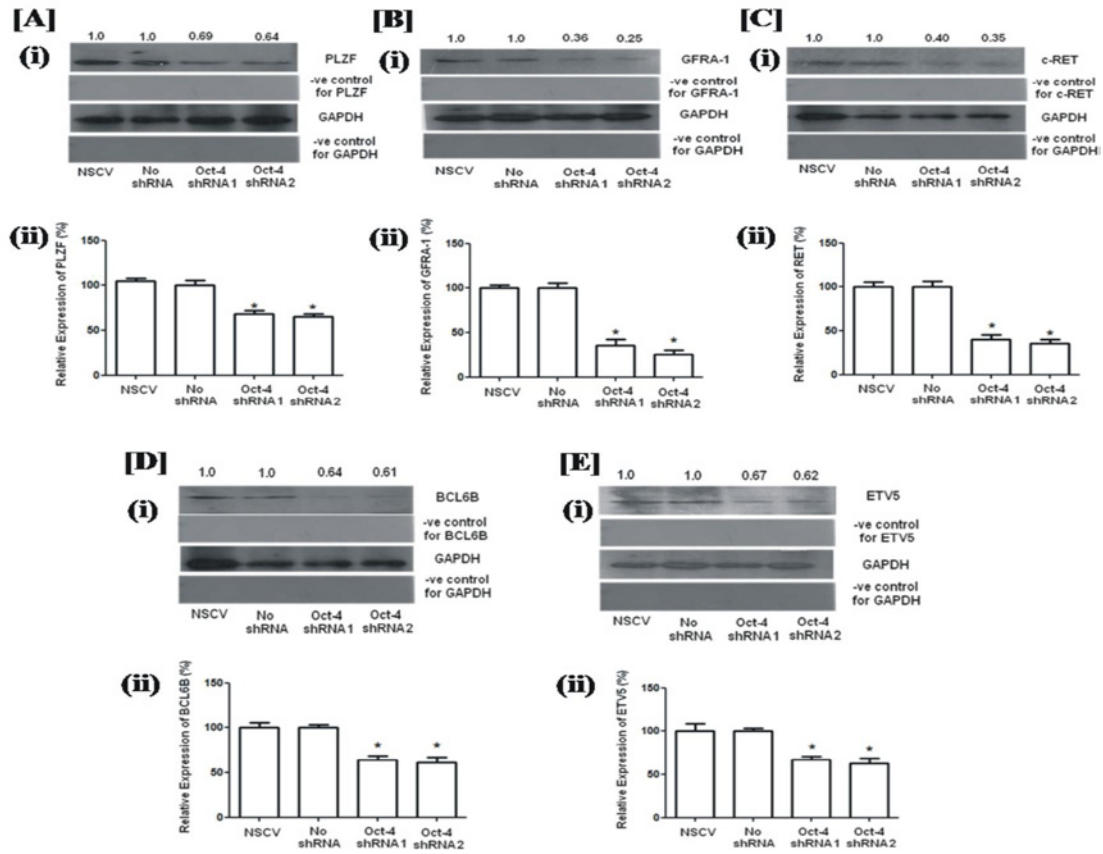

Figure 7: Effect of Oct-4 knock-down on the expression of markers for undifferentiated spermatogonia at the protein level. Western blot analysis of A (i) PLZF, B (i) GFRA-1, C (i) c-RET, D (i) BCL6B, E (i) ETV5 and densitometry analysis of A (ii) PLZF, B (ii) GFRA-1, C (ii) c-RET, D (ii) BCL6B, E (ii) ETV5 in Oct-4 shRNA treated and untreated cells. The replacement of primary antibody with PBS was used as a negative control. Values are the mean ± SD. n=3 different biological replicates performed on three different occasions (*$p<0.05$).

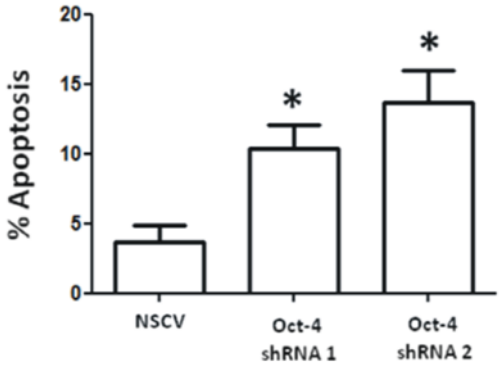

Figure 8: Oct-4 silencing induced 10.33 and 13.67% apoptosis in cultured undifferentiated type A spermatogonial cells when treated with Oct-4 shRNA 1 and Oct-4 shRNA 2. Values are the mean ± SD obtained from three different biological replicates performed on three different occasions, (n=3, *$p<0.05$).

further supported by apoptosis assay results which showed apoptosis of significant percentage of undifferentiated type A spermatogonial cells treated with shRNA when compared to untreated cells.

The results reveal that Oct-4 can be knocked down in undifferentiated type A spermatogonia using shRNA. The PI3K/Akt and ERK1/2-MAPK pathways which regulate the process of self-renewal may get affected directly and/or indirectly due to Oct-4 knock-down in undifferentiated type A spermatogonia. It is known at present that Oct-4 indirectly regulates both these pathways in Embryonic Stem (ES) cells [38,39]. Similarly, it is also known that Oct-4 indirectly controls *Etv5* in ES cells [40] therefore we believe that when Oct-4 was silenced in undifferentiated type A spermatogonial cells, the gene *Etv5* involved in the self-renewal of undifferentiated spermatogonia was down regulated hence, self-renewal of these cells might be affected. Therefore, we speculate that due to Oct-4 repression when cells differentiate into further lineage there is no longer expression of *Gfra-1, Ret* and *Bcl6b* genes in shRNA treated cells. To conclude, Oct-4 does play an important role in deciding the fate of undifferentiated spermatogonia by controlling their proliferation and differentiation.

Probing the molecular targets of Oct-4 is important in unraveling the dynamics of SSC function during spermatogenesis. The shRNA approach would prove to be a useful tool for manipulating SSC function and in turn would help in understanding the mechanisms that control SSC cellular activities.

Conclusion

Our findings show that shRNA mediated knock-down of Oct-4 in undifferentiated type A spermatogonia isolated from 5-6 dpp mice leads to down regulation in the expression of the markers for undifferentiated spermatogonia. Knock-down of Oct-4 further leads to phenotypic differentiation of undifferentiated spermatogonia to more mature germ cells. It also induces apoptosis and decreased proliferation of undifferentiated spermatogonial cells. These findings from our *in vitro* study lead us to believe that Oct-4 plays an important role of keeping spermatogonia in the undifferentiated state and thereby controlling their proliferation and differentiation.

Acknowledgements

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