

## Expression Pattern of OCT-4 and PLZF Transcription Factors during the Early Events of Spermatogenesis in Mice

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### Abstract

Spermatogenesis in mice is typically characterized by self-renewal and proliferation of spermatogonial stem cells (SSCs), which further give rise to mature sperm. The process of SSC self-renewal is regulated by number of genes. Oct-4 (POU family transcription factor) and Promyelocytic Leukemia Zinc Finger (Plzf) transcription factors are very well characterized and are known markers of undifferentiated spermatogonia. They are essential for self-renewal but their pattern of expression during the early events of spermatogenesis in mice has not been reported. Using real time PCR and Western blot we demonstrate marked increase in the expression of Oct-4 and Plzf in the testes of 10 days post partum (dpp) mice compared to 5 dpp both at the transcription and translation levels. The expression was found to be maximum in 10 dpp, declined in 20 dpp and was least in the testes of 35 dpp mice. However, our Immunohistochemistry (IHC) data showed the expression of OCT-4 & PLZF in the undifferentiated spermatogonia of 5, 10 & 20 dpp testes while in the testes of 35 dpp mice expression was seen in the undifferentiated as well as in differentiated spermatogonia. In conclusion, the present study reveals the expression pattern of Oct-4 and Plzf in the early stages of spermatogenesis of mice which sets the basis of spermatogenesis process in adults

**Keywords:** SSCs; Self-renewal; OCT-4; PLZF

### Introduction

Spermatogenesis is a very complex and well orchestrated process involving differentiation of male germ cells to produce mature spermatozoa, and is divided into three distinct stages: the mitotic proliferation of SSCs, meiotic division of spermatocytes, and spermiogenesis of haploid spermatids [1]. In the mammalian testes, spermatozoa are continuously produced in large quantities throughout the life. The foundation of this process is led by self renewing SSCs. The continuity of spermatogenesis is dependent on the self renewing and differentiating capacities of SSCs. During spermatogenesis in adult mice, the stem cell activity resides in a small, primitive set of spermatogonia referred as the undifferentiated spermatogonia corresponding to  $A_{\text{single}}$ ,  $A_{\text{paired}}$ , and  $A_{\text{aligned}}$  ( $A_s$ ,  $A_{\text{pr}}$  and  $A_{\text{al}}$ ) cells [2,3]. Of these, the  $A_{\text{single}}$  are considered to be the SSCs [2]. In the seminiferous tubules, all types of spermatogonia ( $A_{\text{single}}$  to B) are localized on the peripheral basement membrane.

SSCs are a very rare population constituting about 0.03% of the total population of the adult testicular germ cells [4]. In normal seminiferous epithelium, the ratio of 1.0 is maintained between self-renewal and differentiation of SSCs. More self-renewal than differentiation would reduce the seminiferous epithelium to only stem cells and might lead to the formation of a tumor. If differentiation prevailed, the stem cells would deplete themselves, leading to seminiferous tubules with only the supporting Sertoli cells [5]. The self-renewal process is critically regulated by number of genes expressed in undifferentiated spermatogonia such as Plzf, Oct-4, Ngn3, CD-9, Thy-1, Sox3, Gfra-1, c-Ret [6] and also by different signalling pathways (Src kinase, PI3K/Akt pathways etc) [7].

Oct-4 is expressed in the undifferentiated spermatogonia of testes especially in  $A_s$  cells [6]. During development, it is expressed in the unfertilized oocytes and confined to the inner cell mass (ICM) of blastocyst consisting of stem cell population [8]. After implantation, expression is restricted to epiblast compartment until the beginning of gastrulation when it is down regulated progressively in an anterior to posterior manner [9]. At 7.5 days post coitum (dpc) primordial germ

cells (PGCs) are the only cell types which express Oct-4. Expression of Oct-4 is maintained throughout the PGC migration [8-10] and is seen in both the sexes until 13.5 dpc. It is then down regulated in zygotene/pachytene female germ cells at 16.5 dpc while male germ cells still express Oct-4 at this stage [11]. After birth, expression is up-regulated in oocytes within primordial follicles at 12 and 14 dpp and in mature oocytes in adult females [11,12]. Oct-4 expression is found in type  $A_s$  spermatogonia of neonatal and adult testes and has been found to be essential for self-renewal of SSCs [6].

Plzf (Promyelocytic Leukaemia Zinc-Finger) is one of the first transcription factor shown to be required for self-renewal of SSCs [7] and is also co-expressed along with Oct-4 in undifferentiated spermatogonia [13]. Plzf is expressed during embryogenesis and plays a crucial role during limb and axial skeletal patterning. Targeted disruption of Plzf results in a testicular phenotype similar to that of luxoid mutant mice [14]. In the testes, Plzf expression is restricted to  $A_s$ ,  $A_{\text{pr}}$  and  $A_{\text{al}}$  undifferentiated spermatogonia [13]. A possible role of Plzf in spermatogonia could be the maintenance of an undifferentiated state [15], similar to the role suggested for Plzf in haematopoietic precursor cells [16]. Mutations in Plzf have been shown to cause intrinsic defects in self-renewal of SSCs [13,14].

Though, Oct-4 and Plzf are known to be expressed in undifferentiated spermatogonia and are involved in SSC self-renewal, their pattern of expression during the early stages of spermatogenesis

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in mice (5, 10, 20 and 35 dpp; the first wave of spermatogenesis) is not known. The main objective of the study is to evaluate the expression pattern of Oct-4 and Plzf in the 5, 10, 20 and 35 dpp mice when there is maximum alteration taking place in the expression levels of genes.

## Materials and Methods

### Experimental animals

Adult CD1 male and female mice were bred and maintained in institute's mice colony to get the 5, 10, 20 and 35 dpp pups for the study. 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.

### RNA isolation and cDNA synthesis

Total RNA was extracted from the testes of 5, 10, 20 and 35 dpp mice (Five animals for each age group) using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Sigma-Aldrich, St. Luis, MO, USA). First-strand cDNA was synthesized using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Briefly, 5X 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).

### Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Oct-4 and Plzf

Total RNA extracted from the testicular tissue of 5, 10, 20 and 35 dpp mice was reverse transcribed as mentioned earlier and was used for detecting Oct-4 and Plzf expression using specific primers designed with the help of Primer 3.0 online software (Oct-4: NM\_013633; Plzf: NM\_001033324) (Oct-4 Forward: 5'-cgggctgggtgatcctcga-3', Oct-4 Reverse: 5'-ttcacggcattggggcggtc-3'; Plzf Forward: 5'-gcaggagc-cagcaaggcga-3', Plzf Reverse: 5'-gcagagacccaggagggg-3'). Gapdh (NM\_008084) (Gapdh Forward: 5'-aactttgcatgttgaagg-3', Gapdh Reverse: 5'-acacattggggtaggaaca-3') was used as a housekeeping control. Briefly, 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<sub>2</sub>, 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 analyzed on 1.2% (w/v) agarose gel stained with 0.5 mg ml<sup>-1</sup> ethidium bromide (Sigma) and visualized under ultraviolet transilluminator. The product size was approximated using a 100-bp DNA ladder (Bangalore Genei, Bangalore, India). The negative control did not include cDNA in the reaction mixture.

### Q-PCR (Real-Time PCR) analysis of Oct-4 and Plzf in the testes of 5, 10, 20 and 35 dpp mice

The relative expression levels of Oct-4 and Plzf mRNA with respect to Gapdh housekeeping gene were estimated by CFX96 real-time PCR system (Bio-Rad) using SYBR Green chemistry (Bio-Rad). For each primer pair, reaction efficiency was estimated by the amplification of

serial dilution of mouse testicular cDNA pool over a 10-fold range. The amplification conditions for Oct-4 and Plzf 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 65°C for 30 sec, and extension at 72°C for 45 sec. The final extension was carried out for 7 min at 72°C. For Gapdh, the conditions were the same, except that the annealing temperature was optimized at 64°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<sub>t</sub> values generated in each experiment using the CFX Manager software (Bio-Rad) were used to obtain the standard curve, and the cDNA concentrations in the samples were computed and normalized to Gapdh. The relative expression levels in terms of fold change were calculated by 2<sup>-ΔΔC<sub>t</sub></sup> method [17].

### Western blotting

Testicular tissues of 5, 10, 20 and 35 dpp mice (Five animals for each age group) 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 was estimated using the Bradford's method [18]. 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). Electrophoresis was carried out on 10% (v/v) Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel under reducing conditions [19]. 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 (0.01M, pH 7.2) at room temperature (RT) for 2 h. The blots were incubated at 4°C for 18–20 h with anti-OCT-4 rabbit polyclonal (ab19857; dilution 1:200; Abcam, Cambridge, UK), anti-PLZF mouse monoclonal (ab104854; dilution 1:200; Abcam) and anti-GAPDH (as a loading control) rabbit polyclonal (G9545; dilution 1:2000; Sigma) antibodies diluted in PBS (0.01 M, pH 7.2). 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) for OCT-4 and GAPDH and HRP-conjugated goat anti-mouse secondary antibody (A9917; dilution 1:10,000; Sigma) for PLZF. The blots were then washed with PBST (0.1% v/v Tween20) for 10 min each and detected using the ECL Plus™ chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Further, the relative expression levels of the amount of OCT-4 and PLZF were semi quantitatively determined by densitometry (ratios of OCT-4 or PLZF band volume to GAPDH band volume).

### Immunolocalization of OCT-4 and PLZF in the testes of 5, 10, 20 and 35 dpp mice

Immunohistochemical studies were performed on 4% PFA fixed paraffin-embedded testicular sections of 5, 10, 20 and 35 dpp mice. Briefly, the 5 µ paraffin sections were deparaffinized and rehydrated through a graded ethanol series. The endogenous peroxidase was blocked using 0.3% (v/v) hydrogen peroxide for 30 min in dark at RT. Antigen retrieval was done by treating the sections with sodium

citrate buffer (10 mM sodium citrate, pH 6.0) at high power for 5 min in a microwave oven. This was followed by permeabilization step with 0.1% (v/v) Triton X-100 for 10 min. Blocking was done with 5% (v/v) normal goat serum for 1 h at RT. Sections were then incubated with anti-OCT-4 rabbit polyclonal antibody (ab19857; dilution 1:100; Abcam) and anti-PLZF Mouse monoclonal antibody (ab104854; dilution 1:100; Abcam) diluted in PBS at 4°C overnight. Primary antibody was replaced with PBS as negative control. After washing with PBS (0.01M, pH 7.2) three times for 5 min each, the sections were incubated with HRP conjugated goat anti-rabbit secondary antibody (A9169; dilution 1:200; Sigma) for Oct-4 and goat anti-mouse secondary antibody (A9917; dilution 1:200; Sigma) for Plzf for 1 h; the sections were then washed with PBS three times for 5 min each and detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) and counterstained with haematoxylin (Qualigens, Mumbai, India). Representative areas were photographed under DMLA Laser Capture Micro-dissection microscope (Leica, Wetzlar, Germany) at X1250 magnification. In order to count the number of positive cells for OCT-4 and PLZF staining, we counted total of 5 fields per section. The total numbers of sections scanned were ten per age group. The total number of positive cells counted was then divided by the total number of seminiferous tubule counted of the respective day. The SEM was calculated for the average values obtained for each day testicular sample and were considered to be significant if the values were less than 0.05 i.e. ( $p < 0.05$ )

### Statistical analysis

The fold change expression data is represented as mean  $\pm$  SD and number of cells positive for Oct-4 and Plzf is represented as mean  $\pm$  SEM. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA). Multiple comparisons of fold change expression of Oct-4 and Plzf between the testes of 5, 10, 20 and 35 dpp mice was carried out by Bonferroni's multiple comparison post hoc test. All the statistical analysis was done using GraphPad Prism software version 5.0 (GraphPad software, California, 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

### RT-PCR analysis of Oct-4 and Plzf in the testes of 5, 10, 20 and 35 dpp mice

The presence of Oct-4 and Plzf transcripts was studied in the testes of 5, 10, 20 and 35 dpp mice. Bands of expected size corresponding to Oct-4 (277 bp) and Plzf (196 bp) were obtained by RT-PCR with Gapdh as housekeeping control (223 bp) (Figure 1). No bands were seen in the negative control.

### Relative expression levels of Oct-4 and Plzf in the testes of 5, 10, 20 and 35 dpp mice

Relative testicular expression levels of Oct-4 and Plzf quantitated by Q-PCR using Gapdh (internal control) showed that the levels of Oct-4 increased ( $p < 0.001$ ) by almost 3.34 fold ( $3.34 \pm 0.85$ ) (Figure 2a) while that of Plzf by 2.83 fold ( $2.83 \pm 0.89$ ) ( $p < 0.01$ ) (Figure 2b) in the testes of 10 dpp mice when compared with 5 dpp testicular expression. Expression of Oct-4 and Plzf decreased ( $p < 0.001$ ) in the testes of 20 dpp mice and was least in 35 dpp with respect to 10 dpp testicular expression (Figure 2a & 2b). The study was repeated with three different biological replicates of each age group and two technical replicates each time. The similar results were obtained in all biological

replicates. We checked the expression levels of Oct-4 and Plzf in the testes of individual animal as well as in the pooled samples of each age group animals to rule out the animal to animal variation. We did not see any altered pattern of expression in individual as well as in the pooled RNA samples. The expression of Gapdh did not show variation in the expression levels of 5, 10, 20 and 35 dpp testes of mice and hence, was further used as an internal control.

### Expression of OCT-4 and PLZF protein in the testes of 5, 10, 20 and 35 dpp mice

A distinct 42 and 72 kDa size protein bands of OCT-4 (Figure 3a) and PLZF (Figure 3b) respectively were seen in the testicular protein extracts of 5, 10, 20 and 35 dpp mice. We carried out densitometry analysis on these blots and it showed almost 2.25 fold increase ( $2.28 \pm 0.453$ ) ( $p < 0.01$ ) (Figure 3c) in OCT-4 protein expression in the testes of 10 dpp mice when compared to 5 dpp testicular expression while PLZF protein expression was found to be 2.79 fold higher ( $0.352 \pm 0.044$ ) ( $p < 0.001$ ) (Figure 3d) in 10 dpp when compared to 5 dpp testicular expression. In 20 and 35 dpp mice decrease ( $p < 0.001$ ) in the expression of OCT-4 and PLZF was seen in comparison with 10 dpp expression. These results were in agreement with our Q-PCR data, which showed declining expression of OCT-4 and PLZF on day 20 and 35. The study was repeated with three different biological replicates of each age group and two technical replicates each time. The similar results were obtained in all biological replicates.

### Immunohistochemical localization and quantitation of number of cells positive for OCT-4 and PLZF in the testes of 5, 10, 20 and 35 dpp mice

Expression of OCT-4 (Figure 4) and PLZF (Figure 5) was seen in the undifferentiated spermatogonia of 5, 10 & 20 dpp mice (Black

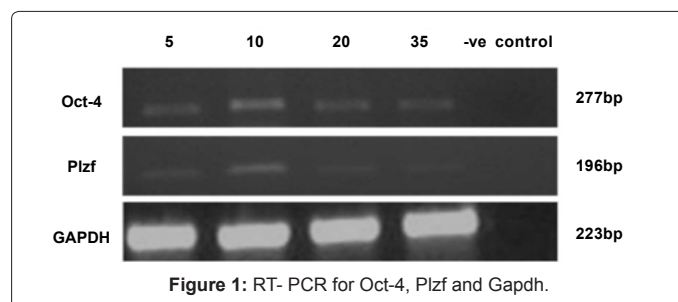


Figure 1: RT-PCR for Oct-4, Plzf and Gapdh.

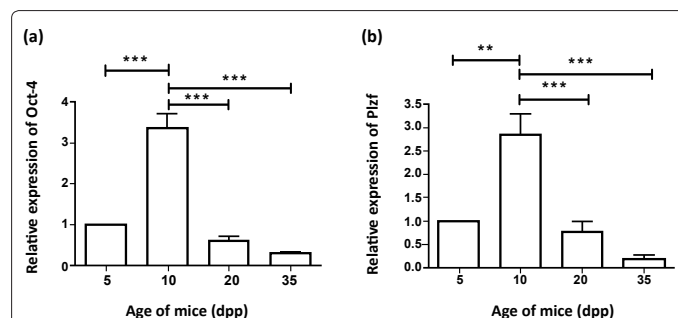
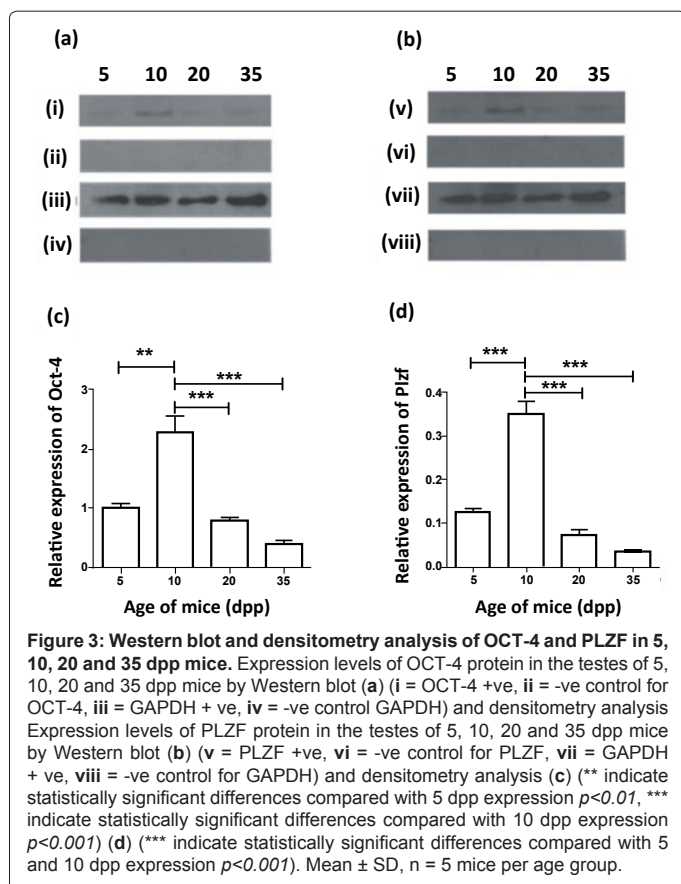


Figure 2: Real-Time PCR for Oct-4 and Plzf. (a) Expression of Oct-4 (\*\*\*) indicate statistically significant differences compared to 5 and 10 dpp expression  $p < 0.001$ ) and (b) Plzf (\*\* indicate statistically significant differences compared with 5dpp expression  $p < 0.01$ , \*\*\* indicate statistically significant differences compared with 10 dpp expression  $p < 0.001$ ) transcript levels in the testes of 5, 10, 20 and 35 dpp mice. Mean  $\pm$  SD, n = 5 mice per age group.



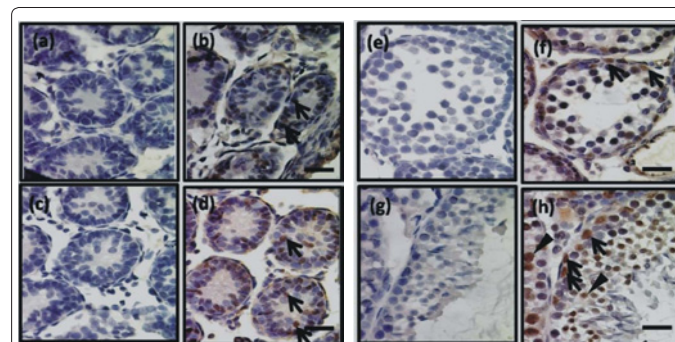
arrows). In the testes of 35 dpp mice, expression was seen not only in the undifferentiated spermatogonia (Black arrows) present at the basement membrane but also in the differentiated spermatogonia (pointed black arrows). We counted the number of positive cells present in the tubules of 5, 10, 20 and 35 dpp testes. The number of positive cells for OCT-4 and PLZF was found to be greater ( $p < 0.05$ ) in the testes of 10 dpp mice compared to 5 & 20 dpp. In the testes of 35 dpp mice, the number of OCT-4 & PLZF positive cells was found to be much higher compared to 10 dpp due to their expression in undifferentiated & differentiated spermatogonia (Figure 6 & Table 1). No staining was seen in the negative control where primary antibody was replaced with isotype control.

## Discussion

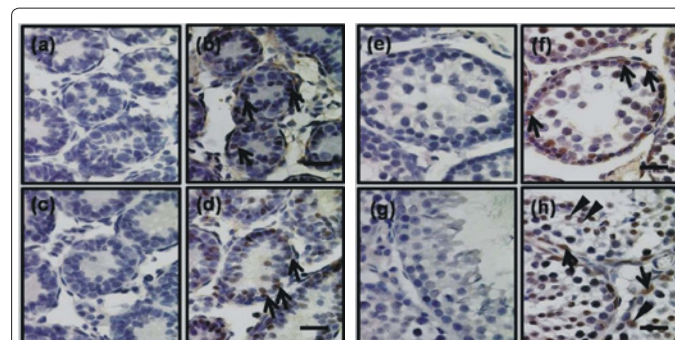
In this study we selected the early stage of spermatogenesis i.e. 5, 10, 20 and 35 dpp mice as it sets the basis for spermatogenesis process. The first wave of spermatogenesis is marked by the appearance of different germ cells at specific day point. At 0 dpp, gonocytes are in the quiescent stage, around 3 dpp gonocytes are converted into SSCs, on 5 dpp proliferation of SSCs starts, 10 dpp first appearance of spermatocytes, 20 dpp marked by appearance of spermatids and 35 dpp first appearance of spermatozoa marking the end of the first wave of spermatogenesis [20,21]. With the appearance of different populations of germ cells, alterations in the expression levels of genes involved in the spermatogenesis takes place during this period. Therefore, it is essential to study candidate genes involved in the process of spermatogenesis. We decided to pick up the genes which are not only involved in the spermatogenesis but also are required for self-renewal process of SSCs, as this population of cells forms the basis of spermatogenesis. We chose

Oct-4 and Plzf genes in this study for two reasons. First, Oct-4 is a known marker for undifferentiated spermatogonia [6] while Plzf is one of the first transcription factors found to be essential for self-renewal of SSCs [7] and both are co-expressed in the undifferentiated spermatogonial cells [13]. Secondly, Oct-4 and Plzf do not come under GDNF pathway which is crucial for self-renewal of SSCs [22]. It is possible that they follow different mechanisms of SSC self-renewal [7] and might be directly or indirectly controlling the number of genes involved in the process of self-renewal. In order to find out the mechanism of their action, first it is essential to undertake the studies on the expression pattern of these genes.

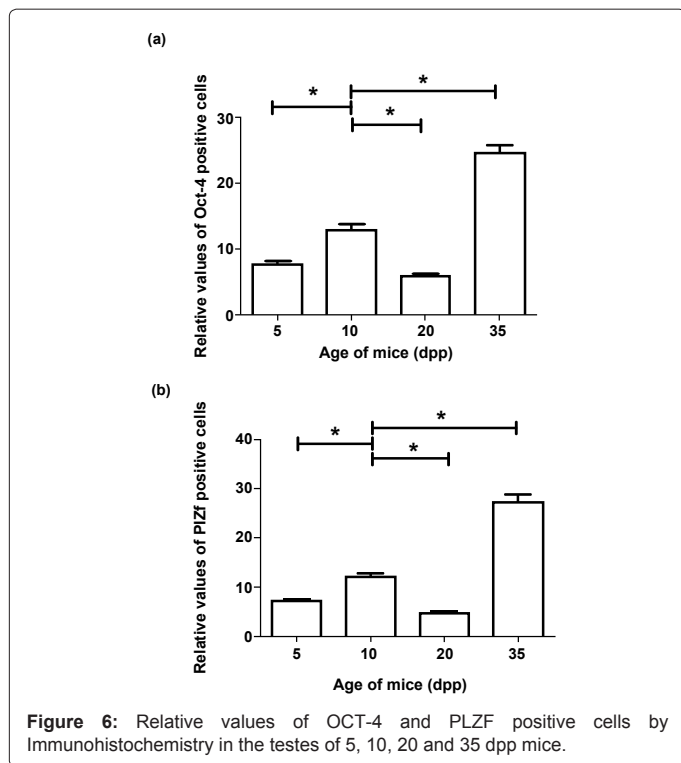
In the present study, the analysis of expression pattern of Oct-4 and Plzf revealed that both are expressed at the gene and the protein level in the undifferentiated spermatogonia of mice in the first week after birth. We further demonstrate that testicular Oct-4 and Plzf mRNA and protein levels are higher in the testes of 10 dpp mice than that of 5 dpp mice. This could be due to the process of SSC self-renewal which starts in the first week after birth and SSCs are the dominant population at this stage in the seminiferous tubules. Thereafter, we observed decreased expression of Oct-4 and Plzf at mRNA and protein level in 20 and 35 dpp testes indicating the appearance of differentiated germ cells viz. spermatocytes, spermatids and spermatozoa from 10 dpp onwards. In other words the number of differentiated germ cells would be in higher number as compared to SSCs and therefore one would expect a possible decrease in the expression of these SSC markers in



**Figure 4: Immunohistochemistry for OCT-4 in 5, 10, 20 and 35 dpp mice.** Arrows indicate expression of OCT-4 in SSCs. (a = -ve control for 5dpp; b = 5dpp OCT-4 +ve; c = -ve control for 10 dpp; d = 10 dpp OCT-4 +ve; e = -ve control for 20 dpp; f = 20 dpp OCT-4 +ve; g = -ve control for 35 dpp; h = 35 dpp OCT-4 +ve) (Scale bar = 8  $\mu$ m).



**Figure 5: Immunohistochemistry for PLZF in 5, 10, 20 and 35 dpp mice.** Arrows indicate expression of PLZF in SSCs. (a = -ve control for 5 dpp; b = 5 dpp PLZF +ve; c = -ve control for 10dpp; d = 10 dpp PLZF +ve; e = -ve control for 20 dpp; f = 20 dpp PLZF +ve; g = -ve control for 35 dpp; h = 35 dpp PLZF +ve) (Scale bar = 8  $\mu$ m).



**Figure 6:** Relative values of OCT-4 and PLZF positive cells by Immunohistochemistry in the testes of 5, 10, 20 and 35 dpp mice.

Age of mice (dpp)	Cells positive for OCT-4 / seminiferous tubule section <sup>a</sup>	Cells positive for PLZF/ seminiferous tubule section <sup>b</sup>
5	7.6 ± 0.6	7 ± 0.32
10	12.8 ± 0.97	12 ± 0.84
20	5.8 ± 0.37	4.8 ± 0.2
35	25.2 ± 2.083	27.2 ± 1.655

<sup>a</sup>Mean Number of Cells positive for OCT-4 ± SEM

<sup>b</sup>Mean Number of Cells positive for PLZF ± SEM [\* (*p*<0.05)]

**Table 1:** Number of positive cells for OCT-4 and PLZF by Immunohistochemistry in the testes of 5, 10, 20 and 35 dpp mice.

day 20 and 35 testicular tissue. However, in our quantitative analysis of Oct-4 and Plzf positive cells in IHC study, we found that there were not only Oct-4 & Plzf positive undifferentiated spermatogonia present but also some of the differentiated germ cells showed the expression of Oct-4 & Plzf. Surprisingly, this finding did not correlated with our real-time PCR and Western blot data which showed the highest expression at 10 dpp and then decline of expression in the testes of 20 & 35 dpp mice. It is still surprising as to why these differentiated germ cells which are expressing Oct-4 & Plzf did not contribute to the total expression at mRNA and protein level by Real-time PCR & Western blot respectively. This finding can't be ruled out & to our knowledge we are the first ones to show that Oct-4 & Plzf expression is not only restricted to the undifferentiated spermatogonia as published by Pesce et al. [11] in 1998. Although, there is only one report recently published which showed the expression of POU3F1 & POU5F1 in sertoli cells of adult mouse testes [23]. We still do not have the answer for such phenomenon and it makes a significant impact to our understanding of the use of Oct-4 & Plzf as important markers of undifferentiated spermatogonia.

Thus far no one has reported this quantitation and pattern of expression of Oct-4 and Plzf in mice testicular tissue during the early stages of spermatogenesis. Earlier reports on the expression of Oct-4

and Plzf are qualitative [11,13,14]. Study by Pesce et al. [11] has shown the expression of Oct-4 in the undifferentiated spermatogonia of 1 dpp, 7 dpp, 17 dpp and the adult testes of mice by IHC [11]. Kokkinaki et al. [24] reported the expression pattern of genes in stem/ progenitor cells. In this study, they observed higher expression of Oct-4 at 6 dpp. This study discusses the expression levels of different genes at the RNA level by microarray but fails to mention anything at the protein level. Similarly, Costoya et al. [14] have showed the expression of Plzf protein in spermatogonia of post natal mice testes of 1 week of age. All these studies show the expression of Oct-4 and Plzf in the testis and do not discuss about the quantitative expression of Oct-4 and Plzf at mRNA and protein level and have not studied the entire first wave of spermatogenesis till 35 dpp. Our study makes a significant contribution in understanding the expression pattern of Oct-4 and Plzf at mRNA and protein level quantitatively during the first wave of spermatogenesis.

In summary, increased expression of OCT-4 and PLZF at mRNA and protein level in the testes of 10 dpp mice gives us some clue that both OCT-4 and PLZF must be playing an important role especially during 5-10 dpp when there is maximum proliferation of SSCs occurring and they might be controlling the genes which are crucial for self renewal of SSCs in the early stages of spermatogenesis after birth. With the appearance of more differentiated germ cells, role of OCT-4 & PLZF might be dispensable. We still do not know what role OCT-4 & PLZF have in differentiated spermatogonia which express OCT-4 & PLZF. This study can also be further extended to delineate the role of these genes in proliferation and differentiation of SSCs thereby determining their fate during the spermatogenesis process.

## Conclusion

In summary, increased expression of Oct-4 and Plzf at mRNA and protein level in the testes of 10 dpp mice gives us some clue that both Oct-4 and Plzf must be playing an important role especially during 5-10 dpp when there is maximum proliferation of SSCs occurring and they might be controlling the genes which are crucial for self renewal of SSCs in the early stages of spermatogenesis after birth. With the appearance of more differentiated germ cells, role of Oct-4 & Plzf might be dispensable. We still do not know what role Oct-4 & Plzf have in differentiated spermatogonia which express Oct-4 & Plzf. This study can also be further extended to delineate the role of these genes in proliferation and differentiation of SSCs thereby determining their fate during the spermatogenesis process.

The authors hereby declare that there are no conflicts of interest (financial or otherwise) in the current study.

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## References

- Zirkin BR (1993) Regulation of spermatogenesis in the adult mammal: gonadotrophin and androgens. In: Desjardins C, Ewing LL (Eds) Cell and Molecular Biology of the Testis. New York: Oxford University Press 166-188.
- de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21: 776-798.
- de Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121: 347-354.
- Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290: 193-200.

5. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, et al. (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287: 1489-1493.
6. Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, et al. (2008) Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells* 26: 2928-2937.
7. He Z, Kokkinaki M, Dym M (2009) Signaling molecules and pathways regulating the fate of spermatogonial stem cells. *Microsc Res Tech* 72: 586-595.
8. Schöler HR, Dressler GR, Balling R, Rohdewohld H, Gruss P (1990) Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J* 9: 2185-2195.
9. Rosner MH, Viganò MA, Ozato K, Timmons PM, Poirier F, et al. (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* 345: 686-692.
10. Schöler HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P (1990) New type of POU domain in germ line-specific protein Oct-4. *Nature* 344: 435-439.
11. Pesce M, Wang X, Wolgemuth DJ, Schöler H (1998) Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mech Dev* 71: 89-98.
12. Palmieri SL, Peter W, Hess H, Schöler HR (1994) Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol* 166: 259-267.
13. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, et al. (2004) Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* 36: 647-652.
14. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, et al. (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* 36: 653-659.
15. Filippini D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, et al. (2007) Repression of kit expression by Plzf in germ cells. *Mol Cell Biol* 27: 6770-6781.
16. Reid A, Gould A, Brand N, Cook M, Strutt P, et al. (1995) Leukemia translocation gene, Plzf, is expressed with a speckled nuclear pattern in early hematopoietic progenitors. *Blood* 86: 4544-4552.
17. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> Method. *Methods* 25: 402-408.
18. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
19. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
20. Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, et al. (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol* 74: 68-85.
21. Zhao GQ, Garbers DL (2002) Male germ cell specification and differentiation. *Dev Cell* 2: 537-547.
22. Hofmann MC (2008) Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol Cell Endocrinol* 288: 95-103.
23. Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR, et al. (2010) The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. *Biol Reprod* 82: 1103-1111.
24. Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, et al. (2010) Age affects gene expression in mouse spermatogonial stem/progenitor cells. *Reproduction* 139: 1011-1020.