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Design and Molecular Characterization of *C-Kit* Transgene Construct during Spermatogenesis in Mice

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Abstract

In the postnatal testis, undifferentiated Type-A Spermatogonial Stem Cells (SSCs) differentiate into Type-B Spermatogonial Cells (SGCs) in the presence of c-*Kit*, which is expressed in a cell/stage specific manner during spermatogenesis in mammals. *c-Kit* is subjected to tight transcriptional control as it is essential not only for germ cells but also for the development of hematopoietic stem cells and melanocytes. Although, *c-Kit* expression is a dynamically regulated process, the molecular basis for its transcriptional regulation during spermatogenesis remains mostly unidentified. The aim of the study is to determine whether or not introduction of *c-Kit* expressing transgene construct into impaired *c-Kit* expressing SSCs isolated from W^v/W^v mutant mice will differentiate and colonize into testis of recipient mice. For this purpose, a 7.4 kb *pG1-Kit*-ORF-GFP transgene construct that contains a 4.4 kb c-*Kit*-ORF-GFP expression cassette was designed and its *in vitro* expression was confirmed by immunofluorescence and flowcytometry analysis. For comparison of the results, three control-GFP constructs, having 1.1 kb (lacking both *c-Kit* promoter and *ORF*), 4.1kb (lacking *c-Kit* promoter) and 1.4 kb (lacking *c-kit*-ORF) were also designed and introduced into SSCs by electroporation (EP). These SSCs were introduced into the testis of busulfan treated recipient infertile mice via rete using Germ Cell Transplantation (GCT) approach. It was observed that SSCs carrying *c-Kit*-ORF-GFP transgene insert were able to colonized and differentiated into other germ cell lineages in the seminiferous tubules of recipient mice as compared to controls. In conclusion, the testis of W^v/W^v mutant mice have the population of SSCs, wherein the function of *c-Kit* signaling is impaired. For the first time we report that SSCs function in the recipient infertile mice could be resumed with the introduction of c-*Kit*-ORF-GFP transgene insert. Thus the present findings may have significant impact in understanding reproductive physiology and potential for novel future therapies in patients with testicular failure.

Keywords: c-*Kit*; *Kit*-GFP transgene construct; SSCs; Germ cell transplantation

Introduction

Spermatogenesis is highly complex, well-orchestrated and efficient biological process in which Spermatogonial Stem Cells (SSCs) undergo self-renewal, proliferation and differentiation to produce virtually unlimited number of spermatozoa [1]. During this process, several genes (*c-myc*, *c-Kit*, *Oct-4*, Androgen Binding Protein (*ABP*), Cyclic-AMP Responsive Modulator (*CREM*), *Protamine-1*, Heat Shock Protein-70 (*HSP-70*), Phosphogylcerate Kinase-2 (*PGK-2*), Transition Nuclear Protein-2 (*TNP-2*) and Azoospermic Factor-1 (*AZF-1*) etc.) are known to be expressed in cell and stage specific manner in the testis [1,2]. Among these genes, great attention has been focused in recent years on *c-Kit*, since it is indispensable right from the gonadal development to the differentiation of SSCs. Lack of or poor expression of *c-Kit* is responsible for abnormal spermatogenesis and infertility in males. In certain cases, infertility has been attributed to functional defects in *c-Kit* [3, 4]. *c-Kit SCF (Stem Cell Factor)* signaling is essential for the survival of SSCs [3,4]. However, the molecular basis of male infertility arising from defects in c-*Kit* remains poorly understood.

c-Kit, a type III Receptor tyrosine kinase (RTK) that has sequence and structural similarities to the platelet-derived growth factor receptor-β-polypeptide (PDGFRB) family of RTKs [5,6]. *c-Kit* is allelic to the W locus on the mouse chromosome-5 [7,8]. The 21-exon *c-Kit* gene encodes 5.15 kb transcript, which translates into a product of ~150 kDa protein with 979 amino acids [8-10]. In the testis of mouse, development, proliferation and migration of primordial germ cells (PGC's) starts at 7.5 day post coitum (dpc) and c-*Kit* mRNA expression is detected in the PGCs, From 15 dpc to 3 day post-partum (dpp), c-Kit expression is markedly reduced which coincides with the period of gonocytes quiescence [3,8]. Synthesis of *c-Kit* mRNA and protein during testicular development is in concordant with the first appearance of differentiating SSCs (A_1-A_4) which occur at 7 dpp.

Studies on the transcription factors that enhances or represses c-*Kit* promoter activity would determine the cellular expression during normal and abnormal spermatogenesis, which will provide valuable clues about the mechanisms underlying the development and differentiation of SSCs. After considering various transcription factors reported in the literature for optimal *c-Kit* activity, we designed germ cell specific *c-Kit* transgene construct. For instance, the transcription of the *c-Kit* is controlled by regulatory sequences located in the 5' flanking region of the gene [10,11]. This region includes both core promoter sequences and also tissue-specific enhancer sequences. The Proximal Promoter Region (PPR) is located 58 bp upstream of translation start site in the mouse promoter region. The PPR contains consensus regulatory binding sites such as SP1, Ap-2 and short GA-rich elements [10-13].

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SSCs are the only adult stem cell that contributes genes to subsequent generations. Recently studies revealed that SSCs have received a great deal of attention due to success in achieving their isolation and maintain them in cultures under undifferentiated condition. Brinster and group who are the pioneers in this area have reported in one of their papers that SSCs can be genetically modified and make them to differentiate into fully competent spermatozoa following transplantation into the recipient mice testis [14]. In the past, several investigators have demonstrated that busulfan treated mice can serve as a good recipient model in Germ Cell Transplantation (GCT) studies [14,15]. In one of these studies it has been demonstrated that, following testicular GCT of SSCs, isolated from W^v/W^v mutant mice were able to colonize the seminiferous tubules in the busulfan-treated mice [15]. However, these cells failed to differentiate due to defects in the KIT/KITL signaling system. In the present study, we used W^v/W^v mutant mice as a source for the isolation of undifferentiated SSCs. We designed 7.4 kb *p*G1-*Kit*-ORF-GFP transgene construct from which a 4.4 kb *c-Kit-ORF-GFP* insert was excised and introduced into SSCs by electroporation (EP). Upon transplantation of SSCs carrying c-*Kit*-ORF-GFP transgene insert into the seminiferous tubule of busulfan treated recipient mice testis, they were differentiated and subsequently colonized. To the best of our knowledge this is the first study revealing the design of testis specific *pG1-c-Kit-ORF-GFP* transgene construct and it's *in vitro* expression and *in vivo* differentiation and colonization in the recipient mice testis.

Materials and Methods

Experimental animals

Male mice pups (n=36) were obtained by crossing C57BL/6 male mice with DBA/2 females. On 30 dpp, the male pups were administered with single injection of busulfan (40 mg/kg body weight) subcutaneously (s.c), to deplete the endogenous testicular germ cells. Post treatment day 30^{th}, two mice were sacrificed by CO_2 asphyxiation; testes were isolated and checked for the presence of *c-Kit* mRNA and protein expression. To determine whether the treatment has any adverse effect on Sertoli cells, expression of specific marker genes (GATA-1 and PEM-1) of these cells were determined. For the isolation of SSCs, W^v/W^v mutant mice were used (Jackson Laboratories, USA). All animals were housed at 25°C with 12L: 12D photoperiod and were given water and a standard diet *ad libitum*. The experimental protocols discussed in this study were approved by the Institutional Animal care and Ethics Committee (IAEC) of the National Institute for Research in Reproductive Health, Mumbai (IAEC# 1/03) in accordance with the guidelines of a committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) established by Govt. of India on animal care.

Cell lines

P815 (c-Kit expressing murine mastocytoma cells) and ES-E14TG2α (c-Kit expressing embryo carcinoma cells) cell lines were procured from National Centre for Cell Science (NCCS), Pune, India, and cultured as described earlier [3]. Bone marrow derived cell line (BMDC) was procured from ATTC and cultured as per the suppliers instructions.

Design of pG1-c-Kit -ORF-GFP transgene construct

For c-kit promoter amplification, genomic DNA was isolated (Quiagen DNA mini Prep) from the testes of adult mice and expected 302bp PCR product was obtained using promoter specific primers (F5′-AGGGAGAGTGCTAGGAGGAA-3′; R 5′-CGCGGTGGCTGCGCT AGA CT-′3). For *c-Kit* gene ORF amplification, total RNA was extracted from the testes of 10 dpp mice and cDNA was prepared using the iScript cDNA synthesis kit. An expected size of 2937bp *c-Kit* gene product (excluding stop codon) was amplified by RT-PCR using gene specific primers (F 5′-ATGAGAGGCGCTCGCGGCGC-3′; R 5′- GGCATCTTCGTGCACGAGCA -3′). Next *c-Kit* promoter and *c-Kit* gene were cloned into *p*MosBlue vector and transformed into *E.coli* competent cells [16]. The presence of the insert was confirmed by colony PCR and validation of the sequences was carried out at the Institutional central DNA sequencing facility. A 7.4 kb *pG1-Kit-ORF*-GFP transgene construct was designed, which contains 4.4 kb c-*Kit* expression cassette (302 bp *c-Kit* promoter, 2937bp of *c-Kit* gene, a 780 bp sequence from the GFP reporter gene, 200 bp SV40 poly-A tail and 200 bp plasmid regulatory sequences). For making the final construct, these transgene elements were orderly cloned in *p*G1 vector (ATCC, # 37305) and confirmed the presence of each insert by DNA sequencing [17]. The Mlu-1 and Apa-1 digested linearized plasmid insert 4.4kb was introduced into SSCs by electroporation (EP). To rule out non-specific expression of c-Kit in SSCs, three control-GFP constructs were designed: (1) control GFP-encoding construct lacking *c-Kit* promoter and *c-Kit*-ORF. This ~1.1 kb insert consists of a 780 bp sequence from the GFP reporter gene, 200 bp SV40 poly-A tail and 200 bp plasmid regulatory sequences, (2) control GFP-encoding construct lacking *c-Kit* promoter. This ~4.1 kb insert consists of 2937 bp *c-Kit*-ORF , a 780 bp sequence from the GFP reporter gene, 200 bp SV40 poly-A tail and 200 bp plasmid regulatory sequences and 3) control GFP-encoding construct lacking *c-Kit* –ORF. This ~1.4 kb insert consists of 302 bp *c-Kit*-ORF, a 780 bp sequence from the GFP reporter gene, 200 bp SV40 poly-A tail and 200 bp plasmid regulatory sequences.

In vitro transient transfection of *pG1-c-Kit*-ORF-GFP transgene construct in P815 and ES-E14TG2α cells

To determine whether the *in vitro* expression efficiency of 7.4 kb pG1-*c-Kit*-GFP transgene fusion construct is under the control of 302 bp *c-Kit* promoter, P815 and ES-E14TG2α cells (1×10^6/well) at ~80 % confluence were transfected using Lipofectamine reagent [3]. At 72 hrs post transfection, P815 and ES-E14TG2α cells were washed with PBS (pH 7.4) and grown on glass cover slips in a 6 well plates, fixed for 10 min in 4% paraformaldehyde-PBS and observed under fluorescent microscope using GFP filter (515 nm). To rule out non-specific expression, ES-E14TG2α cells were transfected with 4.3 kb *p*G1 control vector (3.2 kb vector backbone and a 780 bp sequence from the GFP reporter gene, 200 bp SV40 poly-A tail and 200 bp plasmid regulatory sequences). The fluorescence images were obtained using a digital camera (PM-10SP, Olympus).

Culture of spermatogonial stem cells (SSCs) isolated from W^v/W^v mutant mice after introduction of 4.4 kb *c-Kit*-ORF-GFP transgene insert

Donor undifferentiated SSCs were isolated from W^v/W^v mutant mice testes using a two-step enzymatic digestion followed by laminin selection protocol [18,19]. Our results and previous reports by others [20,21] suggests that the SSCs enriched by this method contain more than 90% undifferentiated SSCs. These cells (~10^5) were resuspended in DMEM containing 10% FBS and transferred into a sterile electroporator cuvette (4 mm). The c-*Kit*-ORF-GFP insert in 10 µl of PBS (2 µg) was introduced into SSCs. Same protocol was followed to introduce all the three control-GFP inserts into SSCs. To enrich control and *c-Kit*-GFP transgene insert introduced SSCs, they were cultured for 48 hrs in the

absence or in the presence of growth factors like leukemia inhibitory factor (LIF), glial cell derived neurotropic factor (GDNF) and bovine fibroblast growth factor (bFGF). To determine the differentiation status, cultured SSCs were analyzed for c-Kit expression by indirect immunofluorescence.

Immunofluorescence evaluation of busulfan treated mice testis

Testicular cross sections of 5 µm were cut from prefixed tissue blocks and deparaffinized. After blocking non-specific binding sites with PBS-BSA, sections were incubated overnight at 4°C with 1:100 dilution of mouse anti-human c-Kit monoclonal antibody (SantaCruz, USA). Following three washings in PBS, the slides were incubated with FITC conjugated goat anti-mouse secondary antibody at a dilution of 1:500 for 1 hr at 37°C. The slides were counterstained with nuclear stain, DAPI. After washing with PBS, slides were mounted with Prolong gold anti-fade reagent (Invitrogen) and images were captured by Laser scanning confocal microscope (LSCM) (Zeiss, 510 META, Germany) (X40) at our Institute Central facility. Slides incubated with secondary antibody alone considered as negative controls.

Flowcytometry analysis

Flow cytometry was performed on a FACS vantage flow cytometer (Becton Dickinson, NJ, USA) to evaluate the surface expression of c-Kit protein in SSCs. Based on initial optimization results, the cells (1×10^5/well) were transfected with 10 µg of 4.4 kb *c-Kit*-ORF-GFP transgene as well as 1.1 kb control insert. Transfected cells were washed twice with DMEM, and analyzed at 72 hrs post- transfection. Excitation of cells was performed with an argon ion laser at a wavelength of 488 nm and the emission of the green fluorescence was detected using a band pass filter of 530/30 nm. Data was collected for a minimum of 10,000 cells per sample. Fluorescence histograms were generated after gating the cell population using cell quest software (http:// facs.scripps.edu). Channels M1 and M2 represented low intensity and high-intensity staining, respectively.

Western blotting

The total protein content of the testicular lysates was determined in wild type and busulfan treated mice using BSA as standard [22]. Equal amount of testicular protein (20 µg/well) was resolved as per the standard protocols using 10% SDS-PAGE and transferred onto nitrocellulose membrane [23,24]. The blots were incubated with mouse anti-human c-Kit monoclonal antibody (SantaCruz, USA) at a dilution of 1:100 in PBS-NFDM followed by incubation with goat anti-mouse HRP conjugated secondary antibody at a dilution of 1:1000 in 1% PBS-NFDM. The same protocol was followed to determine the expression of GFP in *c-Kit*-ORF-GFP introduced and control-GFP introduced contra-lateral testes on day 56 of post GCT using mouse monoclonal anti-GFP antibody (1:100 dilution; Sigma, USA) and peroxidase conjugated goat anti-mouse secondary antibody (1:1000 dilution; Sigma, USA). Normal mouse serum in place of primary antibody was used as negative control.

RNA extraction and RT-PCR analysis

Total RNA was extracted from the testes of control-GFP insert (1.1 kb) and c-*Kit*-ORF-GFP transgene insert (4.4 kb) on post transplantation day 56 to determine the expression of germ cell and Sertoli cell specific marker genes as described earlier [24]. The primer sequences used for various genes are given in table 1. cDNA was synthesized using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, CA, USA). Cyclophilin-A was used as housekeeping gene.

Gene	Gene Accession No	Primer Sequence (5'-3')	Prod Size (bp)
c-Kit	NM_001122733.1	F- ACCCACAGGTGTCCAATTATTC	403
		R-TGGCGTTCATAATTGAAGTCAC	
Rbm-1	NM_001270510.1	F- AACCGAAGTAACATATACTCA	209
		R-ATCTGCTTTCTCCACGACCTC	
Oct-4	NM_013633.3	F-AGCTGCTGAAGCAGAAGAGG	466
		R-TGGGAAAGGTGTCCCTGTAG	
Gfp	M62653	F- AGGACGACGGCAACTACAAG	312
		R-CTGGGTGCTCAGGTAGTGGT	
Gata-1	NM_008089.1	F-TGTGTGAACTGTGGAGCAACGGC	242
		R-AAATGAAGGCCGCAGGCATTGCA	
Pem-1	NM_008818.2	F-AAGAACAGCATGATGTGA	566
		R-TCAAAATCTCGGTGTCGCAAA	
Haploid cell marker genes			
Tnp2	NM_013694.4	F-CGGCCTCAAAGTCACACCAGT	255
		R- AGTCCGTTTCCGCCTCCTGAC	
Hils-1	NM_018792.1	F-TGGAGTATCTAGCACCTGGAGT	218
		R-TCAGAATCACATACGACATGGT	
Leydig cell marker gene			
Lhr	NM_013582.2	F- AATCCCATCACAAGCTTTCAG	215
		R- TGCCTGTGTTACAGATGC	
Spermatocyte marker			
Pgk-2	NM_031190.2	F- GACAATGGAGCCAAGTCCGT	353
		R- GCAGTGCCAAATGCATCGTT	
House keeping genes			
Cyclophilin-A	NM_008907.1	F-AAGTTCCAAAGACAGCAGAAAACT	465
		R-GAGCTACAGAAGGAATGGTTTGAT	
Gapdh	NM_008085.1	F-CCATTCATTGACCTCCACTACA	199
		R-CGTTGCTGACAATCTTGAGAGA	

Table 1: Germ cell specific gene primers were used to confirm the differentiation status of SSCs into other germ cell lineages *in vivo*.

In vivo injection of SSC's carrying c-Kit-ORF-GFP transgene inserts introduced into the seminiferous tubules of busulfan treated recipient mice

A total of 18 testes (right side) from 18 mice were introduced with electroporated undifferentiated SSCs carrying 4.4 kb *c-Kit*-ORF-GFP transgene insert. The 18 contra-lateral testes were divided into three groups each consists of six testes and were injected with control-GFP inserts lacking either *c-Kit* promoter (4.1 kb) or *c-Kit*-ORF (1.4 kb) or both (1.1 kb). To obtain an enriched population of SSCs, they were isolated from all the three control and c-*Kit*-ORF-GFP transgene introduced testes and cultured for 48 hrs in the special medium containing growth factors. SSCs were transplanted into the seminiferous tubules of busulfan treated recipient mice via rete as described previously [21]. Approximately 10-15 µl of the cell suspension was used to fill at least 75–85% of the seminiferous tubules in the right side testes (n=18). Same volume of cell suspension from three control-GFP groups was injected into the contra-lateral testes (n=18). Testes were directly observed under microscope using GFP filter on day 56 of post GCT. To assess the colonization efficiency of transplanted SSCs, mice (n=3/group) on day 56 of post GCT were sacrificed by CO_2 asphyxiation. Testes were collected and subjected to

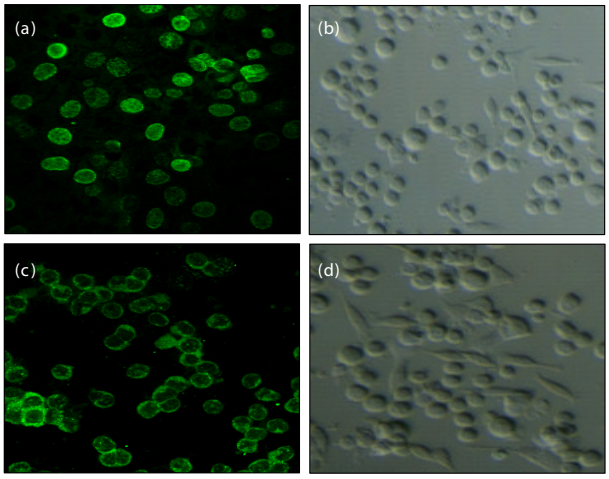

Figure 1: *In vitro* expression analysis of 7.4 kb pG1c-*Kit*-ORF-GFP transgene construct in ES-E14TGα cells **(a)** and P815 cells (c) at 72 hrs post transfection. Light microscopic images of ES-E14TGα cells (b) and P815 cells (d) transfected with 4.0 kb control construct. Figures shown are the representative picture from two different experiments performed on two different occasions (Magnification X 630).

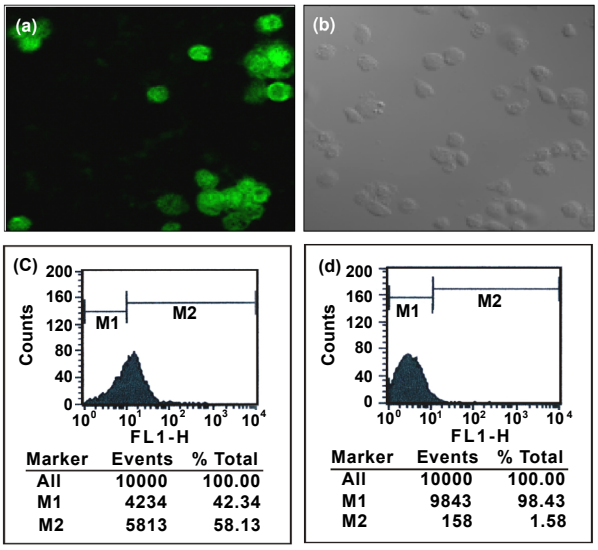

Figure 2: Immunofluroscence analysis of c-Kit-GFP protein expression in differentiated SSCs at 72 hrs post transfection showing surface expression of *c-Kit* protein (a). Light microscopic image of SSCs transfected with 1.1 kb control-GFP construct at 72 hrs post transfection (b) (MagnificationX 630). Flowcytometry analysis of c-Kit-GFP protein expression in differentiated SSCs at 72 hrs post transfection with 4.4 kb c-Kit-ORF-GFP insert (c) and 1.1 kb control-GFP insert transfected SSCs at 72 hrs post transfection (d). Figures shown are the representative picture from two different experiments performed on two different occasions.

serial cryo-sectioning. In a random sections (5 µm) from the middle part of the testis, a minimum of 50 cross sections of the seminiferous tubule were observed directly under a fluorescent stereo microscope (Nikon, model # SMZ1000) using a GFP filter for the presence of SSCs. On post GCT day 56, testes from right side (n=4/group) as well as the contra-lateral (n=4/group) were dissected from the mice and SSCs were isolated for the determination of germ cell specific marker genes as discussed above.

Results

Construction of pG1-*c-Kit*-ORF-GFP transgene construct

A 7.4 kb *pG1-c-Kit-ORF*-GFP fusion transgene construct has been designed. The orientation of the *c-Kit* promoter and c-*Kit*-ORF in pG1-c-*Kit*-ORF-*GFP* vector was confirmed by restriction digestion and DNA sequencing. A 4.4 kb Mlu-1 and Apa-1 digested linearized plasmid insert was used for *in vivo* expression analysis. To rule out any non-specific expression of plasmid regulatory elements and reporter gene, we designed three control GFP-encoding inserts lacking *c-Kit* promoter and *c-Kit*-ORF (1.1 kb) or c-Kit promoter (4.1 kb) and c-Kit –ORF (1.4 kb) as discussed under the section 2.3 in Material and Methods. The GFP protein did not altered the function of c-Kit protein. This has been confirmed by functional assay wherein SSCs carrying 4.4 kb c-Kit-ORF-GFP insert was introduced into recipient infertile mice by GCT.

In-vitro expression of 7.4 kb pG1-*c-Kit*-ORF-GFP transgene construct in P815 and ES-E14TG2α cells

The *c-Kit* gene is known to be expressed by other cell types cells like bone marrow stem cells, mast cells and melanocytes. To determine the expression efficiency and specificity of 7.4 kb pG1-c-Kit-ORF-GFP transgene construct, c-Kit expressing P815 cells and ES-E14TG2α cells were transfected with 10 µl (2 µg) of the construct and analyzed for GFP-driven c-Kit expression at 72 hrs post transfection. The results revealed that 85-90% of P815 cells and ES-E14TG2α cells showed c-Kit expression as compared to the cells transfected with 4.3 kb control-GFP construct, indicating 302 bp *c-Kit* promoter is able to drive specific c-Kit expression (Figure 1). As expected, the bone marrow derived dendritic cells (BMDC) did not show c-Kit expression (Data not shown).

c-*Kit*-ORF-GFP transgene insert introduced SSCs were differentiated *in vitro*

SSCs which carry *c-Kit*-ORF-GFP insert was prepared by introducing 10 µl of sterile PBS containing 2 µg of 4.4 kb linearized insert by electroporation. 10 µl of sterile PBS containing 2 µg of each linearized construct was introduced into SSCs isolated from W^v/W^v mutant mice by electroporation. These SSCs were enriched by culture for 48 hrs as discussed under section 2.5 in Material and Methods. To know whether the electroporated SSCs were differentiated into spermatogonial cells (SGCs), they were divided into two parts, one part has been cultured in the modified medium containing growth factors (LIF, GDNF and bFGF), as expected the SSCs did not differentiate into SGCs as these cells showed lack of c-Kit expression (data not shown). The other parts of SSCs cultured in the absence of growth factors, were differentiated into SGCs which showed significant c-Kit expression on cell surface (Figures 2a and 2b). These results were in agreement with data obtained using flowcytometry, which showed 58.13 % of differentiated SSCs transfected with 4.4 kb c-kit-ORF-GFP insert were expressed c-Kit protein as compared to 1.58% of SSCs transfected with 1.1 kb control insert (Figures 2c and 2d).

Busulfan treatment abrogated c-Kit mRNA and protein expression in recipient mice testis

Busulfan, a DNA-alkylating agent that destroys proliferating cells frequently was used to deplete germ cell population before GCT in recipient mice testes. Immunofluorescence studies revealed that treatment of one month old male mice treated with busulfan resulted complete ablation of testicular germ cells (Figure 3A). *C-Kit* mRNA (Figure 3B) and protein (Figure 3C) expression was undetectable in

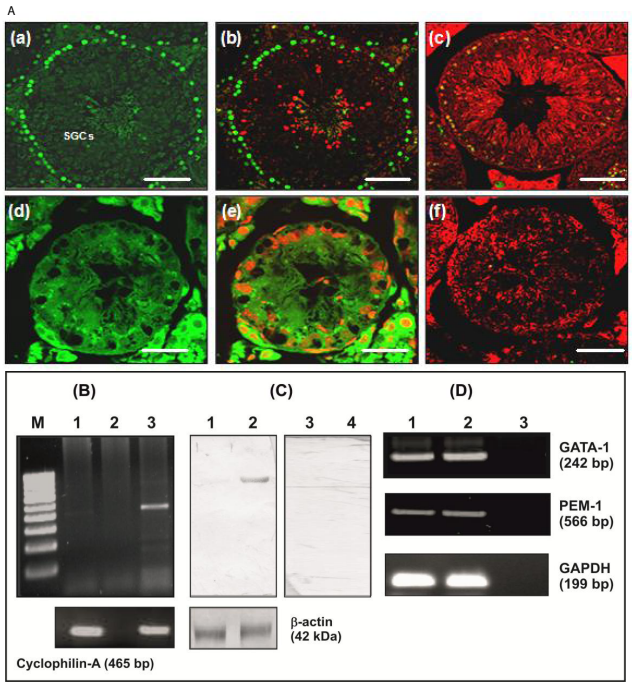

Figure 3: Expression of c-Kit protein in the testis of wide type (a-c) and busulfan treated mice (d-f).(3A).The testis of wild type mice showed normal c-Kit protein expression (Aa,Ab), whereas the expression was absent in the testis of busulfan treated mice (Ad, Ae).a &d FITC; b& e; FITC & PI merge; c, f : respective negative controls wherein primary antibody was replaced with pre-immune serum (**---**Scale bars 50 µm).

(3B). RT-PCR analysis of c-Kit mRNA expression in the testis of busulfan treated (Lane B1) and wild type mice (Lane B3) .c-Kit transcript was observed in the testis of wild type mice . Lane B2: No RT enzyme control. Cyclophiline-A was used as loading control (M = 100 bp DNA ladder).

(3C).Western blot analysis of c-Kit in the testis of busulfan treated (Lane: C1) and wild type mice (Lane: C2). C-Kit protein (150 kDa) expression was seen in the testis of wild type mice. Negative controls (Lanes C3 and C4) where primary antibody was replaced with mouse serum. β-actin was used as loading control.

(3D). RT-PCR analysis of Sertoli cell marker genes (GATA-1 & PEM-1) in the testis of busulfan treated (Lane: D1) and wild type mice (Lane: D2). Transcripts for GATA-1 & PEM-1 genes were seen in both these mice. Lane D3: No RT enzyme control. GAPDH was used as loading control.

the testes of busulfan treated mice as compared to wild type control. However, the expression of Sertoli cell marker genes, GATA-1 and PEM-1 were observed in the testes of busulfan treated mice, indicating Sertoli cell population was not significantly altered in busulfan treated mice (Figure 3D).

SSCs having c-Kit-ORF-GFP transgene insert were colonized and differentiated into other germ cell lineage in the recipient mice testis

Eighteen right side testes (n=18) were injected with electroporated undifferentiated SSCs carrying 4.4 kb *c-Kit*-GFP transgene insert. Of the 18 right side testes directly examined under fluorescent microscope using GFP filter on post GCT day 56, SSCs from 14 testes (78%) showed colonization and differentiation in the seminiferous tubules (Figures 4a-4d). The contra-lateral testes (n=18) were divided into three groups, each consists of six testes. The first group of six contra-lateral testes was introduced with 1.4 kb control-GFP insert lacking *c-Kit*-ORF. Seminiferous tubules of these mice showed *c-Kit* promoter driven GFP protein expression (Figures 4e-4h). Second group of six contra-lateral testes were injected with 4.1 kb control-GFP insert lacking *c-Kit* promoter (Figures 4i-4l). Third group of six contra-lateral testes were injected with 1.1 kb control-GFP insert lacking *c-Kit* promoter & *c-Kit*-ORF and analyzed on day 56 post GCT (Figures 4m-4p). Seminiferous tubules of both group two and three mice did not show GFP protein expression. To support these observations, RT-PCR and Western blot analysis were performed to determine whether the differentiated SGCs were derived from the transplanted SSCs. We observed that germ cells marker genes such as *Oct-4*(466 bp), *c-Kit (*403 bp), *Rbm1 (*209 bp), *Pgk-2(*353 bp*), Hils-1* (218 bp), *Gata-1* (242 bp), *Pem-1*(566 bp), *Lhr* (215 bp) and Tnp2 (255 bp) genes were expressed in the testis of wild type and *c-Kit*-ORF-GFP transgene insert introduced SSCs. However, expression of Oct-*4, c-Kit, Rbm- 1, Pgk-2, Hils-1* and *Tnp-2* genes were absent in the testes of control-GFP insert introduced mice. In contrary, the expression of Gata-*1, Pem-1* (Sertoli cell marker) and *Lhr* (Leydig cell marker) genes was observed in contra-lateral testes, which were introduced with 1.1 kb control-GFP insert (Figure 5).

Further, we determined the expression of GFP mRNA in the testes of recipient mice on post GCT day 56 by RT-PCR. The results revealed that 310 bp GFP transcript was observed in the testis of c-*Kit*-ORF-GFP insert introduced testis but not in the contra-lateral or wild type mice testis. Western blot results showed that anti-GFP antibody identified 172 kDa c-Kit-GFP fusion proteins (Figure 6). Conversely, anti-GFP antibody did not recognize GFP protein in 1.1 kb control-GFP introduced contra-lateral testes. The anti-GFP antibody also did not recognize any GFP related proteins in the testes of wild type mice.

Discussion

Spermatogenesis is characterized by sequential steps of cell proliferation and differentiation of SSCs resulting in the continuous production of spermatozoa. SSCs are known to occupy a special environmental location so called niche, generated by neighboring cells to enable SSCs to undergo differentiation [25]. It is documented that when SSC divide, only one daughter cell remains in the niche as a stem cell and the others would differentiate unless another niche is available. Because Sertoli cells which provide the niches for germ cells, the number and health of these cells is the key factor that decides the rate of SSCs proliferation and differentiation [14].

The results of the present study revealed that SSCs carrying 4.4 kb *c-Kit-GFP* transgene insert were able to colonize in the testes of recipient mice suggesting transgene insert containing 302 bp *c-Kit* promoters is sufficient to drive c-*Kit* expression *in vivo*. The study demonstrated that in 14 out of 18 testes that received SSCs were colonized and differentiated in the testes of busulfan treated infertile recipient mice. In the remaining 4 testes, c-*Kit-ORF*-GFP expression in SSCs was absent may be due to the lack of support from the stem cell niche. However, seminiferous tubules of the contra-lateral testes did not have GFP expressing SSCs, suggesting specificity and genomic integrity of c-*Kit-ORF*-GFP transgene insert. These results were in agreement with earlier reports wherein the authors demonstrated resumption of spermatogenesis [14,26] and the donor haplotype was passed on to the offspring when testicular germ cells from normal mice were transplanted into the testes of busulfan treated infertile mice [21]. Up till now moreover, majority of transplantation experiments performed to date were done with a mixed population of germ cells [26]. To the best of our knowledge, we demonstrated for the first time that transplanted SSCs were colonized and differentiated in the testis of recipient infertile mice.

In the testis, SSCs are located in niches on the basement membrane of

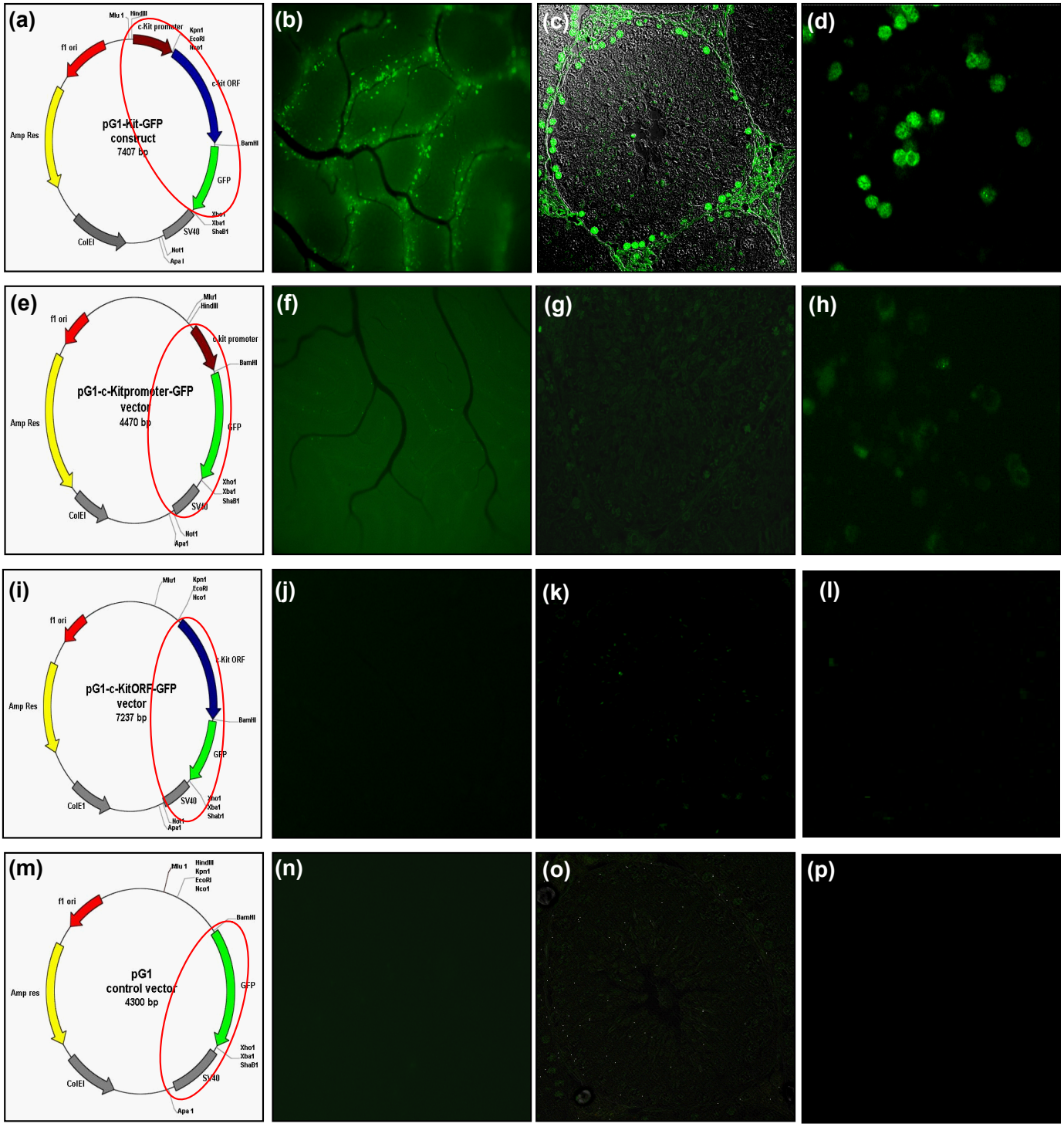

Figure 4: The diagrams (a, e, i, m) showing pG1-c-Kit-GFP-encoding transgene insert (a) and control-GFP inserts (e, i, m) (circled). Testes were directly observed under microscope using GFP filter on day 56 post GCT (b, f, j, n). Cross section of seminiferous tubules on day 56 post GCT showing expression of c-Kit fusion protein by the differentiated SSCs located at the periphery (c,g). However, the testicular cross-sections in control-GFP recipient testis (k, o) did not show the expression of GFP. c-Kit-GFP expressing SGCs isolated from c-Kit-ORF-GFP (d) c-Kit-GFP (h) expressing testes on day 56 post GCT. The 4.1kb and 1.1kb inserts did not show GFP expression. Figures shown are the representative pictures from two different experiments (Scale bar: 50 μm) .

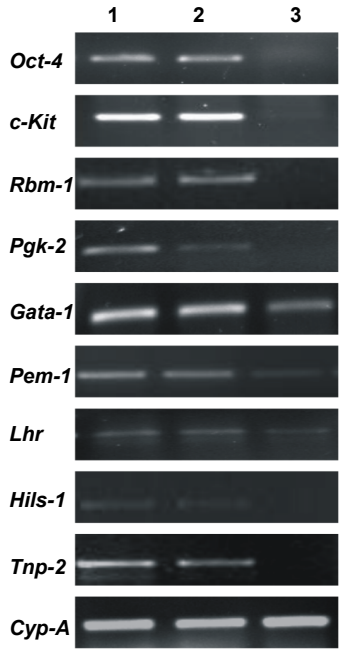

Figure 5: *In vivo* expression of germ cell specific marker genes in differentiated SSCs obtained from the testes of wild type and *c-Kit*-ORF-GFP transgene insert and 1.1 kb control-GFP insert introduced mice testis on post GCT day 56. *Oct-4* (466 bp), *c-Kit (*403 bp), *Rbm1 (*209 bp), *Pgk*-2 (353 bp), *Hils-1*(218 bp), *Gata*-1 (242 bp), *Pem-1-(*566 bp), *Lhr* (215 bp) and *Tnp2* (255 bp) genes were expressed in the testis of wild type and *c-Kit*-ORF-GFP transgene insert introduced SSCs. The expression of *Oct-4, c-Kit, Rbm- 1, Pgk-2, Hils-1* and *Tnp-2* genes were absent in the testes of control-GFP insert introduced mice, whereas the expression of Sertoli cells (*Gata-1, Pem-1*) and Leydig cells (Lhr) markers was observed. Lane: 1 (wild type mice testis), Lane: 2 (*c-Kit*-ORF-GFP insert introduced mice testis) Lane: 3 (control-GFP insert introduced mice testius). Cyclophilin-A (465 bp) was used as an internal loading control. Figures shown are the representative pictures from two different experiments performed on two different days.

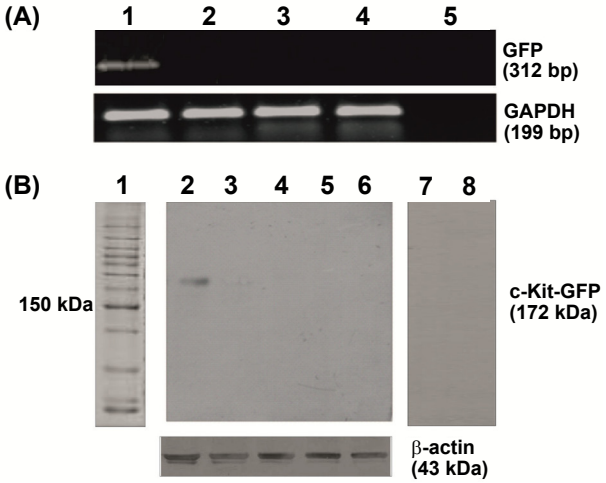

Figure 6: (A).Confirmation of GFP mRNA expression in SSCs of *c-Kit*-ORF-GFP (Lane: 1, 312 bp), 1.1 kb control-GFP introduced (Lanes: 2, 3), wild type (Lane: 4) mice testis and no RT enzyme control (Lane: 5). GFP-mRNA transcript was seen only in the SSCs of *c-Kit*-ORF-GFP insert introduced mice. GAPDH was used as internal loading control. None of the control insert introduced SSCs showed c-Kit-GFP expression (Lanes: 2-5).

(B). Western blot analysis of c-Kit-GFP fusion protein expression in SSCs of *c-Kit*-ORF-GFP (Lane: 2, 172 kDa), 1.1 kb control-GFP insert introduced wild type (lane 5,6) mice testis. GFP protein expression was seen only in the SSCs of *c-Kit*-ORF-GFP insert introduced mice testis. In negative controls (Lanes: 7, 8) primary antibody was replaced with normal mouse serum. β-actin was used as internal loading control. Figure shown is the representative pictures from two different experiments.

the seminiferous tubule enclosed by the basal lamina, the Sertoli cells and the tight junctions between Sertoli cells. The number of these niches would determine the number of SSC survival and function [14,18]. Our RT-PCR results demonstrated that Sertoli cells persist in the busulfan treated mice testis as observed by the expression of Sertoli cell marker genes such as GATA-1 and PEM-1. Therefore, stem cell niche or microenvironment is thought to be formed on the basement membrane surrounded by the Sertoli cells. In their classical experiments, Ogawa et al [21] have demonstrated that male *Sl/Sl*-d mice produce SSCs, but lack stem cell factor (SCF) expression in Sertoli cells. In contrast, the testes of mutant mice W^v/W^v do

not develop SGCs, due to the lack of functional *c-Kit* receptor. Nonetheless, earlier findings suggested that W^v/W^v mice testes have the population of SSCs, wherein the function of c-kit signaling activity is impaired due to point mutation in the tyrosine kinase domain of *c-Kit*. In our studies these SSCs were able to colonize and resume the function of proliferation and differentiation in the presence of c-*Kit*-ORF-GFP transgene construct.

The characterization of the colonized SSCs results revealed that SSCs were differentiated into germ cell lineages as determined by various marker genes of spermatogenesis. Furthermore, we also confirmed the newly differentiated germ cells are truly derived from the transplanted SSCs. Western blot results demonstrated the presence of 172 kDa c-*Kit*-ORF-GFP fusion proteins in the testes of recipient mice and its absence in contra-lateral testes suggests that transplanted SSCs were differentiated in the infertile mice testis. Studies are in progress to determine whether or not the direct introduction of 4.4 kb *c-Kit*-ORF-GFP insert into the testis of W^v/W^v mice will induce differentiation of SSC population. Based on the present observations, we conclude that on a proper migration of donor W^v/W^v SSCs, in a favorable testicular environment can colonize and differentiate into germ cell lineage. This clinically relevant finding raises the possibility for treatment of male infertility.

Conclusion

The present study demonstrated that SSCs obtained from W^v/W^v mutant mice testis can be made functional *in vivo* with the introduction of 4.4 kb c-*Kit*-ORF-GFP transgene insert. Transplantation studies revealed that SSCs carrying this insert were colonized and differentiated in the testes of busulfan treated recipient mice. The strong expression of *c-Kit*-ORF-GFP fusion protein in differentiated SSCs raises the possibility of using these cells to establish a specific cell line that might have germline characteristics. Consistent with our transplantation studies, the present results suggests that a clear role for *c-Kit* in SSCs colonization, proliferation and differentiation during spermatogenesis in mice. If these observations stand the test of the time, one of the causes of male infertility could be suggested and investigated.

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The authors hereby declare that there are no conflicts of interest (financial or otherwise) that could be perceived as prejudicing the impartiality of the research/ work reported in the current study.

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