SHORT REPORT

Differential regulation of gene expression in mouse spermatogonial cells after blocking c-kit-SCF interaction with RNAi

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ABSTRACT

c-Kit, the gene product of the *W* locus is a receptor tyrosine kinase that regulates the survival, growth and differentiation of spermatogonial cells (SGCs). Stem cell factor (SCF), the gene product of the steel (*Sl)* locus is the ligand for c-kit. Normal function of SGCs requires cross-talk between c-kit and SCF through which the receptor-ligand pair regulates the functions of SGCs. The implications of cross-talk between c-kit and SCF in regulating SGC function remains unclear due to the molecular complexity of this interaction. In the present study, we analyzed the interactions between c-kit and SCF in mouse primary SGCs after blocking the c-kit expression by c-kit siRNA and its effect on cell fate were determined using cDNA Expression Array and Real-time PCR. Immunofluorescence (IF) and western blot studies revealed that c-kit protein was detected in SGCs and knocked down to undetectable levels at 24 hr post transfection with 10 nM concentration of c-kit siRNA. We further demonstrated that expression of various genes involved in cell signaling, cell differentiation, apoptosis and cell cycle pathways was altered. SGC functions are affected by SCF signaling through c-kit receptor and this signaling appears to be important to maintain balance between cell proliferation and apoptosis along with the modulation of inflammatory responses of SGCs. To the best of our knowledge, this is the first report that identifies the putative molecular pathways in murine SGCs in response to specific blocking of c-kit-SCF interactions by siRNA. In conclusion, the present study may provide useful insights into siRNA function and hopefully aid in understanding the involvement of c-kit in the early events of SGC activities and spermatogenesis in mice.

KEYWORDS: Spermatogonial cells, c-kit, stem cell factor, RNAi, mouse

INTRODUCTION

Spermatogenesis is a complex process and involves cell division, differentiation and interaction between spermatogonial cells (SGCs) and Sertoli cells (SCs) in the microenvironment of the seminiferous tubule (Yoshinaga et al, 1991). These unique functions are orchestrated by the expression of thousands of genes in the testis (Yu et al, 2003; Hansen et al, 2004). One such gene is c-kit, a 150 kDa transmembrane receptor tyrosine kinase (RTK) whose ligand is stem cell factor (SCF) (Manova et al, 1993). In the testis, c-kit is expressed predominantly by SGCs (Yoshinaga et al, 1991; Manova et al, 1993; Mauduit et al, 1999; Feng et al, 2000) and SCF by SCs (Mauduit et al, 1999). SCF binds to c-kit and leads to receptor dimerization and autophosphorylation of c-kit resulting in activation of downstream signal transduction (Belchman et al, 1995). It is known that SCF/c-kit signaling is required for the survival of differentiating type A spermatogonia (Yoshinaga et al, 1991; Manova et al, 1993; Loveland and

Schlatt, 1997). Natural mutations in c-kit and/or SCF known to arrest primordial germ cell (PGC)/SGC proliferation and differentiation leading to infertility in mice (Sette et al, 2000; Grimaldi et al, 2002). It has been shown that knockdown of c-kit expression in mice during early embryonic development by anti c-kit antibody (ACK2) die as a result of malformed organs (Grimaldi et al, 2002). To the best our knowledge no information is available on specific knockdown of c-kit expression by its cognate RNA and its effect on genes that are required for SGC function.

Since the discovery of SCF as a ligand of c-kit, several studies have contributed to our knowledge on the role of c-kit-SCF signaling in various cellular processes using antibodies to c-kit and SCF (Dym et al, 1995; Mauduit et al, 1999). However, this antibody approach may not be ideal to delineate specific role of c-kit-SCF signaling in SGCs as these antibodies may cross react with locally derived cellular factors, which are yet to be identified. Furthermore, the analysis of genes involved in SGC function in neonates is limited due to the non-availability of efficient germ cell isolation methods. Conversely, the use of whole testes would yield results that may be difficult to interpret because the identified transcripts/proteins can be contributed by a single or multiple cell types. However, no information on is available on specific knockdown of c-kit expression by its cognate RNA and its effect on mouse SGC function *in vitro*. Using this approach, we demonstrated blocking of interactions between c-kit and SCF and its effect on transcriptional changes in SGCs. The present findings envisaged that signaling between c-kit and SCF is crucial for the dynamic balance between cell proliferation, differentiation and apoptosis in the SGCs.

MATERIALS AND METHODS

Animals

Male and female DBA/2 mice (age 10-12 weeks) were maintained in a temperature and humidity controlled room on a 12 hr light/12 hr darkness photoperiod. The mice had free access to food and water. Female mice were naturally mated and observed at 12 hr near the end of pregnancy to record the time at which parturition occurred. The day of birth was designated as day 0. Litter size was adjusted to a maximum of 8 by removing the appropriate number of female pups. The Animal Care and Ethics Committee, National Institute for Research in Reproductive Health, Mumbai had approved the experimental protocols for the study. All the measures taken for the mice were in accordance with approved guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), established by Govt of India on animal care.

Spermatogonial cell isolation

For this study we have used 10 day old DBA/2 male mice and testes were removed, pooled, decapsulated and digested with a mixture of enzymes that included 0.5 mg/ml collagenase-Type-IV, 0.25% trypsin/EDTA and 0.1% hyaluronidase (all from Sigma) dissolved in DMEM (Invitrogen, Germany) supplemented with 50U/ml penicillin and 50 µg/ml of streptomycin (Invitrogen, Germany). SGCs were isolated from resultant mixture of testicular cells as described previously using enzymatic digestion, filtration and differential adhesion to eliminate contaminating SCs, which adhere to culture plates (Kotaja et al, 2004; Prabhu et al, 2006). The cell pellet was resuspended in DMEM supplemented with heat inactivated 10% (v/v) FCS and checked for purity before using for different studies as detailed below. Purity of SGCs was evaluated by observing cell morphology and size under light microscope (x100). To rule out the possible contamination with SCs, immunofluorescence localization of germ cell nuclear antigen-1 (GCNA-1) and c-kit was carried out. Further we also determined the expression of genes such as SGC specific RNA binding motif protein (Rbm) and SC specific Reproductive homeobox-5 (Pem-1) by RT-PCR. Housekeeping gene, Cyclophiline-A (Cyp-A) served as internal loading control. Primers used in this study are shown in Table 1.

Preparation of siRNAs

Two mouse c-kit target-specific double-stranded, short (21bp) plus 2 bp 3' overhangs siRNAs were designed and chemically synthesized by Qiagen (Qiagen, USA). The c-kit and β-actin siRNA sequences homologous to c-kit and β-actin mRNAs were 1240 to 1261 (c-kit siRNA-1), 4239 to 4259 (c-kit siRNA-2) and 1052 – 1071 (β-actin) relative to the first nucleotide of the start codon (Table 1). To ensure the observed changes were not an artifact, β-actin siRNA (positive control), scrambled Cy3 siRNA (negative control) were maintained in parallel to the cells transfected with c-kit siRNAs. Since transfection reagent is known to interfere with siRNA duplexes (Jackson et al, 2006), we also maintained untransfected mock (UTM) controls where cells were exposed only to HiPerFect transfection reagent in DMEM (Table 2).

Cell culture and *in vitro* siRNA transfection

SGCs ($x10^4$) were cultured in DMEM supplemented with 10% (v/v) FCS (Hyclone, Germany), penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen, Germany), mouse recombinant-SCF (mrSCF) (10 ng/ml) (R & D systems, USA), recombinant human leukemia inhibitory factor (rhLIF)(10 ng/ml) (Chemicon, USA) and bovine TGF-α (10 ng/ml) and incubated for 24 hr at 37°C with 5% (v/v) CO_2. The cells were divided into five groups each consists of $5x10^4$/well. Group-1 (UTM control) was incubated with medium containing HiPerFect transfection reagent. Cells in groups 2, 3, 4 and 5 were transfected with c-kit siRNA-1 and 2, Cy3 siRNA and β-actin siRNA, respectively as per the manufacturer's protocol (Qiagen, USA). In brief, transfection complexes were prepared by mixing 3 µl HiPerFect reagent with 10 nM appropriate siRNAs and incubated at room temperature for 25 min. The mixture was then added to the cells in a final volume of 500 µl and incubated for 4 hr at 37°C with 5% (v/v) CO_2. The transfected cells were washed with DMEM and seeded 96 well culture plates at a concentration of 10^4 cells/well and cultured for 24 hr in 500 µl of fresh culture medium.

Immunofluorescence (IF)

A portion of above transfected and control cells (10^4) of four groups were grown on glass cover slips in a 6 well

Table 1. Characteristics of c-kit, β-actin and Cy3 siRNA duplexes and their position in the c-kit and β-actin mRNAs

Target	Accession No	Sequence 5'→3'
c-kit siRNA-1	NM_021011	Sense: 5' - CCG UGA CAU UCA AGC UUU A dT dT – 3' Antisense: 5' –UAA ACG UUG AAU GUC ACG G dA dA – 3'
c-kit siRNA-2	NM_021011	Sense: 5'- CUG UCU AGA AUU UAC UCA AdT dT – 3' Antisense: 5'- UUG AUG AAA UUC UAG ACA GdT dG– 3'
β-actin siRNA	NM_007393	Sense: 5' - UGA AGA UCA AGA UCA UUG CdT dT – 3' Antisense: 5' - GCA AUG AUG UUG AUC UUC AdT dT – 3'
Cy3 siRNA	-	Sense: 5'- UUC UCC GAA CGU GUC ACG UdT dT– 3' Antisense: 3'- ACG UGA CAC GUU CGG AGA AdT dT– 5'

plate as described above. The affect of siRNAs on c-kit protein expression was analyzed by IF at 24 hr post transfection. Briefly, the medium in the plate was aspirated, washed with PBS (pH 7.4) and fixed for 10 min at RT in PBS containing 4% (v/v) paraformaldehyde. Fixed cells were washed briefly with PBS and non-specific binding was blocked using 5% (w/v) BSA in PBS. Mouse monoclonal anti-c-kit antibody (R & D systems, USA) was used at a dilution of 1/100 and incubated for 1 hr at 37^{o}C in a humid chamber. Cover slips were washed in PBS and incubated for 1 hr at 37^{o}C in dark with FITC conjugated goat anti-mouse secondary antibody (Sigma, USA) at a dilution of 1/100.

For β-actin, mouse monoclonal anti β-actin antibody (Sigma, USA) at a dilution of 1/500 and goat anti-mouse secondary antibody conjugated to FITC at a dilution of 1/100 was used. We used mouse myeloma monoclonal antibodies (MOPC-31) (Sigma, USA) as isotype matched controls to rule out non-specific binding of antibody through the Fc-receptors. Same protocol was followed to localize GCNA-1 protein in mouse SGCs. The images were captured by Laser scanning confocal microscopy (LSCM) (Zeiss, 510 meta, Germany) (X 630).

Western blotting

The cells transfected with c-kit, β-actin, Cy3 siRNAs and untransfected mock (UTM) cells were tested for c-kit protein expression. After aspirating the medium from wells, cell extracts from all five groups were prepared by lysing cells (10^4 cells) directly in 24 well plates with hypotonic lysis buffer. Equal amounts of cellular protein (20 μg/lane) was loaded on 10% (w/v) SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, USA). Membranes were then blocked for 1 hr at RT with 5% (w/v) nonfat dry milk in PBS containing 0.1% (w/v) PBS-Tween-20 (PBS-T). The membranes were rinsed with PBS-T and incubated for 1 hr at RT with mouse monoclonal antibodies to anti-c-kit or anti-β-actin (checked against of Cy3 siRNA transfected cells) at a dilution of 1/500. Blots were washed with 0.5% PBS-T and incubated for 1 hr at RT in HRP conjugated goat anti-mouse secondary antibody (Sigma, USA) at a dilution of 1/1000. After washing, bands were detected using substrate, 3'-3' diaminobenzidine (DAB). Intensities of the bands were determined by densitometry scanning (Bio-Rad, USA).Relative expression ratios were calculated (c-kit band volume / β-actin band volume) and normalized to the values obtained with β-actin controls. β-actin siRNA transfected cells were checked for c-kit protein expression.

Adhesion assays

SGCs (10^4/well) 24 hr post transfection were dispensed in 24 well plate pre coated with mrSCF (10 ng/ml). After 2 hr, the plate was washed twice with washing buffer to remove unbound cells. The bound cells were fixed with 4% (v/v) paraformaldehyde (Sigma, USA) and stained with Crystal Violet (Qualigens, India) for 10 min. After solubilizing the dye with Triton X-100, reading was measured at 550 nm using spectrophotometer (Schimadzu, UV-160, Japan).

RNA extraction and cDNA array hybridization

Total RNA was extracted from SGCs using TriPure reagent (Roche Molecular Biochemicals, Germany) as described in manufacturer's protocol. Total RNA was converted into cDNA using modified oligo (dT) primer and amplified using the BD SMART™ PCR cDNA Synthesis kit (Clontech, USA), and labeled using the BD Atlas SMART probe amplification kit (Mouse, 588 cDNAs, Cat # 7853-1; Clontech, USA) in the presence of 50 μCi α32P dATP (Board of Radiation and Isotope Tech-

Table 2. Oligonucleotide primers used for the study

Gene	Accession No	Primer sequence (5' → 3')	Product size (bp)	Annealing temperature
Rbm	NM_011253	F- AACCGAAGTAACATATACTCA R- ATCTGCTTTCTCCACGACCTC	209	56°C
Pem-1	NM_008818	F- AAGAACAGCATGATGTGA R- TCAAAATCTCGATGTCGCAAA	566	55°C
Cyclophilin-A	NM_008907	F- AGC AGC CAT TCC CTT TAA GGT R- ATG GGT TCC GAC GTG CGG GAC	465	59°C
Cathepsin-B	NM_007798	F – GTCACCCTCAGAAATGGTTCTC R - AGATCCATAGGACAACGCTGAT	298	62°C
Cyclin-D3	NM_007632	F - CAGAGCAAGAACCCATACACTG R - CTGCTCTGATGAAGATGAGGTG	278	60°C
CdK-4	NM_009870	F - CTGGAAATGCTGACCTTTAACC R - GTGAACCTCGTAAGGAGAGGTG	250	59°C
Oct-4	NM_013633	F - AGC TGC TGA AGC AGA AGA GG R - TGG GAA AGG TGT CCC TGT AG	466	62°C
Cyclin-A2	NM_009828	F - CACCTCGAGGCATTCGGG R - CGGGTAAAGAGACAGCTGC	371	59°C
Fas-L	NM_010177	F - GAGAATTGCTGAAGACATGACAAT R - GTAGTTTTCACTCCAGACATTGTC	314	60 °C
PI3-K	NM_011083	F - ACCGTCAGCTATTATACCGACAT R - GAGGTTCTTCAGTCCATACTTGG	279	59 °C
P53	NM_011640	F - GCT TCT CCG AAG ACT GGA TG R - GTC CAT GCA GTG AGG TGA TG	143	59 °C
GAPDH	NM_008084	F - GAA ACC TGC CAA GTA TGA TGA C R - ATT GTC ATA CCA GGA AAT GAG C	199	60 °C

-nology, Government of India), CDS primers (complimentary to the array genes, Clontech, Palo Alto, USA), and 2 U of Klenow fragment at 50°C for 30 min. Equal quantity of control and experimental labeled probes were used to hybridize to the Atlas™ mouse cDNA expression array membrane at 68°C (BD Biosciences). Next day washings were done three times in 0.2x SSC/0.5% (w/v) SDS for 20 min and subjected to phosphorImager (Fujifilm, Japan) for 12 hr. Signal intensity of the spots was quantified using Atlas Image 2.7 software. The hybridizations were carried out three times in duplicates. Each time RNA obtained from a separate c-kit knockdown experiment was used.

Real-time quantitative PCR analysis of representative genes

Although differences in gene expression were observed with cDNA array after blocking c-kit binding to SCF, it

was essential to verify these results using other methods. We monitored the mRNA level of selected upregulated (Cathepsin-β, p53, Fas-1, Oct-4) and downregulated genes (Cyclin-D3, Cyclin-A2, PI3-K and Cdk4) noted to be differentially expressed in the Atlas array by Quantitative real-time PCR. Briefly, total RNA was extracted from the mouse SGCs as described above and subsequently cDNA was obtained by random hexamer priming using M-MuLV Reverse transcriptase as per manufacturer's instructions (New England BioLabs, USA). The PCR amplification was carried out with QuantiTect SYBR Green PCR Master Mix (Qiagen, GMBH, Hilden Germany) using 7900HT Fast Real-Time PCR System (Applied Biosystem, USA). Reverse and forward oligonucleotide primers, specific to the chosen candidate genes were designed using Primer 3.0 online software (Table 2).

The homogeneity of the PCR amplicons was verified by running the products on 2% agarose gels. All PCR amplifications were carried out in duplicates and each experiment was repeated thrice to test the reproducibility. cDNAs were tested in triplicate and, in addition, cDNA-negative and 'no template' controls were analyzed to ensure that the signal generated was derived from RNA and not from genomic DNA, primer-dimers or any of the cDNA or PCR reagents. Mean critical time (Ct) values generated in each experiment using the iCycler software (BioRad, USA) were normalized to the housekeeping gene (GAPDH) and were used to calculate cDNA concentrations in the samples. Relative expression ratios were calculated and normalized to the values obtained with GAPDH controls (target gene band volume / GAPDH band volume).

Data analysis and clustering

In order to assess the biological relevance of c-kit-SCF interaction, the upregulated as well as the downregulated genes were subjected to functional analysis with the use of DAVID Functional Annotation Clustering tool (http://david.abcc.ncifcrf.gov/summary.jsp), an online program for viewing and analyzing microarray data representing biological pathways or any other functional grouping of genes. The Genbank accession numbers of the differentially regulated genes were uploaded in the software OntoExpress and the analysis was carried out based on the GO database (Gene Ontology). The annotation tool was used at highest stringency setting to generate clusters of closely related genes. Each cluster was assigned a group Enrichment Score (E-Score) to rank their biological significance. Thus, the top ranked annotation cluster had an E-score (E $\geq$1) that was consistently higher for its annotation members (Table 3).

Statistical Analysis

The significance of differences in c-kit knock down between cells transfected with siRNAs and controls was determined using the Student's t-test. The fold change difference was considered to be statistically significant when $P \leq 0.05$ (ANOVA). The significance of each cluster was calculated in terms of *P*-value using the modified Fisher Exact test.

RESULTS AND DISCUSSION

Characterization of SGCs

In newborn mice, male germ cell precursors undergo self-renewal in the testis between days 1 and 10 postpartum (pp). The isolation of homogeneous population of SGCs is an essential prerequisite for definitive molecular studies of SGC proliferation and differentiation. Isolated SGCs were characterized for their morphology under light microscope (x100). These cells were observed to be 10-15 µm in diameter, spherical in outline with large round nuclei and are comparable with previous reports (Bellve et al, 1977). The average yield of SGCs was found to be 2.82 X 104 cells/testis. Several isolations were performed to render a total of ~1.92 x 10^6. Confocal immunofluorescence images revealed that over 90% of cells showed positive immunoreactivity for GCNA-1 and c-kit proteins. As expected GCNA-1 and c-kit were localized in the nucleus and periphery of the cells, respectively (Figure 1a and 1b). The RT-PCR results revealed that only a 229 bp Rbm transcript was seen in isolated SGCs and not Pem-1 (Figure 1c), suggesting possible absence of SCs contamination.

Immunofluorescence and Western blot analysis of c-kit expression

Immunofluorescence (IF) (Figures 1d, 1e and 1f) and western blot (Figure 2) analysis was carried out to assess the effect of siRNA mediated c-kit gene silencing on c-kit expression in SGCs. The results revealed a significant knockdown of c-kit expression in cells transfected with c-kit siRNA-1 as compared to scrambled Cy3 siRNA and UTM controls. Western blot results are in agreement with IF studies where c-kit siRNA-1 and 2 knockdown the c-kit protein expression. Comparable knockdown of β-actin expression was observed in cells transfected with β-actin siRNA (Data not shown).

Adhesion assay

Adhesion assays were performed to investigate whether c-kit blocking in SGCs by siRNA prevents the binding to SCF. As evident from the results, transfection of SGCs with c-kit siRNA-2 leads to inhibition of binding to mrSCF coated plates. Inhibition by >2 fold over scrambled Cy3 siRNA controls was consistently observed in three independent experiments (Figure 3a). The SGC adhesion was not affected with Cy3 siRNA used in place of c-kit siRNA-2, thus confirming that blocking of c-kit disrupts interactions of SCF to c-kit.

cDNA array analysis of mRNA expression kinetics

We investigated key molecules which get affected by disruption of c-kit-SCF interaction using BD Atlas mouse cDNA expression array. Expression levels of 588 genes were estimated in RNA isolated from SGCs cultured on mrSCF coated plates. Of the 588 genes spotted on the array, about 59 genes showed differential expression of $\geq$ 2 fold between the two groups. Of these, 39 genes were upregulated and 20 were downregulated.

a) Upregulated genes

After blockade of c-kit by c-kit siRNA-2 in SGCs, genes from various families were upregulated. The major group

Table 3. Functional annotation and clustering of differentially expressed genes

Cluster	Biological process	No of genes involved	%	E-score
Upregulated genes				
1	Cellular protein metabolism	17	45.60	1.08
2	Cellular physiological process	26	69.59	1.30
3	Intracellular transport	3	8.05	0.36
4	Cell signaling	14	37.55	2.98
5	Cell differentiation	20	53.65	4.99
6	Immune response	6	16.10	0.51
7	Apoptosis	11	29.51	4.94
8	Regulation of transcription	13	34.87	2.24
9	DNA repair	3	8.05	0.99
10	Neuron differentiation	3	8.05	0.41
11	Lymphocyte differentiation	3	8.05	0.50
Downregulated genes				
1	Regulation of cell cycle	12	65.23	6.98
2	Regulation of transcription	7	38.05	3.5
3	Regulation of cellular physiology	4	21.74	1.43
4	Cellular metabolism	6	32.62	0.62
5	Mitosis	5	27.18	4.76

comprised of the apoptotic genes (Defender against cell death, (Dad-1), Fas associated factor-1 (FAF-1), Nucleotide diphosphate kinase B (NDP kinase B), Interleukin converting enzyme (ICE), DNA damage signaling protein (RAD-23), DNAse-1, Glutathione S-transferase (GST). Along with these, the genes like Oxidative stress induced protein (OSIP), Heat shock proteins-60 and 70 (HSP-60/70), Protein kinase-B, Growth arrest and DNA damage inducible protein-153 (Gadd 153), Transforming growth factor β-Receptor-1 (TGFβR-1) and Interferon-γ receptor (IFNγ-R) were also upregualted. A large number of transcription repressors involved in cell cycle were elevated, notable being p53, retinoblastoma-1(RB-1), POU domain transcription factor (Oct-4) and E2F5. Pim-1 serine/threonine kinase signal transducing transcription factor, Interleukin converting enzyme (ICE), a Cdk inhibitor P27kip, DNAse-1, and Glutathione S-transferase (GST).

b) Downregulated genes

The most remarkable downregulation was that of Phosphoinositol -3 kinase (PI3-K) which falls under the c-kit mediated signaling pathway as seen in the pathway analysis by Onto-expressTM.Cell cycle proteins such as G1/S specific cyclin- D3 (Cyclin-D3), S/G2/M specific cyclin F (Cyclin-F), G2/M specific cyclin-A2 (CCNA-2), G2/M specific cyclin- B1(Cyclin-B1), G1 specific Cyclin-C (Cyclin-C), Cyclin dependent kinase-4 (Cdk4), Cyclin dependent kinase-7 (Cdk7), Mitogen activated protein kinase (MAP-K), c-Myb transcription factor and Ubiquitin-B (UB) were downregulated after blocking of c-kit expression in SGCs (Figure 3c). These differentially

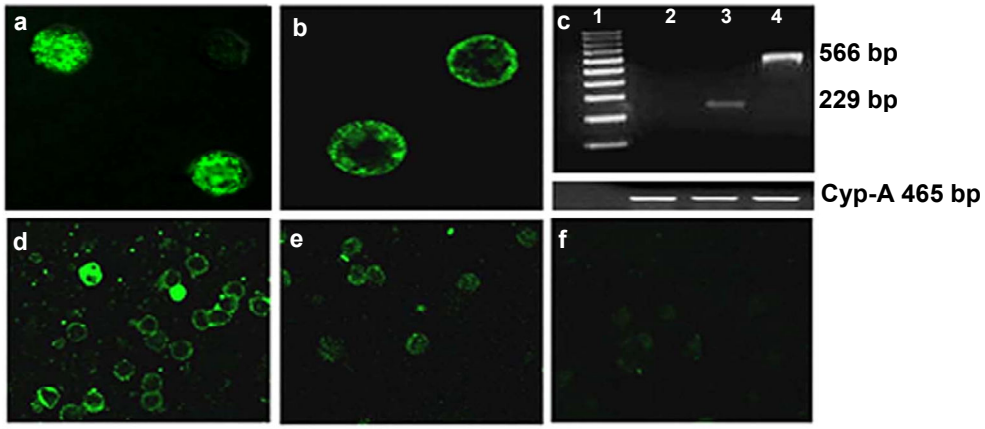

Figure 1. Laser scanning confocal microscope (LSCM) fluorescence images of SGCs. GCNA-1 (a) is localized in the cell nucleus whereas c-kit (b) on the cell membrane (Mag. x1000). RT-PCR analysis of Pem-1 and Rbm genes revealed the presence of Rbm transcript and not PEM-1 in SGCs. (Lane 1= 100 bp marker, 2= Pem-1 in SGCs, 3= Rbm in SGCs, 4= Pem-1 in testes of mice on day 10 pp). LSCM fluorescence images of untransfected mock (UTM) (d), transfected with 10 nM Cy3 siRNA (e) and c-kit siRNA-1 (f) at 24 hr post transfection, showing significant knockdown of c-kit expression in c-kit siRNA transfected cells while it was not affected by scrambled Cy3 siRNA (x630). The figure shown is the representative pictures from three independent experiments.

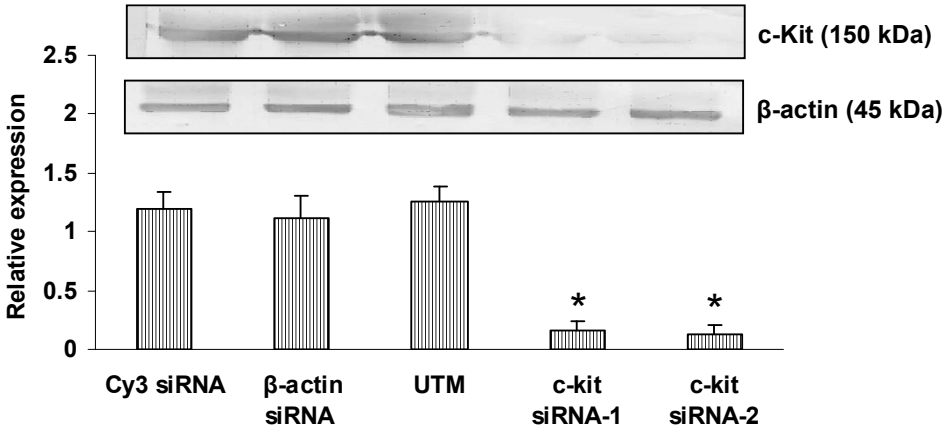

Figure 2. Effect of c-kit siRNA-1 and 2 on c-kit protein expression in SGCs analyzed by western blot. Cellular proteins were separated on SDS-PAGE, transferred onto nitrocellulose membranes and processed for western blot using antibodies to c-kit and β-actin. The Cy3 siRNA and β-actin siRNA transfected cells were checked for c-kit protein expression. Note that c-Kit expression was significantly knocked down with 10 nM siRNAs at 24 h post transfection compared to Cy3 siRNA and UTM controls. β-actin blot confirms equivalent loading of protein sample from scrambled Cy3 siRNA transfected cells. The bars represent the relative expression of c-kit protein normalized to β-actin protein band (* are statistically significant over Cy3 siRNA transfected and UTM controls).

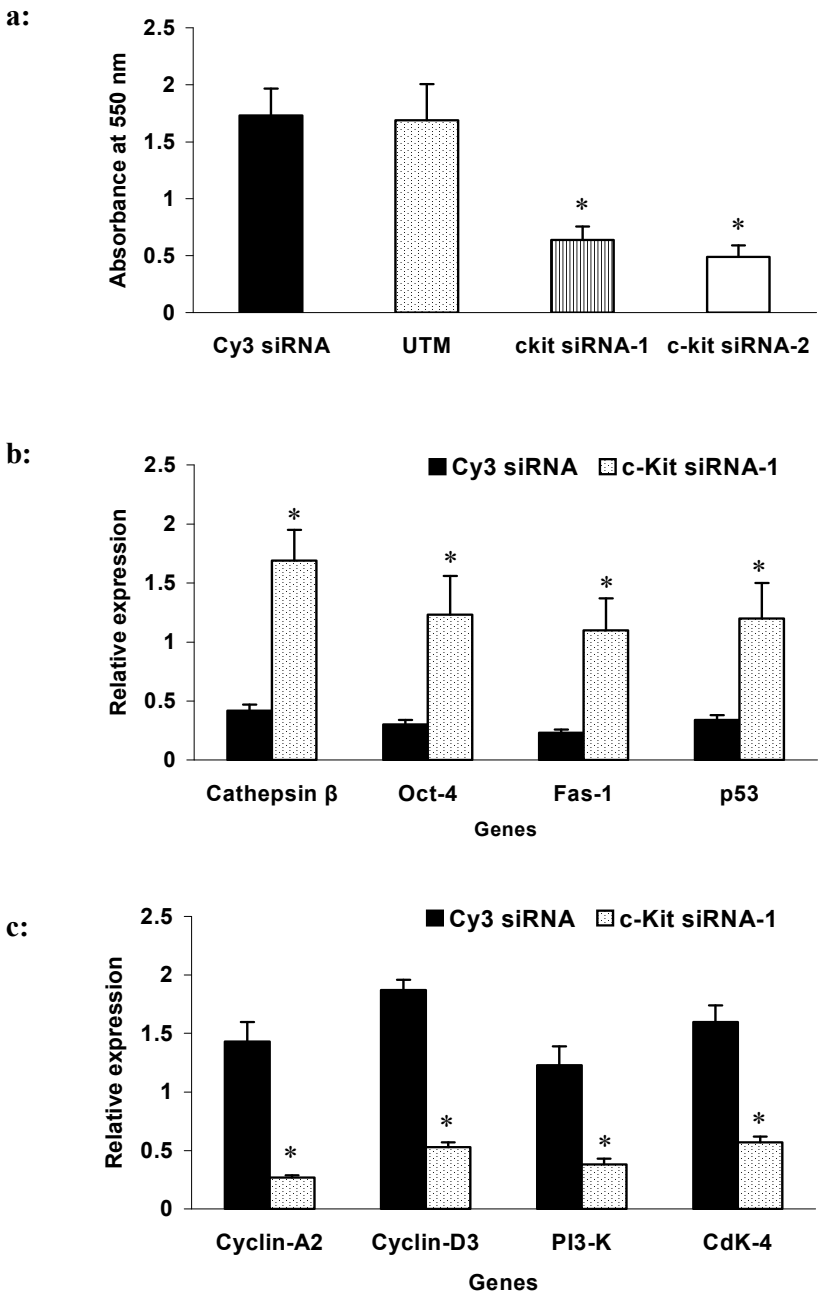

Figure 3. Effect of c-kit knockdown by siRNA-1 on binding of mouse SGCs to SCF (a) and real-time PCR analysis of upregulated (b) and down regulated (c) genes. Transfection of SGCs leads to inhibition of SGC binding to SCF coated plates compared to cells transfected with Cy3 siRNA or UTM controls. Values are the mean ± SD of six observations obtained from three experiments. Quantitative real-time PCR confirmation of cDNA array results of upregulated and downregulated genes. The values represent the relative expression levels of the genes normalized to GAPDH in c-kit siRNA treated vs Cy3 siRNA control. Values are mean ± SD of six samples obtained from three experiments (* are statistically significant over Cy3 siRNA transfected controls).

expressed genes can be classified into basic transcription factors, cyclins, cytoskeleton proteins and growth factors and their receptors.

Validation of array results by Real-time PCR

The expression of 8 differentially expressed genes, 4 upregulated (Cathepsin-B, p53, Fas-1, Oct-4) (Figure 3b) and 4 down regulated (Cyclin-D3, Cyclin-A2, PI3-K and Cdk4) (Figure 3c) was analyzed by real-time PCR in three individual pools of cDNA. The experiment was repeated three times and the results were in concordance with the results obtained by cDNA array analysis. Interestingly, 9 cell cycle genes and 4 apoptotic genes had reduced mRNA levels. Cyclins are the proximal members of the cell cycle regulation assembly, which in turn activate or suppress downstream pathways that comprise molecules

like cyclin A2/B1, C, F and D3 all were downregulated markedly in SGCs. The expression of cyclins and their catalytic partners Cdk4 and Cdk7 have been well studied in murine testis and are present in high levels in the proliferating SGCs (between days 1 and 13 pp) and play a crucial role in self-renewal of these cells (Feng et al, 2000). Cyclin-D2 null male mice are fertile but have reduced testicular size and low sperm count, whereas Cdk4 null mice are sterile (Zindy et al, 2001), suggesting that cyclins in combination with Cdk4 and Cdk7 may regulate G1/S progression in SGCs. More interestingly gene E2F5, which is related to the function of cyclin D3 plays regulatory role in the G1/S phase transition of the cell cycle (Sears and Nevins, 2002) is up-regulated. This may reflect decrease in the proliferative capacity of SGCs. The cell cycle through the G1-S checkpoint is regulated by multiple mitogenic signaling pathways including cyclin D3 and mitogen activated protein kinase (MAP-K) (Gille and Downward, 1999). The present transfection studies revealed that SCF induced c-kit expression in SGCs is completely abolished by c-kit siRNA. C-kit-SCF system is known to modulate germ cell proliferation through D type cyclins (Feng et al, 2000). This data tempted us to hypothesize that proliferation of SGCs requires co-ordinated activity of cyclins, which are regulated by c-kit-KL interaction in SGCs.

The Phosphoinositol -3 kinase (PI3-K) regulatory subunit (p850) was observed to be downregulated in SGCs, which suggests that c-kit-SCF triggers PI3-K signaling. This appears to be the pathway by which SCF to regulate the proliferation of SGCs (Feng et al, 2000). Any alterations in c-kit expression can cause disruption of PI3-K signaling which impairs binding of PI3-K to c-kit leading to male sterility (Blume-Jensen et al, 2000). Myb-B proto-oncogene playing a critical role in controlling the proliferation or differentiation of spermatogonia through the first meiotic prophase (Kwon et al, 2005) was downregulated. Theis repression of Myb-B expression in siRNA transfected SGCs is suggestive of deranged cell proliferation and differentiation.

The molecules like GST and p53 are key decision makers of whether a cell should enter a cell cycle arrest or enter apoptosis (Abrahamson et al, 1995; Xie et al, 2005) were upregulated. Increased levels of p53 in differentiated SGCs have been reported in-vitro and hypothesized to be maintaining the differentiation status of the SGCs. The role of c-kit-SCF signaling in testis is well known, therefore it is likely that this interaction could be important in maintaining the differentiated status of SGCs via p53. However, it remains to be investigated whether this change in p53 expression is a result of direct transcriptional regulation by c-kit or through cyclins.

It is interesting to note that expression pattern of cathepsin family of genes viz., cathepsin B, D, H and L was upregulated in c-kit siRNA treated SGCs suggesting their involvement in cell-cell signaling and differentiation of SGCs. Differential expression of multiple cathepsin mRNAs and their role in testicular development has been reported (Chung et al, 1998). Cross-talk between SGCs and SCs is critical for the control of cathepsin expression in human testis (Gye and Kim, 2004). Functional annotation clustering of the upregulated genes identified a cluster of genes involved in regulation of cellular physiology. Amongst these, Wilms tumor antigen-1 (WT-1), DNase-1, HSPs, and GST were of special importance as these are involved in DNA repair, cell cycle regulation at transcriptional level (Murata et al, 1997). E2F5 known for transcriptional repression is placed farthest downstream in the cell cycle regulatory mechanism and is a major regulator of cell-cycle checkpoints (Gaubatz et al, 2000). Its upregulation further strengthens our idea that blocking of c-kit expression leads to cell-cycle arrest in SGCs.

Beyond these genes which are involved in proliferation, differentiation and cell cycle regulation, another group of molecules which are differentially expressed are genes involved in apoptosis. The cluster of apoptotic molecules Faf-1, Dad-1, NDP-K and RAD-23 were upregulated in c-kit siRNA transfected cells indicating a shift towards apotosis. Together this data led us to believe that the c-kit-SCF interaction is crucial for maintaining the balance between SGC proliferation and apoptosis.

Another striking group of molecules that were differentially expressed in SGCs were that of growth factors and cytokines receptors. Cytokines are critical molecules that are not only known to be involved in proliferation and apoptosis, but are also known to be involved in immunomodulation during spermatogenesis (Hales et al, 1999). The present cDNA array results indicated increased expression of the receptors for EGF-α, TGF-β, IFN- γ in c-kit siRNA transfected cells compared to scrambled Cy3 siRNA treated cells. These cytokine receptors were not clustered under any particular functional category, probably because they are involved in far too many functions and multiple pathways. Molecules like TGF-β and IFN-γ. synthesized in the testis play a crucial role in spermatogenesis and c-kit is known to modulate the expression of these cytokines (Hales et al, 1999). On the other hand, both these cytokines induce germ cell apoptosis (Stranda et al, 2005). These cytokines mediate their actions by binding to their receptors which were upregulated after knockdown of c-kit/SCF signaling. This tempts us to speculate that c-kit-SCF interactions may be altering the cellular response to these cytokines in SGCs. Further studies are required to know the physiological relevance of this finding.

CONCLUSIONS

In conclusion, expression profiling of SGCs revealed that specific regulation of c-kit-SCF interactions, modulate the expression of genes involved in cell signaling, differentiation, metabolism, apoptosis, cell cycle and transcription. With increased understanding of the mechanisms that control these cellular activities, siRNA approach may prove a useful tool for manipulating SGC function. Probing the molecular targets of c-kit-SCF interactions, identified in this study in greater detail will be of importance in unraveling the dynamics of SGC function during spermatogenesis.

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COMPETING INTERESTS

None declared.

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