

Regulated expression of CXCR4 constitutive active mutants revealed the up-modulated chemotaxis and up-regulation of genes crucial for CXCR4 mediated homing and engraftment of hematopoietic stem/progenitor cells

Sharma M¹, Afrin F², Tripathi RP¹, Gangenahalli G¹

SDF-1/CXCR4 axis plays a principle role in the homing and engraftment of hematopoietic stem/progenitor cells (HSPCs), a process that defines cells ability to reach and seed recipient bone marrow niche following their intravenous infusion. However, the proper functioning of CXCR4 downstream signaling depends upon consistent optimal expression of both SDF-1 ligand and its receptor CXCR4, which in turn is variable and regulated by several factors. The constitutive active mutants of CXCR4 (N119A and N119S) being able to induce autonomous downstream signaling, overcome the limitation of ligand-receptor interaction for induction of CXCR4 signaling. Therefore, we intended to explore their potential in chemotaxis; a key cellular process which crucially regulates cells homing to bone marrow. In present study, Tet-on inducible gene expression vector system was used for doxycycline inducible regulated transgene expression of CXCR4 active mutants in hematopoietic stem progenitor cell line K-562. Both of these mutants revealed significantly enhanced chemotaxis to SDF-1 gradient as compared to wild type. Furthermore, gene expression profiling of these genetically engineered cells as assessed by microarray analysis revealed the up-regulation of group of genes that are known to play a crucial role in CXCR4 mediated cells homing and engraftment. Hence, this study suggest the potential prospects of CXCR4 active mutants in research and development aimed to improve the efficiency of cells in the mechanism of homing and engraftment process.

Key Words: HSPCs, CXCR4, active mutants, homing, engraftment, chemotaxis.

Introduction

Hematopoietic stem cell transplantation (HSCT) success primarily depends on the ability of intravenously transfused hematopoietic stem/progenitor cells (HSPCs) in circulation to cross the blood-bone marrow endothelial cell barrier and reach their niche in recipient bone marrow (BM), where they ultimately lodge and repopulate in order to resume hematopoiesis, a process termed as homing and engraftment^[1-2]. However, the low homing and engraftment efficiency especially in context of low availability of donor CD34⁺ HSPCs is a major challenge ahead which limits the clinical applications of HSCT^[3-4]. Moreover, the *ex vivo* HSPCs expansion and manipulation for gene therapy further compromise their homing and engraftment efficiency^[5-6]. In present scenario, specific modification or modulation of key molecular player of homing and engraftment may serve as a possible approach to make the limited number of available HSPCs more efficient in homing and engraftment.

Over the decade, pivotal role of SDF-1/CXCR4 axis has been well established in HSPCs homing and engraftment to BM. Gene knockout studies of SDF-1 and CXCR4 in murine

models have revealed an essential requirement of SDF-1/CXCR4 axis in homing of fetal liver derived HSPCs to BM and their retention and repopulation during embryonic development^[7-8]. Furthermore, the key role of SDF-1/CXCR4 axis in homing and high-level multilineage repopulation of human CD34⁺ enriched cells in BM has been demonstrated using NOD/SCID mice as recipients^[9-10]. SDF-1 binding to its receptor CXCR4 expressed on HSPCs, induces the active conformation of receptor resulting in G protein mediated downstream signaling that regulate cellular and molecular events which elicit cell homing and engraftment. The homing and engraftment efficiency therefore appears to be dependent on the response of HSPCs to SDF-1, which in turn depends upon CXCR4 availability/expression on HSPCs^[11-12]. However, surface expression of CXCR4 on HSPCs is variable and regulated by several factors in BM hematopoietic microenvironment such as cytokines, chemokines, adhesion molecules and proteolytic enzymes^[13,2]. Hence, autonomous signaling of CXCR4 without SDF-1 binding can be a possible approach to overcome the necessity of consistent optimal expression of both ligand and receptor in induction of CXCR4 downstream signaling cascade.

The NYSS is a highly conserved motif in transmembrane three

(TM3) domain of CXC chemokine receptors and play a crucial role by acting as a switch that maintains the dynamic active-inactive conformational equilibrium of receptor^[14]. Conversion of Asn-119 of this motif in CXCR4 to Serine (Ser) or Alanine (Ala) was found to drive the conformational equilibrium of CXCR4 to active state manifested by autonomous downstream signaling and constitutive activity of receptor. Moreover, autonomous coupling of these constitutive active mutants (CAMs) to G protein subunits was shown to further augment by SDF-1 binding, indicating the stabilization of an optimal active conformation of receptor^[15]. Given these facts, the implication of CXCR4-CAMs in a regulated manner has potential prospects in up-modulation of cellular and molecular mechanisms that altogether elicit cell homing and engraftment process, especially when receptor and/or ligand availability is not optimal. In present study, using Tet-on inducible gene expression vector system, we achieved the doxycycline inducible regulated transgene expression of CXCR4-CAMs in hematopoietic stem progenitor cell line K-562 and assessed their potential in cellular process of transmigration/chemotaxis which mediates the transendothelial migration and directed chemotaxis of cells to home and engraft the recipient BM niche. Furthermore, the genome wide differential gene expression profile of these mutants as revealed by microarray analysis provided a better fundamental understanding of their improved migration potential and mode of action by revealing the up-regulation of group of genes which critically regulate the cell homing and engraftment process.

Materials and Methods

Sub-cloning of wild type CXCR4 gene into Tet-on inducible gene expression vector system: Wild type CXCR4-cDNA present in pcDNA3 plasmid vector was kindly gifted by Dr S. C. Peiper (Henry Vogt Cancer Research Institute, University of Louisville, Kentucky). This wild type CXCR4 cDNA of 1.1kb size was sub-cloned into multi-cloning site (MCS) of response plasmid vector pTRE2hyg between Nhe1 and Sal1 restriction sites in sense orientation of *PminCMV* promoter. The sub-cloning of CXCR4 gene was finally characterized by double digestion of recombinant pTRE2hyg (wild typeCXCR4-pTRE2hyg) plasmid DNA by Nhe1 and Sal1 restriction enzymes (Fermentas), which produced two characteristic linear fragments of specific size; 5.3 kb pTRE2hyg and 1.1 kb popped out CXCR4-cDNA (Supplementary Figure 1).

In vitro site directed mutagenesis: The CXCR4-CAMs active mutants; Asn119 to Ser (N119S) and Asn119 to Ala (N119A) were generated by *in vitro* site directed mutagenesis system (Invitrogen) using CXCR4 specific mutagenic primers with wild typeCXCR4-pTRE2hyg plasmid DNA as a template. The specific mutation of N119SCXCR4-pTRE2hyg and N119A CXCR4-pTRE2hyg plasmid constructs were confirmed by commercial DNA sequencing service (Ocimum Biosolutions India Pvt. Ltd.).

Cell lines and stable transfection: Chronic myelogenous leukemia cell line K-562 (ATCC No. CCL-243TM) was obtained from National Center of Cell Science (NCCS), Pune, India. K-562 cells were maintained in Iscove's modified dulbecco's medium (IMDM) (Invitrogen) supplemented with

10% fetal bovine serum (FBS) (Gibco) and antibiotics; penicillin and streptomycin (HiMedia) at concentration of 100 units/ml and 100 µg/ml respectively. The first stable transfection of K-562 cells with pTet-on plasmid DNA was done using lipofectamine-2000 (Invitrogen), followed by selection of Tet stable K-562 cells in IMDM selection media containing 600 µg/ml G418 (Sigma) for 8 weeks. The Tet stable K-562 cells were subsequently transfected with each subset of recombinant pTRE2hyg plasmid constructs (Wild typeCXCR4-pTRE2hyg, N119ACXCR4-pTRE2hyg and N119SCXCR4-pTRE2hyg) as described above, followed by selection of each subset of CXCR4 double stable K-562 cells in IMDM selection media containing 150 µg/ml hygromycin (Sigma) for 8 weeks.

Fluorescence-activated cell sorting assay: Cells were induced by 1µg/ml doxycycline (Sigma). After 48 hours of doxycycline incubation, cells were harvested and washed three times with phosphate buffer saline (PBS). To perform antibody staining, 0.5×10^6 cells were incubated with 10 µg/ml mouse anti-human CXCR4 monoclonal antibody (clone 12G5, BD Biosciences) for 1.5 hr at 4°C. Washed twice with PBS and incubated again with 10 µl of 1:100 FITC-conjugated rat anti-mouse IgG (Sigma) for 1.5 hr at 4°C in dark. Washed twice with PBS and cell fluorescence was analyzed by flow cytometer (BD FACS scan).

Chemotaxis assay: Directed cell migration potential/chemotaxis towards SDF-1 gradient was analyzed *in vitro* as described^[11]. Briefly, 0.5×10^6 cells were induced by addition of 1µg/ml doxycycline in IMDM growth media and incubated for 48 hours. 600 µl IMDM (supplemented with 10% FBS) containing 125 ng/ml SDF-1α (PeproTech) was added to the lower chamber of a Costar 24-wells transwell (pore size 5 µm, Corning, NY). Then, 0.1×10^5 doxycycline induced cells in 100 µl IMDM medium (supplemented with 0.1% FBS) were loaded to the upper chamber and allowed to migrate for 4 hours at 37°C in a humidified CO₂ incubator. After incubation, migrated cells were collected from lower chamber and counted on a hemocytometer under microscope.

Microarray analysis: A minimum of 1×10^6 cells in triplicates were seeded for each set of K-562 cells (control untransfected, wild type CXCR4-pTRE2hyg, N119ACXCR4-pTRE2hyg, N119SCXCR4-pTRE2hyg). The cells were induced by 1µg/ml doxycycline in IMDM growth media with 48 hours incubation in a humidified CO₂ incubator. After incubation, cells were washed twice with PBS and resuspended in 1ml IMDM supplemented with 0.1% FBS and 125 ng/ml SDF-1, incubated for 4 hours in a humidified CO₂ incubator. After incubation, cells were washed twice with PBS and stored in RNA later storage solution (Ambion) to be shipped for microarray analysis. RNA isolation, quality control and hybridization were performed by Genotypic Technologies Pvt. Ltd., Bangalore, India. Briefly, RNA was isolated using RNeasy mini kit (Qiagen) and qualified in nanodropspectrophotometer. RNA sample purity ratio was more than 1.9 for a ratio of 260/280 nm and RIN (RNA integrity number) values were greater than 8.5, as evaluated on bioanalyzer 22100 (Agilent technologies). Following reverse transcription of RNA into cDNA, Cy3 labeled

cRNA was produced by *in vitro* transcription and hybridized to human whole genome array chip (human whole gene expression microarray, 8x60k array, Agilent Technologies). Images were scanned and signal data acquired using feature extraction software (v 10.5, Agilent technologies).

Microarray expression data analysis: Transcripts which were reliably detected in all the replicates were considered for subsequent data analysis. The raw data were processed and normalized by percentile shift normalization method using gene spring GX11 (Agilent Technologies). Following normalization, average signal intensity of probes showing at least 30% change in expression across the three set were computed and ratios (treated/control) were log₂ transformed. Differentially regulated genes were identified based on average log₂ ratio $\geq \pm 0.6$ (± 1.5 fold) and P value < 0.05 . Genes with $> \pm 1.5$ fold change in expression as calculated by Agilent software in all comparisons were selected for further analysis. For additional pathway analysis, the Kyoto Encyclopedia of Genes and Genomics (KEGG) and National Center for Biotechnology Information (NCBI) database were also consulted.

Real time quantitative-polymerase chain reaction (RT-PCR) analysis: RNA (1 μ g) from each cell sample in triplicates (n=3 wild type CXCR4-pTRE2hyg, n=3 N119ACXCR4-pTRE2hyg, n=3 N119SCXCR4-pTRE2hyg) were reverse transcribed to cDNA using superscript c-DNA synthesis kit (Invitrogen) according to manufacturer's instructions. Real time polymerase chain reaction was done using SYBR green quantitative RT-PCR kit (Sigma) following manufacturer protocol. Gene specific primers were designed by Beacon designer software (Supplementary table 1). Amplification of target genes were performed with thermal cycling condition of 95 °C for 10 min followed by 40 cycles of 95 °C for 30 seconds, 52 °C for 60 seconds, 72 °C for 30 seconds in a spectrofluorometric thermal cycler (Staratagene). The relative change in gene expression was calculated in terms of fold change by $2^{-\Delta\Delta CT}$ algorithm [16]. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous reference for normalization of each sample.

Statistical analysis: Results are expressed as mean \pm SD (Standard Deviation). For assay, the statistical significance of differences between means was assessed using student *t*-test considering the P-value < 0.05 as significant. For microarray data analysis, t-test-P-value of fold expression change of gene was calculated using Volcano plot method considering P-Value < 0.05 as significant.

Results

Generation of CXCR4 double stable K-562 cells and doxycycline inducible regulated transgene expression:

The K-562 cell population is a highly undifferentiated hematopoietic stem progenitor cell [17]. Since, this cell line is CXCR4 null at m-RNA transcript level [18], we selected this to avoid the interference of host endogenous CXCR4 gene in transgene expression study of CXCR4-CAMs. The regulated transgene expression of CXCR4-CAMs and wild type gene in K-562 cells was achieved using Tet-on inducible gene expression system [19-20] (Supplementary Figure 2). We developed three different variants of CXCR4 double stable K-562 cells expressing the wild type, mutant 1(N119A) and mutant 2 (N119S) CXCR4 gene under doxycycline dependent manner.

Flow cytometry analysis of each subset of CXCR4 double stable K-562 cells showed doxycycline inducible regulated expression of CXCR4 surface protein as evident by their significant shift in fluorescence intensity as compared to control (without doxycycline induction of CXCR4 double stable transfected cells). This suggested minimal/no leaky expression of CXCR4 transgene in double stable transfected K-562 cells without doxycycline induction (Figure 1).

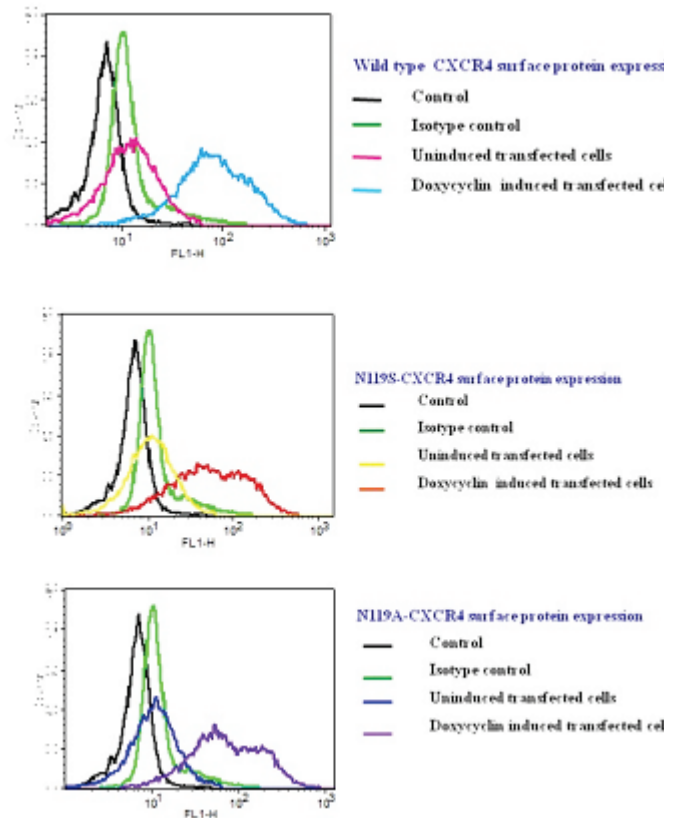


Figure 1: Histogram overlay of flow cytometry of three different variant of CXCR4 double stable K-562 cells showing doxycycline inducible surface protein expression of CXCR4 transgene. (X axis depicts fluorescence intensity in log scale while Y axis depicts cell events).

CXCR4 active mutants revealed enhanced chemotaxis to SDF-1 as compared to wild type:

The directed transmigration potential of K562 cells expressing CXCR4-CAMs towards SDF-1 gradient as compared to wild type gene was analyzed *in vitro* by transwell migration assay. As shown (Figure 2), the chemotaxis of control untransfected K-562 cells towards SDF-1 gradient (125ng/ml) was found to be 1.66 % whereas doxycycline induced wild type CXCR4 double stable K-562 cells was 5.66%. Furthermore, in the comparative analysis among CXCR4 double stable cells, we found significantly increased chemotaxis in the cells expressing CXCR4 mutant 1 (N119ACXCR4) (14.33%) ($P < 0.05$) and mutant 2 (N119SCXCR4) (17%) ($P < 0.05$) as compared to wild type CXCR4 (5.66 %).

Comparative Gene expression profiling of CXCR4 double stable K-562 cells by microarray analysis revealed differential gene expression profile of CXCR4-CAMs: We intended to gain further insights into molecular mechanistic of CXCR4 constitutive active mutants to better understand the

Regulated Expression of CXCR4 Active Mutants in Hematopoietic Stem Progenitor Cell Line-K562

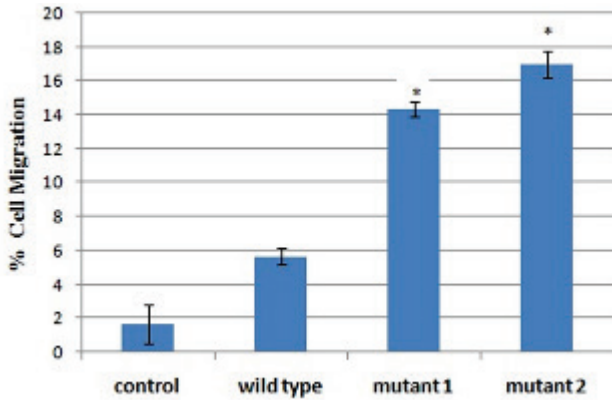


Figure 2: A bar graph depicting the chemotaxis/transmigration of mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) expressing K-562 cells towards SDF-1 gradient (125ng/ml) as compared to wildtype (WtCXCR4) in a transwell assay. The results shown represent the average of triplicates plus or minus Standard Error (SE). Error bars indicate standard deviation and *Indicates $P < 0.05$ with respect to wild type CXCR4.

mode of action for their enhanced cell migration and chemotaxis as compared to wild type. Hence, we analyzed the genome wide gene expression profile of each subset of doxycycline induced CXCR4 double stable K-562 transfected cells by microarray analysis. We compared the gene-expression profile of mutant1 (N119ACXCR4-pTRE2hyg) and mutant 2 (N119SCXCR4-pTRE2hyg) with wild type CXCR4-pTRE2hyg and the relative differences in gene-expression level are represented in terms of expression fold

value on log 2 basis (Table: 1 A-B). The differentially up-regulated genes were classified according to Biointerpretor pathways analysis tool (Genotypic Pvt. Ltd.), based on their association to different pathways; transendothelial migration pathways (PIK3R3, Txk, Cdc42), chemokine signaling pathways (GNG11, CHUK), cytokine-cytokine receptor pathways (IL-7, LIF, VEGFC, IGF-1, HGF), mOTR signaling pathway (PIK3R3, IGF-1, VEGFC), MAPK signaling pathway (MAPK9, MAPK4, MAP3K9, CACNA1E), Phosphatidylinositol signaling pathway (PIK3R3, INPP4B, PIP5K1C, PIP5K1A). Obviously, several differentially expressed genes are associated with more than one biological process. Further, the gene expression profile of differentially up-regulated genes of mutant 1 and mutant 2 was not found 100% similar though some genes were found common among these.

RT-PCR analysis: To verify the microarray results of differentially expressed genes, the gene expression analysis of few numbers of selected genes was performed by real time quantitative-PCR. The PIK3R3, MAPK9, Cdc42, IL-7 and Txk genes up-regulated in CXCR4-CAMs compared to wild type as revealed by microarray analysis, were analyzed through RT-PCR. As shown, the mutant 1 (N119A) and mutant 2 (N119S) expressing K-562 cells have shown a significantly increased gene expression of MAPK9, PIK3R3, IL-7, Cdc42 and Txk ($P < 0.05$) as compared to wild type CXCR4 expressing cells (Figure 3). Hence, these results indicated the congruity between microarray and RT-PCR results.

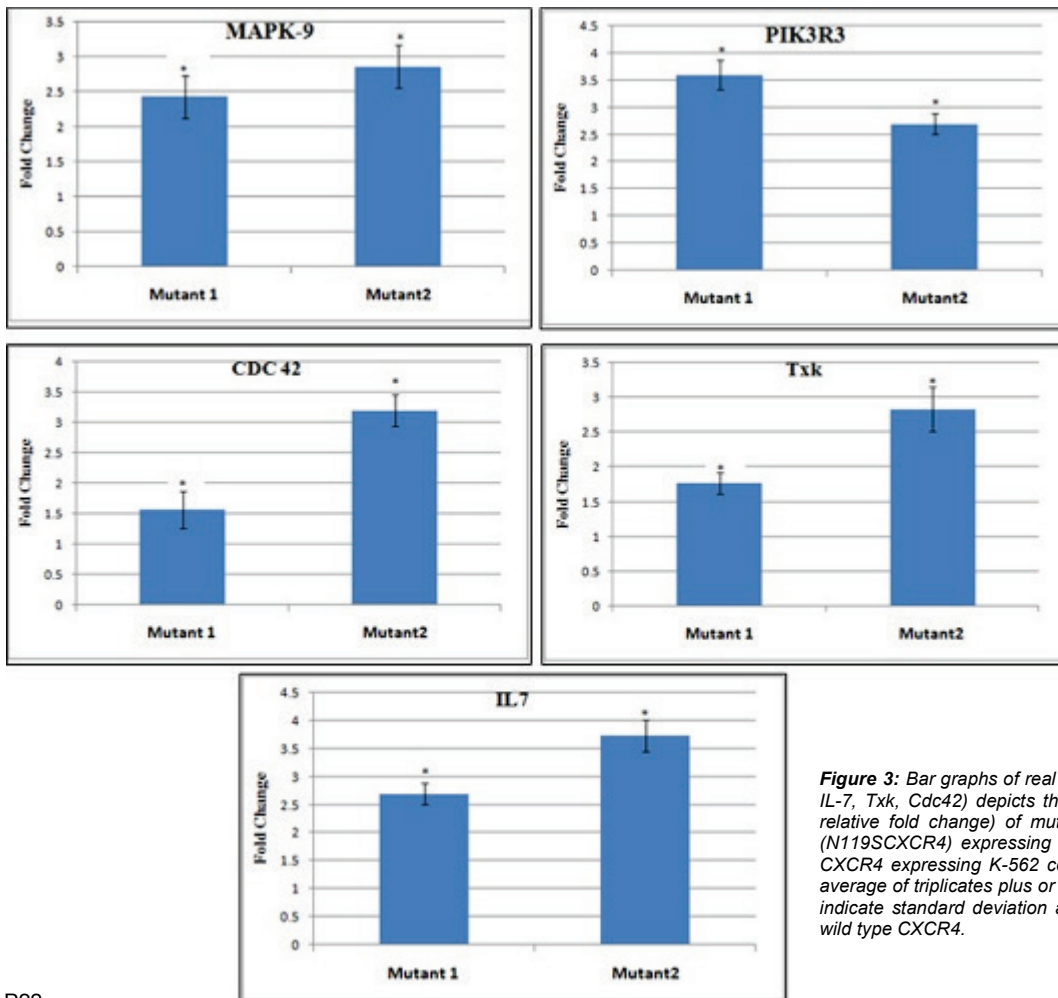


Figure 3: Bar graphs of real time PCR of genes (MAPK9, PIK3R3, IL-7, Txk, Cdc42) depicts the gene expression value (in terms of relative fold change) of mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) expressing K-562 cells with respect to wild type CXCR4 expressing K-562 cells. The results shown represent the average of triplicates plus or minus Standard Error (SE). Error bars indicate standard deviation and * indicate $P < 0.05$ with respect to wild type CXCR4.

Table 1 A: Differentially up-regulated genes in N119ACXCR4 mutant expressing K-562 cells

Gene Name	Gene Symbol	Gene Accession Number	Mean Fold Change
Mitogen activated protein kinase 4	MAPK4	NM_002747	4.96
Calcium channel, voltage-dependent, R type, alpha 1E subunit	CACNA1E	NM_000721	2.10
Mitogen activated protein kinase kinase kinase 9	MAP3K9	AK123430	1.88
Mitogen activated protein kinase 9	MAPK9	NM_001135044	1.64
Inositol polyphosphate-4-phosphatase, type II, 105kDa	INPP4B	NM_003866	3.63
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	PIP5K1C	NM_012398	2.51
Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	PIP5K1A	NM_003557	2.07
Hepatocyte growth factor	HGF	NM_001010931	2.98
Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	PIK3R3	NM_003629	2.17
Cell division cycle 42 (GTP binding protein, 25kDa)	Cdc42	NM_001791	0.87
TXK tyrosine kinase	TXK	NM_003328	1.15
Interleukin 7	IL7	NM_000880	1.91
Vascular endothelial growth factor C	VEGFC	NM_005429	4.18
Insulin-like growth factor 1	IGF1	NM_000618	3.38
Leukemia inhibitory factor	LIF	NM_002309	1.56

Table 1 A: Significantly up-regulated genes in N119ACXCR4 mutant expressing K-562 cells with respect to wild type CXCR4. (Relative mean fold change of each gene is given in log base 2 and P value is <0.05 with respect to wild type CXCR4).

Table 1 B: Differentially up-regulated genes in N119SCXCR4 mutant expressing K-562 cells

Gene Name	Gene Symbol	Gene Accession Number	Mean Fold Change
Guanine nucleotide binding protein (G protein), gamma 11	GNG 11	NM_004126	2.62
Conserved helix-loop-helix ubiquitous kinase	CHUK	NM_001278	1.17
Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	PIK3R3	NM_003629	1.69
Cell division cycle 42 (GTP binding protein, 25kDa)	Cdc42	NM_044472	1.89
TXK tyrosine kinase	TXK	NM_003328	1.72
Interleukin 7	IL7	NM_000880	2.21
Leukemia inhibitory factor (cholinergic differentiation factor)	LIF	NM_002309	2.28
Vascular endothelial growth factor C	VEGFC	NM_005429	3.97
Insulin-like growth factor 1	IGF1	NM_000618	2.29
Mitogen activated protein kinase 9	MAPK9	NM_001135044	1.76

Table 1 B: Significantly up-regulated genes in N119SCXCR4 mutant expressing K-562 cells with respect to wild type CXCR4. (Relative mean fold change of each gene is given in log base 2 and P value is <0.05 with respect to wild type CXCR4).

Discussion

Recent studies highlighted the role of CXCR4 downstream signaling cascaded as a principle molecular regulator of homing and engraftment. Hence, the CXCR4-CAMs (N119SCXCR4 and N119ACXCR4) being able to manifest autonomous signaling and its further up-modulation upon ligand binding, constitutes a promising molecular target for up-modulation of cellular and molecular mechanism of cells in the process of homing and engraftment. In this scenario, the over-expression of wild type CXCR4 gene through viral transduction mediated transgene expression is another approach, investigated earlier by Kahn *et al.*, 2003, which resulted in significantly enhanced homing and engraftment potential of HSPCs. However, the risk of viral vector being virulent and malignant transformation at any time cannot be avoided or overlooked. Also, to execute the enhanced CXCR4 downstream signaling, the over-expressed wild type CXCR4 receptor requires the comparable optimal availability of its ligand SDF-1. However, consistent maintenance of optimally available ligand condition in BM endothelial cells and extra vascular stroma cannot be fulfilled always. Considering this, the employment of CXCR4-CAMs being devoid of above limitations likely offers a better approach, but only if their implication is done in a regulated manner, as the constitutive activated signaling may result in undesirable oncogenic effects. Therefore, we first made an effort to develop a system for transgene expression of CXCR4-CAMs in a regulated manner using Tet-on inducible gene expression vector system. Additionally, this also offers the opportunity to precisely explore and study the transgene expression and its function in mammalian cell system. After achieving the doxycycline inducible regulated transgene surface expression of CXCR4 genetic constructs in the hematopoietic stem progenitor cell line-K562, we further analyzed the chemotaxis of these cells towards SDF-1 gradient *in vitro*. Our results revealed significantly enhanced chemotaxis of the CXCR4 double stable K-562 cells expressive of CXCR4-CAMs as compared to wild type gene. This indicated the likely up-regulation of CXCR4 downstream signaling in these mutants which ultimately regulate the cell motility machinery. However, the percentage increase in cell migration was not found absolutely similar in mutant 1 and mutant 2, as higher migration potential was revealed by mutant 2 than mutant 1. This may suggest that molecular mechanism for their enhanced migration potential might be differential among these two mutants.

Furthermore, the differential gene expression profile of CXCR4-CAMs compared to wild type as assessed by microarray analysis provided better insights into mode of action and responsive behavior of these cells in mechanistic of cell trafficking and homing, by revealing a positive loop of genes related to homing and engraftment. Among these, to envision the likely changes in CXCR4 downstream signaling of both mutants in comparison to wild type, according to KEGG and NCBI database we have classified the differentially up-regulated genes of CXCR4-CAMs into four different groups, based on their relation to classical CXCR4 signaling cascade at protein level. These are genes, whose protein product act as; a) downstream effectors of CXCR4 signaling (Cdc42), b) members of CXCR4 activated downstream pathways (Phosphatidylinositol signaling pathway, MAPK pathway), c) positive regulators of CXCR4 signaling (IGF-1, IL-7), and d) genes whose protein product have established role in cell migration and adhesion but so

far not reported to play a role in activation/induction of CXCR4 downstream signaling (LIF, Txk tyrosine kinase, VEGFC, HGF, GNG11 and CHUK).

Based on the existing information, we further attempt to discuss the role of these genes in context of CXCR4 mediated cell trafficking and homing. The MAPK signaling cascade is a highly conserved module which regulates the various cellular functions including cell proliferation, differentiation, migration and focal adhesion activation. This is one of the important pathways in CXCR4 activated downstream signaling cascade which crucially regulates homing and engraftment of HSPCs in BM^[21-22]. Hence, the up-regulation of genes, protein products of which are known to be positively associated with MAPK pathway (MAPK4, MAPK9, CACNAIE, and MAP3K9) is significant, indicating the likely up modulated potential in the CXCR4 mediated homing and engraftment. In similar way, one of the other important CXCR4 activated downstream pathway is PI3K, which mediate the cell adhesion, transendothelial migration and directed chemotaxis of HSPCs, resulting in homing and engraftment^[2,23]. The up-regulated gene PIK3R3 of this pathway belongs to PI3-Kinases (Phosphoinositide 3-kinases) family of lipid kinases, capable of phosphorylating the 3'OH of the inositol ring of phosphoinositides. They are responsible for coordinating a diverse range of cell functions including proliferation, cell survival, vesicular trafficking and cell migration via acting as a molecular player of PI3K pathway. The other positively associated members of this pathway which found up-regulated in active mutants are; PIP5KIA and PIP5KIC genes^[24-25].

CXCR4 signal transduction crucially depends on the activity of small GTPase of Rho-subfamily to induce the actin polymerization, a cytoskeleton protein remodeling which causes cell polarization and migration, resulting in transendothelial migration and chemotaxis of cells to home BM. The role of Rac-1 and Rac-2 of this family has been well established as downstream effector proteins of CXCR4 signaling to execute this process in HSPCs^[2, 13]. Another important member of this family is Cdc42 gene, protein encoded by which regulates actin polymerization through its direct binding to neural wiskott-aldrich syndrome protein (N-WASP) which subsequently activates Arp2/3 complex, causes the formation of thin, actin-rich surface projections called filopodia leading to cell polarization and transendothelial migration^[26]. However, the role of Cdc42 gene is not much explored so far in the CXCR4 downstream signaling to mediate homing and engraftment, though some recent studies highlighted its role as a downstream effector protein of CXCR4 signaling^[27-28]. In support of recent findings, the Cdc42 gene up-regulation in CXCR4 active mutants, further strengthen its significant role as a CXCR4 mediated downstream effector protein which regulate cell polarization, transmigration and chemotaxis. In this context, the up-regulation of another critical gene we found is Txk tyrosine kinase, a member of the Tec sub-family of non-receptor tyrosine kinases. The role of Txk has been shown in leukocyte transendothelial migration pathways^[29-30], thus suggesting its pivotal role in mutant CXCR4 downstream signaling for their enhanced chemotaxis/migration potential as compared to wild type.

Further, the up-regulation of genes, encoding for different growth factors/cytokines suggest that in the multi molecular BM microenvironment, interaction between different growth

factors and CXCR4 play an important role in homing and engraftment. Insulin like growth factor-1 (IGF-1) is a gene, protein product of which, upon binding to its receptor induces activation of PI3K/Akt and RAS/RAF/MAPK signaling. A recent study revealed that transactivation of CXCR4 by IGF-1 is required for IGF-1-induced cell migration in metastatic MDA-MB-231 cells. IGF-1 induced chemotaxis was inhibited by pretreatment of cells with pertussis toxin (PTX) and RNAi-mediated knockdown of CXCR4 [31]. Similarly, the chemotactic influence of leukemia inhibitory factor (LIF) has been revealed on BM non-hematopoietic cells in young mice as well as in rhabdomyosarcomas and neuroblastomas of BM. LIF/LIF-R axis mediates the phosphorylation of MAPKp42/44, PI-3K/AKT, JAK/STAT pathways and activation of small GTPase Rac-1 [32-33]. Interleukin 7 (IL-7) cytokine secreted by CD4⁺ mature thymocytes has shown to potentiate CXCR4 downstream signaling [34]. Moreover, the other important cytokines that are involved beside SDF-1 in regulating cell trafficking are; hepatocyte growth factor/scatter factor (HGF) [35] and vascular endothelial growth factor (VEGF) [36]. Taken together, thus it is likely that by induction of IGF-1, LIF and IL-7 genes, CXCR4 active mutants may act synergistically with these cytokines to potentiate the downstream signaling pathways such as PI3K/Akt and MAPK which crucially regulate cellular and molecular events of homing and engraftment. Further, a recent study has revealed that mammalian target of rapamycin (mTOR) pathway is activated by SDF-1/CXCR4 axis and mediate cell migration, matrix metalloproteinase (MMPs) production and cell growth. However, mTOR pathway activation has shown to induce not only by CXCR4 but also by cytokines; epidermal growth factor (EGF), HGF, VEGF and tumor necrosis factor-alpha (TNF- α) [37]. Keeping this fact, up-regulation of VEGFC and HGF genes in CXCR4-CAMs indicate a bidirectional correlation between CXCR4 and mOTR pathway, wherein CXCR4 activation directly mediate the induction of mOTR pathway as well as it may induces it indirectly via activation of VEGFC, and HGF.

Hence, the results altogether suggest a likely positive signaling loop of CXCR4 mutants which is more potent/up-modulated than classical wild type via complementary effect of up-regulated genes (Figure 4). It is noteworthy that among these genes, at protein level, while some are established as downstream effectors of wild type CXCR4 signaling, this study also explored some new genes as likely downstream effector proteins of mutant CXCR4 downstream signaling. In addition, up-regulated genes profile among these two active mutants of CXCR4 was found differential wherein only some genes are common among these. This may suggest that enhanced cell migration of both mutants N119A-CXCR4 and N119S-CXCR4 do not mediate via absolutely similar mechanism but their mode of action in downstream signaling is likely different. Nevertheless, it is worth noting that additional studies of these up-regulated genes at protein level may serve as a next phase of research in this direction that would be definitely helpful to explore further the hidden aspects and revealing the additional biological information to elucidate the molecular mechanistic and signaling network of these active mutants.

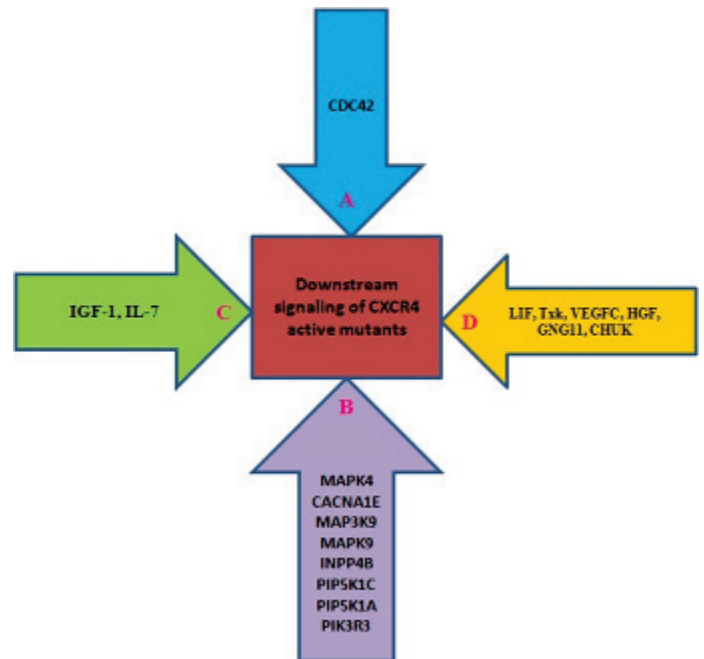


Figure 4: A schematic figure representing the different groups of up-regulated genes based on their relation to the classical CXCR4 signaling at protein level, which altogether may contribute in likely up modulation of mutant downstream signaling. A. as downstream effectors of CXCR4 signaling, B. as positively associated members of CXCR4 activated downstream pathways (Phosphatidylinositol signaling pathway, MAPK pathway), C. as positive regulators of CXCR4 signaling, D. genes whose protein product have established role in cell migration and chemotaxis but so far not reported to play a role in CXCR4 signaling.

Conclusion

Our study demonstrated the CXCR4 constitutive active mutants as potential molecular players having improved efficacy as compared to wild type in cell chemotaxis and transmigration, a key cellular event which critically govern the homing of intravenously infused HSPCs in BM extra vascular niche. Furthermore, the differentially up-regulated genes in CXCR4-CAMs as revealed by microarray analysis provides a better fundamental understanding for cellular and molecular mechanistic of these cells, by revealing the up-regulation of group of genes which are known as crucial regulators of cell trafficking, homing and engraftment. This study is a small contribution which directs our way further in this track that will be definitely helpful to delineate and dissect the response as well as complex-cooperative downstream pathways of CXCR4 mutants. Nevertheless, by developing a system to express the CXCR4-CAMs in a regulated manner and analyze their functional efficiency in a key cellular event of homing and engraftment, as well as identification of positive signaling loop of CXCR4 mediated homing and engraftment, this report enlightened the prospects of CXCR4-CAMs as potential molecular targets in research spectrum aimed to develop new strategies for improvement of HSPCs homing and engraftment efficiency via up-modulation of SDF-1/CXCR4 signaling cascade.

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Abbreviations:

CAMs:	Constitutive active mutants,	HSCT:	Hematopoietic Stem Cells Transplantation
BM:	Bone marrow,	HSPCs:	Hematopoietic Stem/Progenitor Cells
TM3:	Transmembrane three domain,	Asn:	Asparagine
Ser:	Serine,	Ala:	Alanine
SDF-1:	Stroma derived factor -1,	MSC:	Multicloning site
NCCS:	National Center of Cell Science,	IMDM:	Iscove's modified dulbecco's medium
PBS:	Phosphate buffer saline,	KEGG:	Kyoto Encyclopedia of Genes and Genomics
NCBI:	National Center for Biotechnology Information,	RT-PCR:	Real time quantitative-polymerase chain reaction
SD:	Standard Deviation,	IGF-1:	Insulin-like growth factor-1
LIF:	Leukemia inhibitory factor,	IL7:	Interleukin 7
VEGF:	Vascular endothelial growth factor,	TNF:	Tumor necrosis factor
HGF:	Hepatocyte growth factor,	EGF:	Epidermal growth factor
mTOR:	Mammalian target of rapamycin,	MMPs:	Matrix metalloproteinase
FBS:	Fetal Bovine Serum		

Potential Conflicts of Interests:

None

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Additional Information:

Supplementary Information accompanies this article. Supplementary figures and table are linked to the online version of the article

Correspondence to be addressed to:

Dr. Gurudutta Gangenhalli (Scientist F), Head, Stem Cell & Gene Therapy Department, Institute of Nuclear Medicine & Allied Sciences, Defense Research and Development Organization, Lucknow Road, Timarpur, Delhi-110054, India, Phone: 91-11-23905144, Fax: 91-11-23919509.