Hematopoietic stem cell transplantation is today considered a most immediate and effective therapy to reconstitute the impaired hematopoiesis in radiation and chemotherapy compromised BM resulting in restoration of body immunity, as well as in the treatment of hematological malignancy. In addition, this serves as an increasingly effective approach in gene therapy for treatment of various genetic disorders. HSCT is clinically defined by intravenous infusion of HSPCs targeted to reach BM of recipient. Biologically the journey of intravenously infused HSPCs is regulated by process of homing and engraftment, wherein transfused HSPCs in circulation actively cross the vascular endothelial cells barrier and reach recipient BM stroma, in order to lodge and repopulate there. However, the low homing and engraftment efficiency, especially in context of low availability of donor CD34+ HSPCs is a major challenge ahead which limits wide spectrum application and success of HSCT.

Recent studies have highlighted the pivotal role of SDF-1/CXCR4 signaling in regulation of homing and engraftment. SDF-1 binding to its GPCR receptor-CXCR4 expressed on HSPCs induces the conformational change of receptor to active state, making it highly permissible to bind G protein and other downstream effector proteins. These results in activation of CXCR4 downstream signaling cascade, which altogether regulate the cellular events that elicit homing and engraftment. However, the proper functioning of CXCR4 downstream signaling depends on the consistent optimal availability/expression of both SDF-1 ligand and CXCR4 receptor, which in turn is variable and regulated by different factors and conditions of BM microenvironment. The Asn-119 is a highly conserved motif in transmembrane three domain of GPCR and plays a critical role in the induction of CXCR4 signaling through regulation of dynamic conformational equilibrium of receptor from inactive to active state. Conversion of Asn-119 of CXCR4 to Ser (N119S) or Ala (N119A) was found to induce active conformation of CXCR4 manifested by its autonomous signaling resulting in constitutive activity. Furthermore, autonomous coupling of these CXCR4-CAMs to G protein subunits was shown to further augment by SDF-1 binding, suggesting the stabilization of an optimal active conformation. Hence, the autonomous signaling of CXCR4 receptor without ligand binding would not only overcomes the dependence of CXCR4 receptor to its ligand but also overcomes the necessity of consistent optimal expression of both ligand and receptor to interact and induce downstream signaling.
In view of present context, with an aim to establish an approach for up-modulation of cellular and molecular events that elicit homing and engraftment targeting its principle molecular regulator, the present investigation was carried out to imply the CXCR4-CAMs in a regulated manner, and study their response in cellular mechanistic which altogether results homing and engraftment. In the present study, Tet-on inducible gene expression vector system was used to develop a system for transgene expression of CXCR4 in hematopoietic stem progenitor cell line K-562 in a regulated manner under doxycycline dependent target gene induction. The wild type CXCR4 gene was sub-cloned into Tet-on system response component-pTRE2hyg plasmid vector and the CXCR4 constitutive active mutants (N119ACXCR4 and N119SCXCR4) were derived by *in vitro* site directed mutagenesis system using wild type CXCR4-pTRE2hyg recombinant plasmid vector as a template.

The hematopoietic stem progenitor cell line K-562 was selected for transgene expression of CXCR4, as this cell line is negative for endogenous CXCR4 gene expression at m-RNA transcript level thus preventing the endogenous interference of CXCR4 gene in response study of CXCR4-CAMs to evaluate their efficiency in comparison to wild type gene. The three different clone of genetically engineered K-562 cells, expressing wild type CXCR4, mutant 1 (N119ACXCR4), and mutant 2 (N119SCXCR4) were developed by stable transfection of K-562 cell line with pTet-on to make Tet-on stable K-562 cells and subsequent double stable transfection of these Tet-on stable K-562 cells with each clone of recombinant CXCR4-pTRE2hyg plasmid constructs; wild type CXCR4-pTRE2hyg, N119ACXCR4-pTRE2hyg and N119SCXCR4-pTRE2hyg. The immunofluorescence microscopy and flow cytometric analysis of these CXCR4 stable transfected cells using CXCR4 monoclonal antibodies showed the doxycycline induced expression of CXCR4 surface protein reaching peak value at 48 hrs of doxycycline induction. However without doxycycline induction, CXCR4 surface protein expression was not detected in transfected cells thus suggested no leaky expression of target protein without doxycycline induction in stable transfected cells.

In order to evaluate the efficiency of CXCR4-CAMs in the cellular and molecular mechanistic of homing and engraftment in comparison to wild type gene, the response study was done by performing *in vitro* assays to assess the cellular events of endothelial cell adhesion, chemotaxis/directed transmigration potential, binding ability/interaction to extracellular matrix protein fibronectin, all of which happens consecutively to elicit homing and engraftment process.
The functional efficiency of CXCR4-CAMs in directed migration potential (chemotaxis) towards SDF-1 gradient was analyzed by transwell migration assay. Significantly increased chemotaxis was shown by stable transfected cells expressing CXCR4 mutant 1 (N119ACXCR4) (14.33%) and mutant 2 (N119SCXCR4) (17%) as compared to wild type CXCR4 (5.66%). This indicated the up regulation of CXCR4 downstream pathways and effector protein in both of these mutants which regulate cell motility machinery that induces actin polymerization and cell polarization to execute transmigration and chemotaxis.

Next, the adhesion efficiency of CXCR4 active mutants on the endothelial cells in comparison to wild type gene was evaluated by in vitro endothelial cell adhesion assay using human umbilical vascular endothelial cells (HUVECs) monolayer. The CXCR4 stable transfected cells expressing wild type CXCR4, Mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) demonstrated 35%, 66% and 52% adhesion respectively on endothelial cells. Hence, the significantly increased endothelial cell adhesion efficiency of CXCR4 mutant 1 (N119A) and mutant 2 (N119S) as compared to wild type, suggested the improved adhesiveness of integrins receptors on CXCR4 mutants expressing cells to bind their endothelial cells ligands (VCAMs and ICAMs). In light of these encouraging findings, we further evaluated their adhesion response on 4Gy and 8Gy irradiated endothelial cells (HUVECs monolayer) in presence and absence of radio protector (melatonin). Following similar pattern as observed on normal endothelial cells, on 4Gy irradiated HUVECs monolayer, mutant 1 (N119S) and mutant 2 (N119A) expressing cells showed significantly enhanced adhesion of 50% and 39% respectively in comparison to wild type CXCR4 (24%). However, no significant difference was observed in % cell adhesion of each sample on 4Gy irradiated HUVECs monolayer pre-incubated to melatonin radio protector, instead they followed same pattern as observed on irradiated HUVECs monolayer without presence of radio protector. Mutant 1 and mutant 2 expressing cells revealed 56% and 48% cell adhesion respectively which is significantly enhanced as compared to 33% in wild type CXCR4. The adhesion efficiency of cells was found to decrease gradually with increasing dose of irradiation on endothelial cells, as in comparison to 4Gy irradiated HUVECs monolayer, cell adhesion of each sample on 8Gy irradiated HUVECs monolayer was decreased though not significantly. We found 24%, 40% and 35% cell adhesion of wild type CXCR4, mutant 1 (N119ACXCR4), and mutant 2 (N119SCXCR4) respectively. This revealed 10% and 4% decrease in cell adhesion of mutant 1 and mutant 2 respectively as compared to % cell adhesion.
of these mutants observed on 4Gy irradiated HUVECs monolayer, however both the mutants were still retained their significantly increased adhesion potential on endothelial cells in comparison to wild type CXCR4. Similar pattern was observed on 8 Gy irradiated endothelial cells pre-incubated with melatonin radio protector, wherein wild type CXCR4, mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) showed 31%, 44% and 41% cell adhesion respectively. Hence, the radio-protective effect of melatonin was not found very significant to sustain adhesion efficiency of cells as % cells adhesion though found little higher but was not significantly different from cell adhesion on irradiated HUVECs monolayer without melatonin incubation.

Furthermore, we studied the binding efficiency of cells to extracellular matrix protein fibronectin, a molecular process through which transmigration of cells in BM extra vascular stroma and their lodgment in specific niche occurs. Stable transfected K-562 cells expressing wild type CXCR4, mutant 1 (N119ACXCR4), mutant 2 (N119SCXCR4) showed 22%, 49.5%, and 43.6% cell adhesion respectively to fibronectin coated 24 well plate. This revealed significantly increased fibronectin binding efficiency of mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) in comparison to wild type CXCR4 expressing cells. Taken together, the significantly enhanced fibronectin and endothelial cell adhesion efficiency of CXCR4 active mutants suggest up-regulation of their downstream signaling/pathways in comparison to wild type CXCR4 which regulate cell adhesion process.

We attempted to gain further insights into molecular mechanistic of CXCR4 active mutants to elucidate their enhanced efficiency in cell chemotaxis/transmigration and adhesion potential. Genome wide microarray gene expression profiling of genetically engineered K-562 cells has allowed a better fundamental understanding of mode of action of CXCR4 active mutants by revealing up regulation of group of genes that regulate homing and engraftment process. Comparative analysis of microarray results revealed the differential gene expression profile of stable transfected cells expressing mutant 1 (N119ACXCR4) and mutant 2 (N119SCXCR4) compared to wild type CXCR4 expressing cells. These results revealed the up regulation of genes associated with leucocytes trans-endothelial migration pathways, chemokine signaling pathways, cytokine-cytokine receptor interaction, mOTR signaling pathway, Phophotidyl inositol and MAPK pathway in CXCR4 active mutants.
Among these, to envision the up regulation of activated downstream signaling in CXCR4 active mutants in comparison to wild type, based on reference of KEGG and NCBI data base, we classified the up regulated genes into four major groups, in a way they related to CXCR4 signaling. These are genes whose protein product are known to act: as downstream effectors of CXCR4 signaling (Cdc42), as members of CXCR4 activated downstream pathways (Phophotidyl inositol pathway, MAPK pathway), as positive regulator of CXCR4 signaling (IGF-1, IL-7), and the genes whose protein product have established role in cell migration and adhesion but so far not reported to play a role in activation of CXCR4 downstream signaling (LIF, mOTR signaling, Txk tyrosine kinase, VEGFC, HGF). Moreover, these data revealed that gene expression profile for up regulated genes of mutant 1 (N119A) and mutant 2 (N119S) is not 100% similar though some genes are common among these. Altogether, gene profiling study further indicated that up-regulation of downstream signaling of CXCR4-CAMs as compared to wild type is a result of induction of positive regulators and downstream effector proteins of CXCR4 signaling cascade instead of suppression of its negative regulation. Finally, based on present investigation it can be concluded that:

- Regulated expression of CXCR4 constitutive active mutants (N119SCXCR4 & N119A CXCR4) induce significantly enhanced cells adhesion of genetically engineered cells on extracellular matrix protein fibronectin as well as on normal and irradiated endothelial cells compared to wild type.

- N119S & N119A mutants of CXCR4 not only induce the improved adhesion, but also increase the chemotaxis and transmigration potential of cells towards SDF-1 gradient compared to wild type.

- CXCR4-CAMs stable transfected cells induces the differential gene expression profile in comparison to wild type CXCR4, wherein up-regulation of group of genes positively related to CXCR4 signaling suggested the up-modulation of downstream signaling in CXCR4-CAMs compared to wild type.

- Synergistic action of up-regulation of genes, acting at protein level as CXCR4 downstream pathways member, downstream effector protein and positive regulator such as MAPK pathway, PI3K pathway, Cdc42, Txk tyrosine kinase, IGF-1, IL-7 is likely
potentiate the activation of integrins, focal adhesion proteins and actin polymerization resulting in enhanced cell adhesion, chemoatxis and transmigration potential of mutant expressing cells.

- Up-modulation of CXCR4 signaling cascade by regulated expression of CXCR4 active mutants resulting in their improved chemotaxis and adhesion potential, implies the beneficial role of CXCR4-CAMs in cellular and molecular mechanistic of homing and engraftment.