Introduction
Bone marrow cells are primarily involved in two processes; the production of blood cells, and the formation of bone that houses the hematopoietic tissue. Hematopoietic stem cells (HSCs) are responsible for maintaining all blood lineages throughout the lifetime of an individual, and have been utilised in clinical treatments of various malignant and non-malignant disorders (Copelan et al., 2006). Some HSC grafts however, due to their limitation in numbers restrict their application to pediatric patients, for instance from umbilical cord blood (UCB) source (Gluckman et al., 1997). Expanding HSCs in vitro or improving their homing efficiency would overcome this hurdle (Bernstein et al., 2012). As the HSC niche regulates HSC function in vivo, it is believed that additional insights in the regulation of HSCs by their niche may identify novel ways to manipulate HSCs and enhance their clinical use (Dellatore et al., 2008). HSCs, the origin of blood cells reside in the bone marrow. HSCs self-renew and differentiate into a variety of specialized blood cells. Under specific situation they can mobilize in the peripheral blood. In the hematopoietic system, stem cells are heterogeneous with respect to their ability to self-renew. HSCs constitute 0.05% of total mouse bone-marrow cells, and can be divided into three different populations: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors without detectable self-renewal potential (Morrison and Weissman, 1994). HSCs originate sequentially in which the long-term HSCs give rise to short-term HSCs, which in turn give rise to multipotent progenitors (Morrison et al., 1997). A major thrust of basic HSC research has been identifying and characterizing these cells.

HSC reside in bone marrow microenvironment called “niche” which regulates and maintains its stemness. Bone and marrow are linked with HSCs, and the primitive HSCs are located proximal to the endosteal surface of trabecular bone. Numerous studies have been carried out to understand the signaling molecules involved in self-renewal and
differentiation of HSCs. These signaling molecules are believed to be involved in maintenance of stem cells *in vivo*. HSCs can undergo both symmetric and asymmetric division. In symmetric division two copies of the parent stem cells are produced; whereas in asymmetric division it leads to self-renewal of the parent stem cell as well as production of a daughter cell with decreasing stemness and capability of differentiating along specific lineages. Asymmetric division is most common, by which stem cells maintain their number at the same time generates differentiated progenies. This property of HSC is more attractive because it manages both tasks with a single division. The fate of HSC is determined by both intrinsic and extrinsic signals. In other words, these signals decide whether HSCs stay in quiescent stage or maintain their properties (self-renewal and differentiation) or undergo apoptosis.

HSC niche plays a key role in their expansion. Niches are local tissue microenvironments that maintain and regulate HSCs. Osteoblastic niche maintains long-term hematopoietic stem cells (LT-HSCs) in quiescent (G₀) state (Gong *et al.*, 1978; Nilsson *et al.*, 2001; Zhang *et al.*, 2003; Kiel *et al.*, 2005; Wilson *et al.*, 2006). Stem cells remain attached to the niche cells through many cell surface molecules (Arai, 2004; Trumpp *et al.*, 2010). Niche protects HSCs from myelo-suppressive stresses. According to Wilson *et al.* (2008), dormant or quiescent HSCs are activated and undergo self-renewal, following asymmetric division of cells, in response to any stress or stimulation with granulocyte colony-stimulating factor (G-CSF). Self-renewal division of HSCs generates a large number of transiently amplified progenitors and matured cells for replenishment of the loss of bone marrow (BM) cellularity. Once marrow regeneration is completed, activated HSCs return to dormancy (Wilson *et al.*, 2008). It has been reported that in response to the combination treatment of cyclo-phosphamide (CY) and G-CSF, the endogenous HSCs proliferate about
10-fold prior to the mobilization in the peripheral blood (Morrison et al., 1997; Wright et al., 2001). In another study by Bodine et al. (1996) the repopulation ability of HSCs was shown to be significantly improved by treatment of mice with G-CSF and stem cell factor (SCF). The rapid expansion of HSCs following above treatments suggested that most of these cells had entered in the cell cycle (Morrison et al., 1997).

In management of hematological malignancy, often combination of radiation and chemotherapy is given to the patients, which may severely affect the hematopoietic system. This may affect other vital organs also. The hematopoietic system is reconstituted by transplanting BM cells, especially HSCs. How these donor HSCs respond to the ablated BM environment is not clearly understood. Earlier studies showed that in humans as well as in mouse, bone marrow chimera eventually fails in the long run. This could happen due to either or combination of (a) rapidly dividing donor HSCs become defective in engraftment on osteoblastic niche, (b) osteoblastic niche loses control over donor-HSCs due to competition with endogenous cells for the space, thus these cells are engrafted in the vascular niche and slowly egress from the marrow environment after differentiation, and (c) asymmetric self renewal property of donor HSCs is lost. The divisions of LT-HSCs and short term (ST)-HSCs are considered to be related with the cell cycle status, capable of long-term and short-term engraftment potential, respectively. The quiescent LT-HSCs are responsible for long-term engraftment; whereas cells exit from the $G_0$ phase (ST-HSCs/multipotent progenitors) are engrafted for the short-term (Fleming et al., 1993; Gothot et al., 1998; Habibian et al., 1998). The difference between these cells was linked with marrow homing capacity. However, a recent study showed that ST-HSCs are also capable for long-term multi-lineage engraftment in an irradiated host (Liu et al., 2012).
Other disorders like tumor formation can also be taken care by the HSC microenvironment. The tumor ecosystem is a dynamic, complex, and evolving environment. Interactions between tumor cells and the surrounding host components provide multiple options for therapeutic targets. Because one of the major functions of the HSC niche is to induce growth arrest in its occupants while supporting their self-renewal (Taichman, 2005), tumor cells that metastasize to bone may be seized by HSC niche to make them dormant and stay that way for years. Although the mechanisms that regulate the induction and release of tumor dormancy are poorly understood, strategies that target the HSC niche to mobilize disseminated tumour cells (DTCs) using agents that mobilize HSCs would open up new possibilities to eradicate incurable DTCs. Once a cancer patient's tumor spreads to distant organs, such as bone, the survival rate of that patient drastically declines. Although significant progress has been made in the early diagnosis and treatment of localized tumors, we are still losing the battle against metastatic disease.

Recently, Napoli et al. (2008) targeted vascular niche and showed that parathyroid hormone (PTH) in concert with granulocyte colony stimulating factor (G-CSF) increases blood flow, capillary density, and circulating progenitor cell numbers and decreases apoptosis, fibrosis, oxidative stress, and inflammation in ischemic muscles. Administration of PTH externally greatly enhanced the beneficial effect of G-CSF for angiogenesis. Furthermore, concentrations of hematopoietic resident antigen-specific B cells were also increased in bone marrow after combined therapy.

On the other hand; the osteoblasts also produce hematopoietic growth factors and are activated by parathyroid hormone (PTH) or the locally produced, PTH-related protein, through the PTH/PTHrP receptor (PRP). With increase in PTH level there was an increase in frequency of HSC cells. Therefore, increased number of HSCs in the bone marrow following PTH treatment can be mobilized into the peripheral blood circulation. This shows that different component of the hematopoietic stem cell niche supports the HSCs in its proliferation and maintenance.
In this study, it was observed that there is reversibility between dormancy and proliferation of donor HSCs during marrow regeneration. In the competitive environment, host cells initially proliferated; later were found to be compromised in the presence of highly proliferating donor cells. It was also reported that mouse BM stromal cells transiently expressed thousands to million folds of hematopoietic growth factor genes compared to normal mouse stromal cells. This induction of growth factor genes was commenced with the proliferation of HSCs. As per the microarray data, it was identified that few genes of the stromal compartment were up regulated with time. The present study also demonstrated the importance of the key molecules identified in the stromal compartment that are believed to be responsible for protecting HSCs from apoptosis. Among the key molecules identified from the microarray analysis we choose Amphiregulin and SCGF of the stromal compartment to study their role in proliferation and differentiation of HSCs. Amphiregulin (AREG) is a ligand of the ErbB pathway and it gets activated when it binds to the Epidermal Growth Factor receptor (EGFR). The binding of AREG to EGFR triggers the auto phosphorylation of the EGFR intracellular tyrosine kinase domain, thereby activating various pathways like; MEK/ERK1/2 and PI3K/AKT. AREG is known to be involved in cell proliferation and differentiation, cell metabolism, cell migration, survival and regulation of cell cycle. AREG harbors a mitogenic activity to cells and is involved in several biological processes like nerve generation, bone formation, mammary duct formation, as well as the outgrowth and branching of several tissues such as lung, kidney and prostate (Hurbin et al., 2002).

On the other hand, stem cell growth factor (SCGF) is a novel hematopoietic cytokine which aids in HSC proliferation. Multiple growth factors synergistically stimulate proliferation of primitive hematopoietic progenitor cells. COS-1 cells transiently produce SCGF which supports growth of hematopoietic progenitor cells through short-term cultures of bone marrow cells. Expression of SCGF mRNA is restricted to myeloid cells and fibroblasts, suggesting that SCGF is a growth factor functioning within the hematopoietic microenvironment (Hiraoka et al., 1997).
Aims and objectives
1. To study the symmetric and asymmetric stem cell division in irradiated and transplanted mice

The aim of this study was to understand the proliferation status of donor-derived LSK cells and possible competition with that of the host, when crude BM cells (CD45.2) or purified donor HSCs (LSK: Lin^Scal"^cKit") cells were transplanted in irradiated hosts (CD45.1). The cell division status of donor and recipient LSK cells was to be determined. Another perspective of the study was to understand the implication of the microenvironment induced changes on division status of donor and recipient LSK cells.

2. To identify the key molecules and their potential role involved during the symmetric and asymmetric division.

The second part of the investigation was to identify the molecules of the microenvironment which may be responsible for the LSK cells proliferation and maintenance.

3. To mimic the in vivo conditions in vitro.

After identification of the molecule of the microenvironment, an in vitro study was to be performed to elucidate the role of the novel molecules in hematopoiesis by gene knockdown study.
Review of literature
4. Review of Literature

4.1. Stem Cells

Since 1868, when Ernst Haeckel coined the term “stem cell” (Haeckel et al., 1868) till today scientists have been trying to determine the exact potential of these cells. The stem cells were discovered in the early 1900’s when it was found that blood cells are generated from a smaller population of cells. For long, it had been the topic of debate whether the so-called stem cells exist or not. After the unsuccessful trials of oral administration of bone marrow cells to anaemia and leukemia patients, researchers demonstrated that mice with defective marrow could be restored to normal by infusing the donor bone marrow into the blood stream. Pioneering work by James Till, Ernest McCulloch, and others in the 1960s (Till et al., 1961, Becker et al., 1963, Till et al., 1964) terminated the long standing debate about the existence of a common hematopoietic stem cell (HSCs). Today it is an established fact that the ability of an embryo to diversify and form all adult tissues, which regenerate throughout life is due to the presence of adult stem cells.

Stem cells are those cells that have the ability to self renew and differentiate into other cells without compromising with the self-renewal potential. On the basis of developmental potential and ontogeny, stem cells are classified as: totipotent (can give rise to all embryonic and extra-embryonic cell types), pluripotent (can give rise to all cell types of the embryo proper), multipotent (able to give rise to a subset of cell lineages), oligopotent (able to give rise to a more restricted subset of cell lineages than multipotent stem cells), and unipotent (able to contribute only one mature cell type).

Stem cells being the cardinal cells of the body and have the property to self renew and differentiate into various types of specialized cells. These properties of stem cells play an important role in organogenesis during embryonic development and tissue regeneration. Stem cells reside in a specialized, regulatory environment termed the “niche” which dictates how they will generate, maintain and will repair tissues (Schofield et al., 1978; Xie
and Spradling, 2000). Broadly stem cells are classified into two types: embryonic stem cell and adult stem cell. Adult stem cells are present in most of the self-renewing tissues, including the skin, the intestinal epithelium and the hematopoietic system. The best-characterized adult stem cell is the hematopoietic stem cell (HSC) present in the bone marrow. Hematopoietic stem cells (HSCs) first arise in the yolk sac developing blood vessels from the wall of the embryonic aorta and arteries. HSCs produced in the yolk sac and in the embryonic truncal arteries migrate to and transiently colonize the embryonic liver (EL), and thereafter the bone marrow (BM), their permanent site of residence. At the moment, the origin of HSCs is still controversial; one of the main hypotheses being that they are generated by hemogenic endothelial cells (ECs). Oberlin et al. (2010) proved definitively, the endothelial origin of HSCs arise within the embryo. It was also found that some of the HSCs present in the EL were co-expressing vascular endothelial (VE)-cadherin, an endothelial marker, CD45, a pan-hematopoietic marker, and CD34, a common endothelial and hematopoietic marker, which demonstrates that these HSCs bearing a dual hemato-endothelial phenotype were endowed with remarkably high self renewal and proliferative potentials. Therefore, these studies strongly suggest that at least a part of the murine hematopoietic systems arise from an endothelium-like ancestor.

The bone marrow is a complex organ containing many different hematopoietic and non-hematopoietic cell types. Hematopoiesis occurs within the medullary cavity, surrounded by shell of vascularized and innervated cancellous bone. Minute projections of bone (trabeculae) are found throughout the trabecular zone of bone, such that many cells in this region are close to the bone surface. The interface of bone and bone marrow is known as the endosteum, and this is covered by bone-lining cells that can differentiate into bone forming osteoblasts. Bone-resorbing osteoclasts are also present at the endosteum. Arteries carry oxygen, nutrients and growth factors into the bone marrow, before feeding into capillaries and then sinusoids, which coalesce to form the venous circulation. In addition to bone marrow, HSCs can also be found at low levels in extra medullary tissues such as
spleen and liver throughout adult life (Figure 1). When bone marrow hematopoiesis is impaired by age, cancer or myeloablation, expanded numbers of HSCs can engage in extramedullary hematopoiesis in the spleen. HSCs reside around sinusoids in the red pulp of the spleen, but not in the white pulp, which contains lymphocytes and antigen-presenting cells.

In bone marrow, majority of the HSCs are in quiescent state. Approximately, 7-20% of the cells are in the S/G2/M phase (dividing phase) of the cell cycle and the remaining are in G0/G1 phase. Phenotypically, HSCs are defined as a cell population expressing Lin\(^{-}\) Sca1\(^{+}\) ckit\(^{+}\) in mouse (Morrison & Weissman, 1994; Spangrude et al., 1988; Ikuta & Weissman, 1992; Fleming et al., 1993) and Lin\(^{-}\) CD34\(^{+}\) in humans (Baum et al., 1992; Craig et al.,
HSCs are defined functionally by their ability to mediate LT-HSC repopulation (Figure 2) of all blood-cell lineages (known as long-term repopulating (LTR) activity) and to form colony forming units in the spleen after transfer to lethally irradiated recipients. Different in vitro assays followed to determine HSCs activity include LTC-IC (long-term culture-initiating cell) and CAFC (cobblestone area-forming cell).

**Figure 2: Characteristics of LT-HSCs and ST-HSCs** (Source: Wilson et al., Nature 2006) HSCs are defined functionally by their ability to mediate long-term repopulation of all blood-cell lineages (known as long-term repopulating (LTR) activity) and to form colony forming units in the spleen after transfer to lethally irradiated recipients. Assays to assess HSC activity in vitro include LTC-IC (long-term culture-initiating cell) and CAFC (cobblestone area-forming cell) assays. All LTR HSCs are contained in the lineage-negative (Lin)− stem-cell antigen 1 (SCA1)−KIT− (LSK) subset that comprises ~0.5% of bone marrow. 100 LSK cells are sufficient for multi-lineage LTR activity. Additional markers to further subdivide the LSK population into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), which have limited self-renewal activity, have been identified and are summarized in the figure. LTR activity is also enriched in the population of bone-marrow cells with low-level staining of rhodamine 123 (Rho). In addition, functional adult LTR HSCs can also be isolated by their ability to actively efflux the DNA-binding dye Hoechst 33342. This characteristic is designated as side-population (SP) ability.

The bone marrow consists of the hematopoietic cells and the non-hematopoietic (stromal) cells, these stromal cells play an active role in the stem cell regulation (Russell et al., 2005). HSCs undergo three major transitions during the cell cycle. The beginning of S phase is marked by the onset of DNA replication, the start of mitosis is accompanied by
breakdown of the nuclear envelope and chromosome condensation, and the metaphase-to-anaphase transition is characterized by the segregation of sister chromatids. Cyclin-dependent kinases (CDKs) trigger the transitions from G1 to S phase and from G2 to M phase by phosphorylating distinct sets of substrates. D-type cyclins complexed with CDK4 or CDK6 are thought to regulate events in early G1 phase; the cyclin E-CDK2 complex is required to initiate S phase; cyclin A, together with CDK1 or CDK2, is then responsible for the continuation of S phase and for entry into mitosis; and the B-type cyclins complexed with CDK1 promote entry into mitosis. Cyclin-CDK complexes phosphorylate retinoblastoma protein (Rb) family members and thereby inactivate their transcriptional co-repressor function. The resulting activation of E2F-dependent gene transcription leads to the production of proteins that are required for DNA replication and other aspects of cell cycle progression. CDK inhibitors (CKIs) inhibit the activity of cyclin-CDK complexes, resulting in suppression of the cell cycle (Figure 3). In hematopoietic stem cell biology, Bromodeoxyuridine (BrdU) retention assay or Hoechst 33342 (Ho) and pyronin Y (PY) staining are done to determine the cell cycle status of the cells (Roy et al., 2012). BM niches regulate the stem cell division of HSCs and based on their adhesion to niche the divisional symmetry is decided.

When both daughter cells remain adherent to the endosteal niche, the stem cell pool increases in size, whereas when one or both progeny migrate away from the Osteoblastic niche, they undergo terminal differentiation (Spradling et al., 2001). Utilizing the time-lapse microscopy; Punzel et al. (2003) showed the regulation of asymmetric stem cell division. According to their group, the microenvironmental cells induce the HSCs to enter active cell cycling and initiate asymmetric cell division without reduction in stemness of colony-forming cell.
Figure 3: Molecular mechanisms of cell cycle regulation. (Source: Matsumoto et al., Biochimica et Biophysica Acta 2012). Cells undergo three major transitions during the cell cycle. The beginning of S phase is marked by the onset of DNA replication, the start of mitosis is accompanied by breakdown of the nuclear envelope and chromosome condensation, and the metaphase-to-anaphase transition is characterized by the segregation of sister chromatids. Cyclin-dependent kinases (CDKs) trigger the transitions from G1 to S phase and from G2 to M phase by phosphorylating distinct sets of substrates. D-type cyclins complexed with CDK4 or CDK6 are thought to regulate events in early G1 phase; the cyclin E–CDK2 complex is required to initiate S phase; cyclin A, together with CDK1 or CDK2, is then responsible for the continuation of S phase and for entry into mitosis; and the B-type cyclins complexed with CDK1 promote entry into mitosis.

In 1978, Schofield predicted that in addition to differentiation-inducing microenvironments, there was also a specific hematopoietic stem cell niche, which "fixed" the stem cells in place and prevented their maturation, allowing them to proliferate at the same time retain their stemness. Once the stem cell progeny left the stem cell niche they proceeded to differentiate (Schofield, 1978).

4.2. Concept of HSC Niche and its function

A primary function of the niche is to anchor stem cells and maintain them at quiescence state. However, to maintain stem-cell homeostasis, the niche not only provides a shelter to
protect the stem cell from physiological stress or challenges but also restrains it from differentiation (which could exhaust the stem-cell pool) and overproduction (which could result in tumor formation). Therefore, niche keeps stem cells in a dynamic balance between self-renewal and differentiation. In short, the niche regulates stem-cell activity (Schofield, 1978). Hematopoietic stem and progenitor cells reside in a niche that consists of cellular and non-cellular components (Figure 4). Cellular components include stem or progenitor cells, stromal cells, neurons, immune cells, osteoblastic cells, osteoclasts, and endothelial cells, as well as the progeny of stem or progenitor cells. These cellular niche components regulate stem and progenitor cells directly through cell–cell interactions or indirectly by modifying non-cellular components.

Figure 4: Known components of the HSC niche: (Source: Levesque et al., Bioessays 2013) Components of the extracellular matrix (ECM) including collagen, fibronectin, and laminin contribute to the physical microenvironment comprising diverse biomechanical interactions.
During the past 25 years, studies on the bone marrow microenvironment have been predominantly based on either microscopy studies (light and ultrastructural; Lichtman, 1981) or through *ex vivo* culture process, especially using Dexter long-term bone marrow cultures, which were capable of long-term support of hematopoiesis (Dexter *et al.*, 1973, Dexter *et al.*, 1977). In these studies, the bone marrow stromal cells were defined as fibroblasts, reticular cells, endothelial cells, adipocytes and osteoblasts. However the exact identity of the cell type(s) comprising the hematopoietic stem cell niche remained unknown.

4.3. Homeostasis maintenance of HSCs by the niche

The capacity of adult stem cells to self-renew and differentiate is crucial for tissue homeostasis. The stem cell population will go down if cell differentiation overwhelmed self-renewal. Similarly, unchecked stem cell self-renewal would expand the stem cell population excessively, risking tumorigenesis (Jones *et al.*, 2004). An important function of the stem cell niche, therefore, is to regulate the balance between cellular self-renewal and differentiation. One mechanism that ensures this balance is the control of asymmetric/symmetric stem cell division. **Asymmetric division** means that stem cells divide into 2 daughter cells; one daughter cell remains in the niche as a stem cell and the other leaves the niche to produce a large number of progeny (Figure 5). **Symmetric division** means that stem cells divide into two identical daughter cells, both remaining in the niche as stem cells. Switching between symmetric and asymmetric division can occur in multiple stem cells that occupy the same niche under different physiological conditions (Watt & Hogan 2000; Fuchs *et al.*, 2006).
Figure 5: Modes of Hematopoietic stem cell division: Asymmetric and Symmetric Division (Source: Morrison et al., Nature 2006)

Anatomically, the hematopoietic stem cell niche may be of two types: an osteoblastic niche (located near osteoblasts) and a vascular niche (near the sinusoids) (Figure 6A). The HSC microenvironment dictates the HSC's symmetric or asymmetric division with the influence of local factors. When a lineage mechanism prevails the HSCs divides such that one daughter is in connections to the niche, while the other is not (Figure 6B).
Figure 6: A. Types of Niche (Source: Mitsiadis et al., Cell 2007); B. Niche structure (Source: Allan Spradling et al., Nature 2001).

Niche cells (green) underlying a basement membrane signal to stem cells (red) to block differentiation and regulate division. When a lineage mechanism prevails (lower mitotic cell), the stem cell divides such that one daughter retains its connections to the niche, while the other (yellow) becomes untethered and begins to differentiate. When a population mechanism prevails (upper mitotic cell), stem cell division may be either symmetric (shown) or asymmetric (not shown), as determined by local factors.

4.4. Osteoblastic Niches

Osteoblastic cells are located on the inner surface of the endosteum in the bone marrow. These cells are believed to be derived from the mesenchymal stem cells. Primarily osteoblasts are involved in the bone formation and they are present in dynamic equilibrium with osteoclasts (bone resorbing cells) which are also produced near the endosteum (Troen B et al. 2003). Bone lining endothelial cells is heterogeneous in their degree of differentiation, and only small number of these cells is actually bone-synthesizing osteoblasts.

4.5. Osteoblastic niche and HSC maintenance

HSCs can be classified into two types, viz., long-term and short-term HSCs. Long-term HSCs (LT-HSCs) lead to hematopoiesis for several months or even to a lifetime. Short-term HSCs (ST-HSCs) have limited reconstitution ability only to few months (Morisson et
LT-HSCs are maintained primarily as quiescent low-cycling cells, (Figure 7) while ST-HSCs are actively cycling (Yang et al., 2005).

Figure 7: Hematopoietic cell hierarchy; (Source: Wasnik et al., International Review of Cell and Molecular Biology 2012) A simple outline of the cellular hierarchy is described in the figure. LT-HSC (long-term HSCs) is at the top of the hierarchy and considered most primitive having capability of long-term self-renewal. LT-HSC gives rise to ST-HSCs (short-term repopulating HSCs), which further differentiates to MPPs (multipotent progenitors). The self-renewal capacity decrease as the cells differentiates from LTHSC to ST-HSCs and then to MPP and later on become more restricted toward certain lineages. Finally, MPPs give rise to unipotent cells which are committed progenitors of specific cell lineages, and these unipotent cells terminally differentiate as mature cells of different cell types of blood. The cellular hierarchy is identified and established by using specific monoclonal antibodies against cell surface markers by fluorescent-activated cell-sorting assays.

The role of osteoblasts in maintaining the HSCs has been the area of focus for long time (Rodan & Martin, 1981). The osteoblastic cells maintain the HSCs in quiescence, the resting phase. Earlier many studies have been done exhibiting the role of osteoblasts in regulating the hematopoiesis (Rodan & Martin, 1981) but only in recent time it has been made clear that osteoblasts are essential component of the HSC niche in adult bone marrow. Taichman et al. (1998) first proposed that osteoblasts support HSCs. They have
demonstrated that primary and transformed murine osteoblast cell lines secrete various cytokines like SCF and thrombopoietin that regulates the stem cell function. Further studies by Zhang et al. (2003) & Calvi et al. (2003) suggest the involvement of osteoblasts for homing of hematopoietic stem cells. In BMP receptor type I knockout mice model it has been shown, that with increase in the spindle shaped N-cadherin CD45'osteoblastic (SNO) cells, which HSCs number is increased suggesting a direct correlation between the two cell types.

Again, over-expression of the osteoblastic cells specific parathyroid hormone related protein (PTHrp) results in increased number of osteoblasts which in turn increases the HSC population (Calvi et al. 2003). In another study utilizing the transgenic mice model the osteoblastic cells are severely compromised due to the specific expression of Thymidine Kinase, which is associated with fall in HSC population indicating a direct correlation between osteoblastic cells and HSCs. All aforementioned studies emphasize on the involvement of osteoblasts in maintenance of the LT-HSCs.

While studies of osteoblast expansion have demonstrated an increase in HSCs, studies of osteoblast decrease have not consistently shown to reduce HSC number. Visnjic et al. showed that deletion of osteoblasts by gancyclovir in mice transgenic for thymidine kinase expressing osteoblasts, causes reduced marrow and increased extramedullary hematopoiesis (Visnjic et al., 2004). This was accompanied by a reduction in absolute number of primitive hematopoietic cells in the bone marrow and increased number in spleen and liver (Visnjic et al., 2004). However, osteoblast dysfunction in the biglycan deficient mouse did not result in the change in marrow HSC (Kiel et al., 2007). Therefore, either specific subsets of osteoblasts contribute to niche or osteoblasts are not required for niche function (Figure 8).
Review of literature

Figure 8: Illustration of endosteal zone and central marrow zone. (Source: Xie et al., Nature 2010)  
a. The endosteal zone is the inner bone surface and is under homeostasis. HSCs residing in this zone normally receive inhibitory signals from osteoblastic cells but are also exposed to vascular signals. In the central marrow zone, vascular signals might be dominant.  
b. When bone marrow, including HSC, is damaged by irradiation, the endosteal environment transiently converts into a stimulatory environment. This might be due to both reduction in osteoblastic inhibitory and increase in vascular stimulatory signals.

4.6. Factors present in the bone marrow osteoblastic niche which are important for HSCs maintenance

HSCs endosteal niche secrete of various factors required for HSCs self renewal and differentiation. These include growth factors, chemokines, adhesion molecules etc. (Heissig et al., 2002, Aveill et al., 2004, Kopp et al., 2005, Yin & Li, 2006) as shown in the following Figure 9.
Osteopontin (OPN): OPN is an acidic glycosylated phosphoprotein, expressed by the osteoblasts. The role of OPN in regulation of HSC population in bone marrow has been well documented (Denhardt & Guo, 1993). Nilsson et al. (2005) has demonstrated that OPN is also involved in HSC trans-marrow migration. In addition, OPN has been postulated to act as a negative regulator of HSCs by actively maintaining their quiescence (Nilsson et al., 2005).

Angiopontin (Ang): Angiopontin family of growth factors is composed of four members that bind to the Tie-2 tyrosine kinase receptor and are important modulators of angiogenesis. Angiopontin-1 expressed in the osteoblasts, has been shown to enhance the ability of HSC to remain in the quiescent stage. Tie-2 interaction with angiopontin-1 leads
to long term engraftment of HSCs. Furthermore, this pathway also supports SCID-mouse repopulating cell (SRC) activity in human CD34+ cell in culture (Nakamura et al., 2007).

**Thrombopoietin (TPO):** TPO is synthesized by the bone-marrow stroma and osteoblasts (Guerriero et al., 1997). TPO and its receptor myelo-proliferative leukemia virus oncogene (Mpl) are shown to modulate the HSC number. Studies in TPO or Mpl knock-out mouse showed decrease in HSC population (Solar et al., 1997), thereby, emanating its role in maintenance of HSC population.

**Steel factor:** Also known as stem cell factor (SCF), functions by binding to ckit, a tyrosine kinase receptor expressed in all HSCs. Expression of defective ckit leads to a decrease in repopulating HSCs, although it may not be necessary for HSC generation (Ikuta & Weissman, 1992). Studies have shown that it play a critical role in preventing apoptosis in HSCs (Hassan & Zander, 1996). Membrane bound SCF is expressed on osteoblasts, which helps in bonding of HSCs and hematopoietic progenitor cells (HPCs) on the niche (Kinashi & Springer, 1994). It can activate very late antigen 4 (VLA4) and very late antigen 5 (VLA5), thus it has been considered that membrane-bound SCF can affect the adhesive properties of the endosteal niche by modifying the functional state of specific integrins (Kovach et al., 1995).

**p53:** p53 plays critical roles in triggering senescence, apoptosis and cell cycle arrest in virtually all cell types (Vousden & Lane, 2007). Recently, Liu et al. (2009) highlighted its role in hematopoietic stem/progenitor cells and have shown that HSC quiescence is impaired due to the absence of p53. Function of p53 is found to be essential for the enhancing stem cell quiescence.
Serpina1 (Serpi): Serpina1 a serine protease inhibitor is one of the several molecules secreted by the osteoblastic cells. Serpi1 is primarily involved in hematopoietic stem cell maintenance thus indicating its role in HS regulation through Osteoblastic niche (Kuiperij et al., 2009).

Wnt (Wingless type): Wnt signaling has been shown as an important signaling pathway in stem cells self renewal and its maintenance (Reya et al., 2003). Wnt ligand may act in two ways; β catenin dependent pathway (canonical pathway) or β catenin independent pathway (non canonical pathway). In the canonical pathway receptor activation leads to the stabilization of β catenin, this accumulates and translocates in the nucleus to activate target gene expression in concert with transcription factors such as Tef and Lef (Willert et al., 1998). Hence, signaling through the Wnt canonical pathway is centred on the β catenin activity. Non canonical ‘Wnt5a’ supports HSC repopulation by inhibiting canonical Wnt signaling in HSC and maintains HSCs in a quiescent state (Nemeth et al., 2007). Non canonical Wnt Signaling has been proposed to be mediated through intracellular calcium release and kinase activation (Kuhl et al., 2000). Recently, it has been shown that Wnt inhibited microenvironment created by the osteoblasts specific over expression of Dkk1 (Dickkopf-1), results in the increase of the number of proliferating HSCs and the reduction in the ability to reconstitute the hematopoietic system of irradiated recipient mice. This indicates that micro-environment related Wnt/ β catenin activity is crucial for the maintenance of HSC quiescence (Heather et al., 2008). Wnt proteins act by binding to the frizzled family of seven-pass trans-membrane proteins (Wodarz et al., 1998) as well as to proteins of the low density lipoprotein receptor related protein family, LRP5 and LRP6 (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The necessacity of Wnt
pathway for HSC maintenance was uncoupled as expression of the Wnt inhibitor axin (scaffold protein) leads to inhibition of HSC proliferation and viability of cells in vitro (Reya et al., 2003; Willert et al., 2003).

**Notch ligand:** Notch signaling has been identified as a key factor in inhibiting HSCs differentiation by maintaining them into quiescence state. Notch proteins are highly conserved cell surface receptors that regulate development (Artavanis-Tsakonas et al., 1995). Notch is a single-pass trans-membrane receptor that is activated when its extra cellular domain interacts with the ligands of delta and serrate families. Mammals have four notch receptors (Notch 1-4), which binds to five different ligands (Jagged 1-2, Delta like 1-3-4) (Radtke et al., 2004). Expression of a notch 1 (N1) in murine bone marrow progenitors leads to increase in HSCs self-renewal. Recently it has been identified that Jagged1 expressing osteoblast regulated HSCs homeostasis through notch 1 activation (Calvi et al., 2003). The role of notch1 in HSC maintenance has also been exemplified by over-expression of activated notch1 in recombination activating gene1-deficient HSCs which results in increased generation of HSCs both in-vitro and in-vivo (Stier et al., 2002).

**Cell adhesion molecules:** Maintenance of HSCs homeostasis also depends on physical attachment of cells with other supporting stromal cells. Adherin junctions are bridges between the two cells that are formed by homotype interactions between transmembrane proteins called cadherins. Studies have shown that the association of HSCs with osteoblasts is facilitated through cadherin mediated cell adhesions (N-cadherin) (Zhang et al., 2003).
**N-cadherin** is expressed by both spindle-shaped N-cadherin\(^{CD45^{-}}\) osteoblastic cell (SNOs), and a subset of LSK HSCs (Zhang et al., 2003) but recent study argues its involvement in regulating HSCs (Wilson et al., 2004). Involvement of Tie2/Ang-1 signaling has been shown to induce \(\beta1\)-integrin and N-cadherin dependent HSC adhesion (Arai et al., 2004). MPL/THPO signaling also up-regulates \(\beta\)-integrin in LT-HSCs (Yoshihara et al., 2007). Furthermore, studies also showed that the conditional inactivation of c-Myc induces excessive expression of integrins and N-cadherin in HSCs, and thus Myc-deficient HSCs are not able to proliferate and detach from the niche due to uncontrollable cell adhesion (Wilson et al., 2004). Above studies suggest that main function of these interactions would be to maintain HSCs in close association to cells in the endosteal bone marrow niche and also regulate cell cycle quiescence of HSCs in the niche.

**mKirre** is another membrane protein that has been identified to support growth of HSC in vitro (Ueno et al., 2003). In addition, some of the pleiotropic cytokine like IL-10 was also reported to have additive effect on HSC growth and self renewal (Kang et al., 2007). The other reported cytokine IL-6 when used in combination with SCF greatly helps in the ex-vivo expansion of HSCs (Lyman et al., 1993; Small et al., 1994).

**CXC-chemokine ligand 12** (CXCL12) is secreted by reticular cells that reside perivascularly as well as near the endosteum. (Sugiyama et al., 2006). CXCL12 helps in to regulating HSC migration and localization in the bone marrow (Peled et al., 1999, Petit et al., 2002). The biological effects of CXCL12 are mediated by its capacity to induce motility, chemotaxis and adhesion, as well as to induce secretion of matrix metalloproteinases (MMPs) and angiogenic factors [such as vascular endothelial growth factor (VEGF) by cells expressing its receptor, CXC-chemokine receptor 4 (CXCR4)].
CXCR4 deficiency affects the HSC number in the bone marrow and HSC activity upon transplantation (Zou et al., 1998).

4.7. Vascular niche and its relation to Hematopoietic stem cells

The vascular niche with relation to HSCs can be divided into two distinct parts: an embryonic part and an adult part. The embryonic part has been explored extensively, in which interactions of HSCs and endothelia cells are tightly linked. During embryogenesis, hematopoietic stem cells originate in the developing aorto-gonado-mesonephros region (Shalaby et al., 1997; Choi et al., 1998; Godin et al., 1995; Medvinsky et al., 1996). Shalaby et al. (1997) showed that mice lacking Flk1, a tyrosine kinase expressed on endothelial progenitor cells, failed to develop both vascular endothelium and blood islands during embryogenesis. Choi et al. (1998) subsequently demonstrated via gene tracing studies that vascular endothelial and hematopoietic cells arise in vitro from a common precursor cell, the hemangioblast. The onset of definitive hematopoiesis was shown by different investigators to occur at the site of the dorsal aorta at E10.5–11.5 within the aorto-gonado-mesonephros region (Godin et al., 1995; Medvinsky et al., 1996). Several complementary studies using lineage tracing experiments in both mice and zebrafish have subsequently shown that HSCs arise from hemogenic endothelium within the ventral aspect of the dorsal aorta (Zovein et al., 2008; Bertrand et al., 2010; Eilken et al., 2009). According to various reports, the transcription factor Runx1 critically regulates HSC generation from aortic endothelial cells (Chen et al. 2009; Lam et al. 2010). HSCs, which arise from hemogenic endothelium, migrate to the fetal liver and to the fetal bone marrow and are capable of self-renewal and multilineage differentiation. In adults, endothelial cell form the inner line of blood vessels in the body. In case of bone marrow, they form a
barrier between the developing hematopoietic cells and the blood. They are therefore the initial site of entrance of all blood cells into the bone marrow from the circulation and also the final place whereby blood cells leave the bone marrow to enter the bloodstream (Sipkins et al., 2005; Winkler and Levesque, 2006). The possibility of a perivascular zone serving as a regulatory niche for stem cells is derived from two studies. In vivo imaging of primitive hematopoietic cells in animals over time revealed that they localized to specific subsections of the marrow microvasculature where cells persisted or increased in number over a 70 day interval (Sipkins et al., 2005). These data suggested the perivascular site as a potential niche for HSC, although the percentage of HSCs was found to be low when characterized (Figure 10).

![Figure 10: Endosteal and vascular niches are sub-compartments of a single niche.](Source: Lilly et al., Stem Cells International 2011) HSCs located at the endosteum are more quiescent and have a greater self-renewal capacity due to a variety of cytokines, adhesion molecules, and hypoxia. HSCs located close to the sinusoid endothelium have reduced self-renewal capacity and are cycling more rapidly, due to higher oxygen levels and SCF. However, nearly all HSCs reside adjacent to CAR cells and in close proximity to the sinusoid endothelium. The cells of the vascular niche communicate with cells of the endosteal niche, and the subtle balance of factors from these sub-compartments governs the behaviour of the HSCs.
The discovery of signaling lymphocyte activation molecule (SLAM) antigens specific markers to HSCs enabled histological assessment for HSCs residence in the marrow microenvironment (Kiel et al., 2005). These studies indicated that the majority of HSCs were in the perivascular region with only a minority (~16%) at the periendosteal region.

4.8. Vascular niche and HSC maintenance

The first evidence of vascular regulation of hematopoiesis was reported by Knospe et al. (1972) where they showed hematopoietic regeneration in areas of curetted bone marrow of adult mice in relation to sites of BM sinusoidal vascular regeneration. The BM sinusoidal vasculature is radiosensitive but regenerates and reorganizes within 3–4 weeks following sublethal exposure (Chute et al., 2007). However, doses at or above 20Gy, complete repair and regeneration of the BM sinusoidal vasculature does not occur (Shirota & Tavassoli, 1992). Importantly (Rafii et al. 1994; 1995) showed that primary human BM endothelial cells (ECs) support the proliferation and differentiation of human CD34^ cells in culture and produced several hematopoietic cytokines. Interestingly, (Davis et al. 1995) described the expansion of human BM CD34^ progenitor cells in culture with a porcine brain microvascular EC line, suggesting that ECs from non-hematopoietic tissues could also support HSC growth in vitro. Subsequent studies demonstrated that the porcine brain microvascular EC line was capable of supporting the maintenance of non-human primate (baboon) HSCs in vitro as well as human cord blood repopulating cells as measured in a xenograft transplantation model (Brandt et al., 1999; Rosler et al., 2000). These studies provided foundation for several important questions to be addressed regarding the role of ECs in regulating hematopoiesis and, more specifically, HSC fate.
4.9. Factors present in the bone marrow endothelial niche which are important for HSCs maintenance

Bone marrow endothelial niche comprise of various factors required for HSCs self renewal and differentiation. These include various secreted ligands and their receptors, growth factors, cytokines, adhesion molecules etc. as shown below in Figure 11.

![Figure 11: Molecular interactions and physicochemical gradients in the vascular niche](Source: Wasnik et al., International Review of Cell and Molecular Biology 2012)

**SDF-1** released from endothelial cells promotes HSC trans-endothelial migration. Likewise, high levels of SDF-1 on osteoblastic-cell surfaces guide HSC homing to the niche. Activation of adhesion molecules, such as VLA-4 and leukocyte function antigen 1 (LFA-1), is also necessary for these movements (Kopp et al., 2005).

**Pleiotrophin (PTN):** PTN is a heparin-binding growth factor, expressed by sinusoidal endothelial cells within the bone marrow vascular niche and regulates the maintenance of
the HSC pool *in vivo*. It is a secreted component of the vascular niche that regulates HSC self-renewal. BM sinusoidal ECs uniquely express a secreted protein, PTN that regulates the maintenance, regeneration, and retention of HSCs in the vascular niche. It represents a unique target for pharmacologic approaches to modulate HSC function *in vivo*. Genetic deletion of PTPRZ, a receptor for PTN; that is expressed by HSCs, causes a significant expansion of the HSC. PTN is an important paracrine factor for HSCs within the vascular niche. It has a function in regulating hematopoietic regeneration PTN has an important potential clinical application for anti-PTN in mobilizing HSPCs in patients undergoing stem cell transplantation.

*E-selectin*: HSCs express E-selectin–binding cell-surface glycosphingolipid ligands. E-selectin is a cell adhesion molecule expressed exclusively by bone marrow endothelial cells in the vascular HSC niche. It is a potent driver of HSC proliferation *in vivo*. Transient administration of a small synthetic E-selectin antagonist blocks proliferative signaling by the vascular HSC niche, promoting HSC quiescence, HSC self renewal and increased survival after treatment with chemotherapeutic agents *in vivo*. The expression of E-selectin is greatly increased during the recovery phase after irradiation. The *de novo* expression of E-selectin ligands on tumor cells is thought to aid their homing and engraftment into the bone marrow. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance (Winkler *et al.* 2012).

*Vascular Endothelial Growth Factor (VEGF)*: VEGF-3 is expressed exclusively by the sinusoidal endothelial cells (SECs). The differential expression of VEGFR3 can differentiate SECs from arterioles. The main receptors for VEGF mediate EC proliferation, survival, and migration and are expressed on SECs. Under stress conditions such as
thrombocytopenia, SDF-1 and VEGF activate MMP-9, which converts membrane-associated Kit ligand into soluble Kit ligand and in turn promotes HSCs entry into the cell cycle, mobilization to the vascular niche, and differentiation.

4.10. Interaction between Osteoblastic niche and Vascular niche

Although the osteoblastic and vascular niches are different from each other in many facets, there are some common components in both of them. CAR cells, reticular cells which have been demonstrated to maintain the stem cell pool and regulate HSC trafficking by CXCL12/CXCR4, have been shown to be present in both the osteoblastic and the vascular niche, indicating that both share some common characteristics (Sugiyama et al., 2006). Similarly, the major component of vascular niche – the endothelial cells are found to be present in almost entire bone marrow, could be spotted in osteoblastic region where they help in osteogenesis and vasculogenesis (Guerrouahen et al., 2011; Nakamura et al., 2010).

The role of Notch signaling and Ang1-mediated regulation of HSCs has been demonstrated in both niches (Figure 12). Studies conducted in the recent past have shown that Annexin II (Anx II) is expressed by both OBs and endothelial cells. While Anx II-mediated adhesion between HSCs and OBs has been studied, Anx II-mediated endothelial cell-HSC interaction has to be investigated (Jung et al., 2007).
4.11. Hematopoietic stem cell mobilization

Release of hematopoietic stem/progenitor cells (HSC) from the bone marrow into peripheral blood following treatment with chemotherapy, radiation-therapy and/or cytokines or due to some other stress is termed mobilization. Bone marrow cells also enter peripheral blood in response to stress signals during injury and inflammation of hematopoietic and non-hematopoietic tissues (Kollet et al., 2003; Massa et al., 2005; Lemoli et al., 2006). Mobilization of HSCs is a two-way process, having movement of HSCs both into and out of the BM. Transmigration of cells from the vasculature into the BM is not a very well-understood process, but CXCL12 signaling has been shown to be involved in the process. The Rho family proteins Rac1 and Rac2 are activated in response
of the CXCR4-CXCL12 signaling, and inhibition of these proteins affects the homing and transmigration to the BM. The calcium-sensing receptors, CaRs expressed on HSCs guide the cells to migrate to the Ca^{2+}-rich endosteal region. Recent studies demonstrate the role of nestin^{+} cells in the HSCs homing and migration, and the nestin-negative mice show 90% decrease in homing to BM as compared to wild type (Mendez-Ferrer and Frenette, 2007).

HSCs migrate from the BM into the circulation throughout life and can be detected in the peripheral blood in low numbers (Wright et al., 2001). The migration of HSC is increased during infection and inflammation and in response to myeloablation and cytokines such as G-CSF. It has been suggested that HSC migration is one of the mechanism for the repair of tissue damage (Hannoush et al., 2011).

The mechanism of HSC mobilization includes at least three systems of interaction — the interaction between VLA-1 and VCAM-1, between SCF and its receptor, c-kit, and between CXCR-4 and SDF-1. Disruption of these interactions involves the action of proteases such as neutrophil-derived MMP-9, which cleaves membrane-bound SCF (Heissig et al., 2002). MMP-9 also seems to play a role in disruption interactions between the chemokine GRO-α and its ligand CXCL-2, with synergistic effects on G-CSF mobilization (Pelus et al., 2004). The SDF-1/CXCR-4 axis is integral to HSC mobilization, and disruption of this axis by a CXCR-4 antagonist, plerixafor, is being investigated as an agent for mobilizing HSC for clinical use (Salman and Lazarus, 2011). Other proteases within the BM microenvironment appear to act to disrupt both SCF-1/CXCR-4 and the VLA-1/VCAM-1 interactions in vivo.

The release of HSCs not only occurs during mobilization but is also observed during homeostasis, when a small number of HSCs are constantly released into the circulation (Wright et al., 2001). Although their precise physiological role remains unclear, they might...
provide a rapidly accessible source of HSCs to repopulate areas of injured bone marrow (Lapidot et al., 2002). Alternatively, circulating HSCs might be a secondary consequence of permanent bone remodelling that causes constant destruction and formation of HSC niches, therefore requiring frequent re-localization of HSCs (Figure 13).

Figure 13: Model illustrating the quiescent endosteal niche and the active erivascular HSC niche after stimulation with G-CSF or depletion of onocytes/macrophages. (Source: Ehninger et al., The Journal of Experimental Medicine 2011). Upon stimulation with G-CSF, which binds G-CSFR on monocytic cells, the monocytes/macrophages disappear. As a consequence of their missing supportive activity, OB activity is decreased and nestin\textsuperscript{+} MSCs no longer express high levels of SCF, VCAM1, Ang-1, and CXCL12. Because CXCL12-mediated activation of the CXCR4 receptor on HSCs is a critical niche retention signal, HSCs get mobilized into the periphery via entry into the sinusoids. Alternatively, the CXCR4/CXCL12 axis can be inhibited by the clinically used mobilizing agent AMD3100. Additionally, G-CSF stimulates the SNS, which contributes to HSC mobilization.

Despite of intensive research carried out on hematopoietic stem cells we understand little about the stemness of HSC and its regulation for self renewal and differentiation. The structure and localization, as well as the molecular and cellular basis for niche activity, has long remained unanswered. It is only recently that the concept of a stem-cell niche has been supported by data, first in invertebrates and more recently in mammals.
HSC niche plays a key role in HSCs expansion. At present, *in vitro* expansion and maintenance of HSCs are limited which in turn limit the wide use of HSCs in therapeutics. Thus attempts are made in this study to explore the bone marrow microenvironment in detail. Global gene expression patterns in stem cells can unravel the molecular pathways behind self-renewal. If the stem cell population can be subjected to self-renew continuously *in vitro* by the addition of novel molecules in the culture media, it may help in improving the *ex-vivo* expansion of the culture. Considerable availability of large amount of stem cell population *in vitro* mimicking the *in vivo* environment could meet the therapeutic needs.
Materials and methods
5.1 Materials

5.1.1 Animals

Six to eight weeks old C57BL6/J mice (male) and C57BL6-SJL mice (female) were used in this study as donor and recipient, respectively. Mice were procured from the Jackson Laboratories (Barharbor, ME, USA) and maintained in the institute's experimental animal facility. During the experiment, mice were kept in an isolator, fed with autoclaved acidified water and irradiated food ad libitum. All animals were kept at 24±2°C under 14hrs light and 10hrs dark cycle and used as per the National Guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA). All experiments were conducted as per procedures approved by the Institutional Animal Ethics Committee at National Institute of Immunology (NII), New Delhi.

5.1.2. Cell Lines

M210B4 cell line (Murine Bone marrow fibroblast cell line) and HEK293T cell line (Human Embryonic Kidney 293 cells) were obtained from American Type Culture Collection (ATCC), Manassas, VA.

5.1.3. Vectors

Gag, Pol, Rev and Tat containing packaging plasmid psPAX2, VSV-G expressing envelop vector pMD2.G and pLVTHM deposited by Didier Trono’s Lab were obtained from the Addgene Plasmid repository, Cambridge, USA.

5.1.4. Antibodies and secondary conjugates

Biotinylated antibodies against Mac-1, purified anti-mouse Sca-1; PECy-5 conjugated anti mouse c-Kit, APC conjugated anti mouse CD45, CD45.2; PerCP efluor 710 conjugated anti
Mouse CD135 (Flk2) were procured from eBiosciences (San Diego, CA, USA). FITC conjugated anti mouse Scal, APC conjugated anti mouse c-Kit and streptavidin conjugated APC-Cy7 were procured from BD Biosciences (San Jose, CA, USA). Biotinylated anti mouse lineage cocktail and streptavidin microbeads for magnetic labelling were purchased from Miltenyi Biotech (Gladbach, Germany). Antibody against GFP was procured from Clontech Takara Bio Company (Country view, USA). Anti-goat, anti-rat, anti-mouse and anti-rabbit Ig antibodies labeled with fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE) were purchased from Jackson Immunologicals (Pennsylvania, USA). Anti-mouse Ig, anti-goat and anti-rabbit antibodies labeled with Alexafluor 488 or Alexafluor 594 or Alexafluor 555 and ProLong® anti-fade kit were obtained from Molecular Probes Inc. (Eugene, OR, USA). Apoptotic marker Annexin V conjugated with FITC was obtained from BD Biosciences. Antibody against Ki67; proliferation marker and anti osteopontin (OPN) antibody; osteoblastic niche marker was procured from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

5.1.5 Enzymes
DNA restriction enzymes (EcoRI, AgeI, Sall) and DNA ladder (100bp and 1 Kb) were purchased from New England Biolabs (Massachusetts, USA). Taq DNA polymerase was obtained from Bangalore Genetic India Pvt Ltd. (Bangalore, India).

5.1.6 Chemicals and reagents
Antibiotic mix for mammalian cell culture, Sequebrine, Hoechst 33342 (Ho), Pyronin Y (PY), Propidium Iodide (PI), Bovine Serum Albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI), Poly-L-lysine, Mayer’s Haematoxylin, Tri Sodium Citrate, Saponin, 5-bromo-2'-deoxyuridine (BrdU), and Histopaque were procured from Sigma Chemicals.
Materials and methods

Co. (USA). Luria Bertani (LB) medium was purchased from Becton Dickinson, Microbiology Systems (USA). Iscove's Modified Dulbecco's Media (IMDM), Roswell Park Memorial Institute (RPMI-1640), Dulbecco's Modified Eagle Medium with high glucose (DMEM), Lipoectamine, and Trizol were purchased from Invitrogen (CA, USA). Fetal calf serum (FCS) was procured from Biological Industries (Kibbutz, Israel). Chemicals used for histology were of analytical grade, and unless otherwise stated were obtained from Qualigens Fine Chemicals (Mumbai, India). Formamide was purchased from Amersham Biosciences (UK). Absolute Ethanol and Paraffin wax were purchased from Merck (India) Ltd. (Mumbai, India). RNAqueous Micro RNA isolation kit was purchased from Ambion® Life Technologies (California, USA). High capacity cDNA Reverse transcription kit and Power SYBR Green for real time PCR was procured from Applied Biosystems (Foster City, CA, USA).

5.1.7. Primers for Real Time PCR

The following primers were designed using Primer Designing Tool of National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), checked for secondary structure formation by Gene Runner Version 3.05 (Copyright, Hastings Softwares Inc.) and synthesized by Sigma-Aldrich. The primers for relative quantification Real Time PCR were synthesized such that the forward and the reverse primers spanned different exons.
### Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5'—Sequence—&gt;3'</th>
<th>Reverse Primer 5'—Sequence—&gt;3'</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphla</td>
<td>CACCCCTGCTAACTCCTTGG</td>
<td>CAGCATTATGACGACCAGTG</td>
<td>111</td>
</tr>
<tr>
<td>Areg</td>
<td>ACAGCGAGGATGACAAAGGAC</td>
<td>CTGTGATAACGATGCCGATG</td>
<td>115</td>
</tr>
<tr>
<td>FL3L</td>
<td>CTGTGCTGTCTGTGAGT</td>
<td>AAGCAGGTGGTCAGTCAA</td>
<td>113</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACGGCCGCACTCTTCTGTGCA</td>
<td>CAGGCACCCCAATACGGCCAA</td>
<td>100</td>
</tr>
<tr>
<td>IL3</td>
<td>ACTGATGATGAAAGGACC</td>
<td>CAGATGTAGGGCAGGCAA</td>
<td>151</td>
</tr>
<tr>
<td>IL6</td>
<td>GAAATGATGGATGTACC</td>
<td>GGCTTGTCTTTTCTTGT</td>
<td>133</td>
</tr>
<tr>
<td>Jagged2</td>
<td>CCTGGCGGGAAGCTGATAGCTG</td>
<td>CCAAGCAGGCTAAGCAGTGTG</td>
<td>118</td>
</tr>
<tr>
<td>Rac1</td>
<td>GTTCCTCTGCTCCCCCTCTGT</td>
<td>GCAAAGCGTACAAAGGTTCC</td>
<td>112</td>
</tr>
<tr>
<td>SCF</td>
<td>TGTTCTGCTACCTGTGACCT</td>
<td>CCTCCAGAGTCTGCTGCG</td>
<td>122</td>
</tr>
<tr>
<td>SCGF</td>
<td>CACAGGTCTCTCTCTCTC</td>
<td>AGTACCAGGCTAGCGCTTC</td>
<td>127</td>
</tr>
<tr>
<td>TnfB</td>
<td>GTGCGCTATGTCTCAGGCTCT</td>
<td>GTCTGGGCCCATAAGAICTGAT</td>
<td>144</td>
</tr>
<tr>
<td>Vangl2</td>
<td>ATCAAGACGAAGCTGCCATT</td>
<td>CTCTTGCTTTCCCTCCCTTG</td>
<td>120</td>
</tr>
<tr>
<td>VEGF</td>
<td>CCTGGCTCTGCTCTTACCC</td>
<td>AAGGCACTCACACACACACGC</td>
<td>117</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>CACCCAGGGTCAAGAACA</td>
<td>GCACCCAGAAGGATAGGCAG</td>
<td>150</td>
</tr>
<tr>
<td>Wnt9b</td>
<td>GAGAGGAAGCAGGACCCTGA</td>
<td>ACAGGAGCGTGACACACCAT</td>
<td>135</td>
</tr>
</tbody>
</table>
5.1.8. shRNA sequence

The following shRNA sequences were designed using the standard shRNA designing method. The target sense sequence was obtained from the Broad institute “The RNAi consortium” (TRC) depository.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence 5'-----Sequence-----3'</th>
<th>Reverse sequence 5'-----Sequence-----3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areg</td>
<td>CCGTGAGCAATCCCATGAGTTATCTGATTCCTTCTGACG</td>
<td>AAATCGGTAGCACAAAAACGAATGAAGTCGACG</td>
</tr>
<tr>
<td>SCGF clone 1</td>
<td>CCGTGAGCAATCCCATGAGTTATCTGATTCCTTCTGACG</td>
<td>AAATCGGTAGCACAAAAACGAATGAAGTCGACG</td>
</tr>
<tr>
<td>SCGF clone 2</td>
<td>CCGTGAGCAATCCCATGAGTTATCTGATTCCTTCTGACG</td>
<td>AAATCGGTAGCACAAAAACGAATGAAGTCGACG</td>
</tr>
</tbody>
</table>

5.1.9. shRNA construct used for knockdown study

i. Rac1 shRNA construct: Five different sets of Rac1 shRNA clones in the form of bacterial glycerol stocks were procured from Sigma-Aldrich, USA. shRNA was cloned in pLKO.1-puro vector under the control of U6 promoter (RNA polymerase III promoter).

ii. SCGF and Areg shRNA construct: shRNA sequence for the desired gene was designed and cloned in a pLKO.1-puro vector backbone under the control of U6 promoter (RNA polymerase III promoter).
5.2 Preparation of buffers and other reagents

5.2.1. Phosphate Buffered Saline (pH7.4)

Dulbecco’s PBS (dry powder without Calcium and Magnesium ions, TS1006) was purchased from HiMedia Laboratories, Mumbai, India. The media powder was dissolved in MQ water and sterilized by autoclaving and stored at 4°C until use.

5.2.2. Luria Bertani (LB) broth

25 g of LB powder was dissolved in 1 litre double distilled water. The medium was sterilized by autoclaving at 15lbs/square inch for 20 min.

5.2.3. Luria Bertani (LB) agar

40 g of LB powder was dissolved in 1 litre double distilled water. The medium was sterilized by autoclaving at 15lbs/square inch for 20 min. Ninety millimeter dishes were made by pouring LB agar. These LB plates were used for streaking bacterial cultures and picking the clones grown on them.

5.2.4. Cell culture media

Dry powder of IMDM, RPMI-1640 and DMEM high glucose was reconstituted in MQ water. Sodium bicarbonate was added at a concentration of 3 g, 2g and 3.7 g per litre of medium respectively, filter sterilized and stored at 4°C.

5.2.5. MACS buffer

1X PBS (pH 7.2) containing:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5%</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

Filter sterilized, degassed and stored at 4°C.
5.2.6. Reagents for DNA gel
PCR products were run in 1.6% to 2.5% agarose gels in TBE buffer.

10X TBE (pH 8):
- Tris Base: 108 g
- Boric Acid: 55 g
- EDTA: 9.3 g
- Double distilled water: 1000 ml

5.2.7. Reagents for Immunocytochemistry

5.2.7.1. PBS-Azide
1X PBS containing 0.1% sodium azide

5.2.7.2. Blocking Buffer
1X PBS containing:
- BSA: 1%
- Sodium azide: 0.1%

5.2.7.3. Washing buffer
1X PBS containing:
- BSA: 0.5%
- Sodium azide: 0.1%

5.2.8. Reagents for Immunohistochemistry

5.2.8.1. Paraformaldehyde (PFA)
100 ml 1X PBS containing 4 gm of PFA dissolved at 56°C water bath for 1 hr.
Materials and methods

5.2.8. Permeabilization Buffer

1X PBS containing:

- BSA 1%
- Saponin 0.1%
- Sodium azide 0.1%

5.2.9. Flow cytometry

1X PBS containing:

- BSA 1%
- Sodium azide 0.1%

5.2.10. Sodium citrate buffer (pH 6.0) for antigen retrieval

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri-sodium citrate (dihydrate)</td>
<td>2.94 g</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Dissolved Tri-sodium citrate and adjusted pH to 6.0 with 1N HCl. Added 0.5mL of Tween-20 and mixed well. Stored at 4°C until use.

5.2.11. Gey's buffer for RBC lysis

1000 mL Solution A:  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>35.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.85 g</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>1.5 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.119 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>Gelatin</td>
<td>25 g</td>
</tr>
<tr>
<td>1% Phenol Red</td>
<td>1.5 mL</td>
</tr>
</tbody>
</table>
Materials and methods

1000 mL Solution B: MgCl₂·6H₂O 4.2 g
MgSO₄·7H₂O 1.4 g
CaCl₂ 3.4 g

1000 mL Solution C: NaHCO₃ 22.5 g

To prepare Gey's buffer, each solution was autoclaved separately. To prepare 100mL buffer, 20mL solution A, 5mL solution B and 5mL solution C was added in 70mL of autoclaved double distilled water.

5.3. Methods

5.3.1. Isolation of bone marrow cells and Hematopoietic reconstitution

The femurs and tibiae of 6-8 weeks old C57B6/J mice were excised under sterile conditions. The connective tissue around the bones was carefully removed. The bone marrow cells (BMCs) were isolated by flushing the marrow cavity with IMDM supplemented with 3% FCS. The bones were further crushed using collagenase (0.03%) and dispase (2 U/ml) enzymes and incubated in a 37°C water bath for 15 mins with constant shaking. The flushed cells were mixed with the cells obtained from crushed bones. The cells were centrifuged at 1200 rpm for 5 min at 4°C and the pellet was treated with Gey's solution for 90 seconds (5 mL/mice) for erythrocyte lysis. The reaction was stopped by adding 45 mL 1X PBS. Cells were pelleted by centrifugation and resuspended in complete medium. The viable cells were counted by trypan dye exclusion method using a standard haemocytometer. Three different doses (2.5, 5, and 10 x 10⁶ per mice) of these cells were transplanted in Ly5.1 mice through lateral tail vein injection. Prior to transplantation, mice were irradiated at 700 cGy (1.96 cGy/s) in two splits of 3 h interval.
Mice were sacrificed at different time points for phenotypical analysis of LSK and lineage committed cells.

5.3.2. Competitive marrow repopulation assay
Primary (1°) transplantation was carried out as mentioned earlier. After 10 and 15 days of transplantation, mice were sacrificed and BM cells were isolated as usual. Donor cells (Ly5.2) from 1° mice were separated by positive selection method using a one-step magnetic activated cell sorting (MACS) method (Miltenyl Biotec, Bergisch, Gladbach, Germany). In brief, cells were incubated with biotinylated CD45.2 (Ly5.2) antibody (eBiosciences) for 15 min at 8°C. Excess antibody was washed out, cells resuspended in MACS buffer and further incubated with streptavidin microbeads (Milteny) for 15 min at 8°C. Finally, washed magnetically labeled cells were positively selected in a MS column and then eluted by flushing with MACS buffer. The purity of the cells was determined by staining with anti-CD45.1/FITC. Four doses (10, 30, 100, 300 x 10^3) of sorted cells were transplanted in an irradiated (700 C Gy) 2° host (Ly5.1). After 30 days of transplantation donor-derived LSK cells were analyzed by FACS.

5.3.3. Cell cycle analysis of LSK cells
5.3.3.1. Ho and PY staining method
Cell cycle analysis was performed by staining with Hoechst 33342 and Pyronin Y (Sigma, St Louis) dye (Shapiro et al., 1981). Staining of cells was done by incubating 1 x 10^6 cells/500 μl of 1% FCS containing IMDM with 5 μl Ho33342 dye (10 mg/ml) at 37°C for 1 hr. Cells were washed, resuspended in the same medium and further staining with PY (1.6 μg/ml) by incubating at 37°C for 1 hr. Ho-PY stained cells were washed twice and staining with antibodies Sca-1/FITC, biotinylated lineage antibody cocktail,
c-Kit/PECy5, CD45.2/APC or CD45.1/APC for 30 min in ice. Cells were washed three times with PBS containing 0.5% BSA and further incubated with streptavidin-APCCy7 for an additional period of 30 min. Washed cells were analyzed with a customized FACS Aria III (BD Biosciences, San Jose, CA) using specific band-pass filters.

5.3.3.2. BrdU Ip Injection and staining

BrdU staining was done for macroscopic and microscopic analysis of stem cell compartment. For macroscopic analysis, after 24 hrs of transplantation (as described above) mice were given pulse of BrdU for 10 days. Pulse was given by single injection (100 mg/Kg body weight) through intra-peritoneal route and feeding (1 mg/ml) through drinking water which was changed every third day. Following pulse of BrdU, it was chased for 30 days by withdrawing the DNA analogue from drinking water. Mice were sacrificed at 10 and 30 days of transplantation, BM cells were harvested and the incorporation of BrdU in donor and recipient-derived LSK cells was determined by flowcytometric analysis. BrdU staining was done following the method published in the literature. The Donor and recipient LSK cells were sorted and stained for anti Brdu antibody (ebioscience, San Diego, CA). The sorted cell population was incubated with primary antibody against BrdU (1:200) directly conjugated with FITC for 1 hr. The desired fraction of sorted donor and recipient LSK cells were fixed in 4% para-formaldehyde for 30 min, followed by denaturation with 2N HCl + 0.5% Triton X-100 for 15 -20 min. This was followed by neutralization using 0.1 M borate buffer. Washed cells were stained with anti-BrdU IgG/FITC antibody (BD Biosciences) and analyzed with a customized FACS Aria III (BD Biosciences) using specific band-pass filters.
Materials and methods

5.4. Immunocytochemistry

Sorted BMCs (Recipient LSK) were coated over a limited area on a poly-L-Lysine coated glass slide using a Cytospin 2 apparatus (Shandon Southern Products, Cheshire, UK). They were then fixed with 4% Paraformaldehyde at 4°C for 15 min followed by permeabilization with 0.1% saponin in case of intracellular antigen. The cells were stained for the markers with the primary antibodies for 1 hr at 4°C followed by staining with the Alexafluor 488 conjugated secondary antibodies for 1 hr at 4°C. Nuclear staining was carried out with DAPI at 10 μg/ml concentration for 5 minutes in the dark. The cells were then mounted with ProLong® anti-fade Molecular Probes Inc. (Eugene, OR, USA) and analyzed by Zeiss LSM 510 META confocal laser-scanning microscope using a Plan-Apochromat 63 ×/1.4 oil objective. LSM 510 software was used for acquisition of images. The images were processed by Zeiss LSM Image browser, version 4.2.0.121.

5.5. Immunohistochemistry

Engraftment of donor stem cells on the endosteal niche was identified by IHC. GFP-expressing crude BM cells were transplanted to irradiated C57BL/6J mice. After 10 days of transplantation, mice were sacrificed and tibia and femurs were isolated. Bones were cleaned, fixed in 4% PFA and decalcified in 5% formic acid. Five micron bone tissue cryosections (Thermo-Shandon E, USA) were stained with mouse anti-GFP, mouse osteopontin and anti-Sca-1 antibodies. Alexa Fluor 488/594/555 secondary antibodies were used to identify the specific proteins. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Sections were imaged with a Zeiss LSM 510 META confocal laser-scanning microscope using a Plan-Apochromat 63 ×/1.4 oil objective. LSM 510 software
Materials and methods

was used for acquisition of images. The images were processed by Zeiss LSM Image browser, version 4.2.0.121.

5.6. Bone marrow chimera formation

Six to eight week old CD45.1 C57BL/6J female mice were irradiated at 700 cGy and transplanted with $10 \times 10^6$ unfractionated bone marrow cells from CD45.2 C57BL/6J male mice (Streetz et al., 2008). The mice were housed at pathogen-free conditions and fed sterile water and food. Peripheral blood mononuclear cells were collected from bone marrow transplanted C57BL/6J female mice and stained with CD45.1-PECy-5 and CD45.2-APC and analyzed using FACS to ascertain the percentage of CD45.2 cells to determine the extent of chimerism even after re-irradiation at the end of seventh month.

5.6.1. Isolation of mononuclear cells from peripheral blood (BMCs)

The isolated BMCs were overlaid on Histopaque (Sigma, USA) at room temperature such that the ratio of Cells and Histopaque was 1:1. It was then centrifuged at 400 g for 30 min without brakes and acceleration at 25°C. The buffy coat containing the mononuclear cells was carefully pipetted out. The cells were then washed with 1X PBS by centrifuging at 300 g for 10 min at 25°C. The mononuclear cells thus obtained were further used for flow cytometry based analysis.

5.7. Cell Sorting

5.7.1. Donor and recipient specific LSK sub-populations from BMCs for BrdU analysis by FACS

The BMCs were stained with donor specific marker CD45.2 antibody conjugated with APC and recipient specific marker CD45.1 antibody conjugated with PE Cy5 separately. Both
the sub populations were further stained with Sca1 conjugated with FITC, cKit conjugated with PECy5/ APC and biotin conjugated lineage for 30 min at 4°C. Streptavidin conjugated APC Cy7 was used for 30 min at 4°C.

Both the donor and recipient LSK sub-populations were sorted in FACS AriaIII (BD, USA) using a 70 μm nozzle at a sheath pressure of 45psi and a flow rate of 3000 events per second in the "4-way purity" mode. The two sorted fractions were further stained with BrdU to determine the proliferating status of the cells.

5.7.2. CD45 negative cell fraction for microarray analysis by MACS

CD45 Negative (CD45-) cells were sorted from the BMCs of the recipient mice (Irradiated and transplanted as mentioned before) at various days post transplantation. A single-step magnetic-activated cell sorting technique using MACS LS Column (Milteny Biotech) was done. CD45- cells having a cell density of 1-2 x 10^6 cells obtained from all the time points were stored in RNA later (Ambion 7040). RNA isolation and microarray analysis was outsourced to Genotypics (Bangalore, India). Single color hybridization was performed using Cy3 on an Agilent platform. Genespring software was used for the data analysis. Experiments using mouse primary cells were conducted according to procedures approved by the Institutional Animal Ethics Committee.

5.8. Flow cytometry

BMCs were stained by incubating with directly fluorochrome conjugated primary antibodies at 4°C for 30 mins followed by washing with BSA containing 1XPBS. For biotinylated and purified primary antibodies an additional incubation at 4°C for 30 min was done with fluorochrome labelled streptavidin and fluorochrome labelled secondary
Materials and methods

antibody respectively. Cells were analyzed by flow-cytometry (FACS Aria III, BD Biosciences, San Jose, CA, USA). The antibodies used are described in Materials section.

5.9. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). The cells were pelleted at 600g for 5 min at 4°C, the supernatant removed and the pellet resuspended vigorously in TRIZOL® Reagent. Chloroform (0.2 ml of per ml of TRIZOL® Reagent) was added and shaken vigorously for 15 seconds. The samples were incubated for 2 to 3 min at room temperature and then centrifuged at 12,000g for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by addition of 0.5 ml of isopropyl alcohol per ml of TRIZOL® Reagent, incubation at room temperature for 10 min and centrifugation at 12000g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed once by addition of 1 ml 75% ethanol per ml of TRIZOL® Reagent, followed by vigorous mixing by vortexing and centrifugation at 7500g for 5 mins at 4°C. The RNA pellet was briefly dried and dissolved in RNase free water and stored at -80°C. Total RNA was isolated from sorted cells using the RNAqueous Micro RNA isolation kit (Ambion® Life Technologies, USA).

The RNA isolated by both the methods was treated with DNase (Ambion® Life Technologies, USA) to remove contaminating genomic DNA. The RNA sample was treated with 1U of DNase in the 1X DNase buffer for 20 mins at 37°C. The DNase inactivating reagent provided with the kit was used to inactivate the DNase. The RNA
treated with the DNase was quantified using a NanoDrop microvolume Spectrophotometer (Thermo Scientific, DE, USA). The RNA preparations having OD$_{260}$/OD$_{280}$ value $\geq 1.8$ were considered to be of good integrity and used for cDNA synthesis.

The cDNA was synthesized with the High capacity cDNA Reverse transcription kit from Applied Biosystems. The 20 $\mu$L reaction mixture was made with 2 $\mu$L of 10X RT Buffer, 0.8 $\mu$L of 25X dNTP mix (100 mM), 2 $\mu$L of 10X RT Random Primers, 1 $\mu$L of Multiscribe™ reverse transcriptase, RNA and the remaining volume was made up with nuclease free water. The reaction conditions were 25°C for 10 min, followed by 37°C for 120 min and 85°C for 5 mins to inactivate the enzyme. The cDNA thus synthesized was used for further experiments.

5.10. Relative quantification by Real Time PCR

Real-time qPCRs were performed by means of SYBR Green technology and Eppendorf Realplex instrument (Hamburg, Germany). For each sample, the Realplex software plotted an amplification curve by relating the fluorescence signal intensity (ΔRn) to the cycle number. The Ct was defined as the cycle number at which a significant increase in the fluorescence signal was first detected (the higher the starting copy number, the lower the Ct).

For relative quantification the real time PCR was carried out for the genes of interest (GOI) in various template cDNA samples with the calibrator depending on the experiment. Each PCR reaction was carried out with a control House Keeping Gene (HKG) for each template. The PCR reaction parameters were as follows: (I) reaction mix- template DNA mixed with 12.5 $\mu$L 2X Master Mix (Applied Biosystems), 100 nM each primer in a final
volume of 25-μl; (2) PCR cycles: 10 min at 95°C, 40 amplification cycles (95°C for 15 seconds, 60°C for 60 seconds) followed by a dissociation cycle to obtain the melting curve. The relative fold change in expression of the gene of interest (GOI) in the sample with respect to the calibrator was calculated as follows,

\[
\text{Fold Change} = 2^{[\text{CT GOI SAMPLE} - \text{CT HKG SAMPLE}] - [\text{CT GOI CALIBRATOR} - \text{CT HKG CALIBRATOR}]} 
\]

\[
= 2^{-\Delta\Delta CT} 
\]

All the reactions were carried out in triplicates and those reactions with a \(\pm 0.5\) standard deviation values for the Cts were not considered.

5.11. Cell-Cell Interaction study

This study was conducted by both in vitro and in vivo experiments. In vitro experiments were conducted in two ways: (i) recipient and donor cells were co-cultured (contact) in 6-well plates, and (ii) cells were cultured by separating them (non-contact) using 3-μm cell culture insert (Millicell, Millipore Corporation, Billerica, MA) in 24-well plates. In co-culture experiments, 2 x 10⁶ irradiated (700 cGy) BM cells of CD45.1 mouse were cultured with same number of unirradiated CD45.2⁺ cells in 2 ml of medium (IMDM supplemented with 5% ESCertified FCS). In control experiments, CD45.2⁺ cells were not used. In non-contact experiments, 0.5 x 10⁶ /300 μL irradiated CD45.1-mouse BM cells were taken in the lower chamber, whereas 0.2 x 10⁶ /300 μL of non irradiated CD45.2⁺ cells were taken on the insert and cultured. The control experiments were devoid of CD45.2⁺ cells. After day 1 and 2, cells in the lower chamber were analyzed for apoptosis by staining with CD45.1 and Annexin V (Apoptosis Detection Kit, BD Pharmingen) antibodies. Another fraction of cells were fixed with 4% para-formaldehyde for 30 min, and then permeabilized using 0.1% saponin. The washed cells were stained with CD45.1 and Ki67 specific
antibodies to determine the proliferation of host cells. Finally, the labelled cells were analyzed with a customized FACS Aria III using specific band-pass filters.

In vivo studies were conducted by transplanting $3 \times 10^4$ sorted CD45.2LSK cells in each mouse. Mice without and with transplantation were sacrificed on day 3, 5 and 10 for analysis of necrotic (PI staining) and apoptotic (Annexin V staining) cells of CD45.1LSK compartment. Furthermore, these cells were stained with anti-cyclin A antibody (Santa Cruz) for immune-cytochemical analysis. Cells were also subjected to real-time RT-PCR analysis for p21 expression.

5.12. Maintenance of cell lines

M210B4 cells were maintained in RPMI supplemented with 10% heat inactivated fetal calf serum at 37°C in a humidified 5% CO$_2$ incubator. HEK293T cells were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum at 37°C in a humidified 5% CO$_2$ incubator.

5.13. Cloning of ShRNA in a pLKO.1-puromycin vector

5.13.1. Ultra competent E.Coli cell preparation by Inoue Method (Inoue H. et al., 1990)

E.coli DH5α cells were plated on Luria Bertini (LB) agar plate with the help of sterile glass beads and grown overnight at 37°C. A single colony was picked and inoculated in 6ml of LB broth and cultured overnight at 37°C with shaking at 220 rpm. One ml of primary culture was used as an inoculum for a 100ml of pre-warmed LB broth (secondary culture). This culture was grown at 25°C with shaking at 220 rpm till the cell density reached the O.D$_{600}$ at 0.2-0.3. The bacterial culture was then aseptically transferred into sterile, ice-cold, 50 ml falcon tube and the culture was cooled to 0°C for 10 min. The cells were then harvested at 4000 rpm (Sorvall Super T21, Kendro Laboratory Products, Germany) for
10 min at 4°C under sterile conditions. The supernatant LB medium was completely aseptically removed. The pellet was resuspended gently (by swirling) in 80 ml of ice-cold Inoue transformation buffer. The cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. Supernatant was again completely aseptically removed, ensuring not a single drop of medium was present. Cells were again resuspended gently in 20 ml of Inoue transformation buffer (0°C). 1.5 ml of DMSO was added, swirled to mix the bacterial suspension and incubated on ice for 10 min. Aliquots of 100 μl each were prepared in pre-chilled sterile cryo vials. The aliquots were immediately snap-frozen by immersing them in liquid nitrogen before storing at -70°C until used.

5.13.2. Transformation: transforming competent cells with plasmid DNA
Competent cells were thawed on ice and mixed with 20-40 ng plasmid DNA of interest per 50 μl of competent cells aseptically and incubated on ice for 30 min. The cells were given heat shock at 42°C for 90 s and immediately chilled on an ice bath for 5 min. For recovery, 800 μl of LB broth was added to the cells followed by shaking 37°C with at 220 rpm for 45-60 mins. The cells were then plated on LB agar plates supplemented with the desired antibiotic with the help of sterile glass beads.

5.13.3. Maintenance of bacterial cultures and plasmid isolation
Single colonies of transformed bacteria were inoculated in LB broth with appropriate antibiotic and cultured at 37°C with shaking at 220 rpm for 12-14 h. Bacterial stocks were made in 15% glycerol (from autoclaved 80% glycerol stock), snap-frozen in liquid nitrogen and stored at -70°C. Plasmids were isolated from 6 ml or 500 ml LB broth cultures (inoculated from above glycerol stocks) using QIAGEN Mini or Maxi kits, following the instructions provided by the manufacturer.
5.13.4. Agarose gel electrophoresis and gel extraction

Agarose gel electrophoresis was performed using submarine gel electrophoresis system (Sharp et al., 1973). Plasmids and DNA fragments were resolved based on size on 1% Tris borate EDTA (TBE) gels at 100-120V. For extraction of desired fragment from the gel for transfection, plasmid DNA was digested with suitable restriction enzymes and resolved on a 1% TBE agarose gel. The desired fragment was excised with minimum agarose using a clean scalpel and eluted in 30 - 50µl of sterile filtered MQ water using QIAquick gel extraction kit (QIAGEN, Germany) following the manufacturer’s instructions. Yield of purified fragment was quantified by measuring the absorbance at 260 nm spectrophotometrically.

5.13.5. pLKO.1-TRC Cloning Vector

5.13.5.1. A.2 Map of pLKO.1

pLKO.1 is a replication-incompetent lentiviral vector obtained from The RNAi consortium (TRC). pLKO.1 once introduced into cells (via direct transfection, or by lentiviral particles); the puromycin resistance marker encodes in pLKO.1 allows for stable selection.

Map of pLKO.1 containing an shRNA insert. The original pLKO.1-TRC cloning vector has a 1.9kb stuffer that is released by digestion with AgeI and EcoRI. shRNA oligos are cloned into the AgeI and EcoRI sites in place of the stuffer.
5.13.5.2. Designing shRNA Oligos for pLKO.1

Small hairpin RNAs (shRNAs) generated using an oligonucleotide DNA sequence. The desired target shRNA sense sequence was derived from the TRC depository. A hairpin loop sequence is located between the sense and antisense sequences on each complementary strand.

5.13.5.3. Cloning Oligos into pLKO.1

The pLKO.1-TRC cloning vector containing a 1.9kb stuffer was released upon digestion with EcoRI and AgeI. The shRNA oligo was flanked by sequences that are compatible with the sticky ends of EcoRI and AgeI. Forward and reverse oligos were annealed and ligated into the pLKO.1 vector, producing a final plasmid that expresses the shRNA of interest.

5.13.5.4. Annealing Oligos

Oligos were resuspended in dd H₂O to a concentration of 20 μM as below:

5 μL Forward oligo

5 μL Reverse oligo
Materials and methods

5 μL 10x NEB buffer 2
35 μL ddH₂O

The reaction mix was incubated for 4 min at 95°C in a PCR machine followed by gradual cooling to room temperature. This will take few hours, but it is important for the cooling to occur slowly for the oligos to anneal.

5.13.5.5. Digesting pLKO.1 TRC Cloning Vector

The pLKO.1 TRC-cloning vector was digested with EcoRI and AgeI as below and incubated at 37°C for 2 hrs.

6 μg pLKO.1 TRC-cloning vector (maxiprep or miniprep DNA)
5 μL 10x NEB buffer 1
1 μL AgeI
1 μL EcoRI

The digested product was purified with Qiaquick gel extraction kit and eluted in 30 μL of ddH₂O and the DNA concentration was measured spectrophotometrically.

5.13.5.6. Ligation and Transformation into Bacteria

A standard T4 ligation method was followed

2 μL annealed oligo
20 ng digested pLKO.1 TRC-cloning vector
2 μL 10x NEB T4 DNA ligase buffer
1 μL NEB T4 DNA ligase
to 20 μL ddH₂O

The whole mix was incubated at 16°C for 16 hours followed by transformation. Two micro-litre of the ligation mix was transformed into 25 μL competent DH5 alpha cells,
following manufacturer’s protocol and plated on LB agar plates containing 100 μg/mL ampicillin.

Schematic procedure for Transfection

5.13.5.7. Screening for Inserts

Plasmids were screened by restriction enzyme digestion and PCR amplification by using gene specific primer pairs.

5.14. Lentiviral Transduction and selection using puromycin

The packaging plasmids psPAX2, pMD2.G and the transfer vector pLVTHM obtained as stab culture from Addgene Plasmids repository were streaked on LB Agar plates containing 100μg/mL Ampicillin (Sigma Aldrich, USA). Single colonies were picked and further cultured in Ampicillin containing LB Broth. Plasmids were isolated using Qiagen Maxi Prep Kit and were used for transfection after linearizing and checking their size.

Co-transfection of the packaging plasmids and transfer vector was performed with Lipofectamine-2000 (Invitrogen, USA). HEK cells were plated at the density of 0.2 million cells per well and cultured overnight in antibiotic free medium. A mixture of 750 ng of
Materials and methods

psPAX2, 250 ng of pMD2.G and 1 μg of pLVTHM plasmids was added to 500 μL of Opti-MEM (Gibco, Invitrogen, USA). Six microlitre of Lipofectamine-2000 for each microgram of DNA was mixed with 500 μl of Opti-MEM. Both the solutions prepared were mixed and incubated at room temperature for 20 min. They were then added to the cells in a well and cultured overnight. The media was replaced with fresh serum containing medium after 6 hrs. The virus containing supernatant was collected and filtered using a 0.45 micron filter at 24 hrs and 48 hrs and preserved at -70°C and used for viral transduction after thawing.

For transduction, M210B4 cells were plated at a density of 0.2 million cells per well of a 6-well plate and cultured till they reached a confluency of 70-80%. The medium was replaced with undiluted viral supernatant with 10μg/mL Sequebrene (Sigma Aldrich, USA) and the cells were kept overnight at 37°C in a humidified 5% CO₂ incubator. The viral supernatant was replaced with DMEM supplemented with 10% heat inactivated fetal calf serum after 24 hrs of transfection. Selection was done using 3 μg/ml puromycin for three days followed by maintenance medium using 1 μg/ml puromycin concentration.

5.15. Paraffin block preparation and tissue sectioning

Bone tissue was fixed in 10% buffered formalin for 24 hrs was decalcified using 5% formic acid for 7-10 days, until soft. Tissue was washed with running tap water for 15 min. Tissue was dehydrated in by placing the fixed tissue for 30 min each in increasing grades of 50%, 70%, 90% and 100% isopropanol. Tissue was then immersed in a mixture of xylene and isopropanol (1:1) for 30 min. This was followed with immersion in Xylene for 3 hrs with 3 changes. The tissue was then placed in Paraffin wax at 60°C for 3 hrs. The tissue was embedded in paraffin wax in a suitable mould and 5 μm sections were cut with microtome (MRS3500, Histo-Line Laboratories, Milan, Italy).
Materials and methods

The sections were deparaffinised by immersing the slides in the series of reagents in the reverse order of paraffinization. The slides were kept for 5 min each in Xylene, 100%, 90%, 70%, 50% isopropanol. Slides were finally rinsed in double distilled water. The deparaffinised slides were further stained with Haematoxylin & Eosin for tissue pathology and used for immunohistochemistry after antigen retrieval.

5.16. Haematoxylin and Eosin staining for tissue pathology

After rinsing with double distilled water the deparaffinised tissue sections were kept in Mayer's Haematoxylin (Sigma, USA) for 10 min to stain the nucleus. This was followed by washing the slides in running tap water for 15 min to remove the excessive staining. The slides were then dipped in 1% Eosin solution (Qualigens Fine Chemicals, India) for 5-10 seconds followed by immersing in 90% and 100% isopropanol for 5 min each. The slides were then kept in Xylene: Isopropanol (1:1) for 5 min and Xylene for 5 min. Finally the sections were mounted in DPX (Qualigens Fine Chemicals, India) and sealed for making permanent slides.

5.17. Statistical Analysis

Results of multiple experiments were reported as the mean ± Standard Error Mean (SEM). One-way ANOVA was used to calculate the significance between the two experimental groups.
Results and discussion
6. Results

6.1. Donor derived LSK cells are predominantly amplified during marrow regeneration

In order to examine the proliferation kinetics of donor-derived LSK cells and possible competition with that of host, crude BM cells (CD45.2) were transplanted in irradiated hosts (CD45.1). We observed increase of LSK cells in BM of the host with the increase of cell dose (Figure 1). The early detection of donor LSK cells (within 24 h of transplantation) was made possible at a dose of $10 \times 10^6$ cells. Henceforth, all experiments using crude cells were conducted using the same dose. Both CD45.1 and CD45.2 antibodies did not have any cross reactivity (Figure 2A, 2B). BL6/J-SJL (Recipient: CD45.1) mice were sacrificed post irradiation and transplantation. BM cells were isolated and both CD45.1 and CD45.2 antibodies were used to stain the BMCs obtained from the transplantated mouse model (Figure 2C). Dot Plot shows no cross reactivity of the CD45.1 and CD45.2 antibody. Few double positive CD45.1 and CD45.2 cells were obtained but out of which 81.9% were positive for Mac1. To study host cells-derived hematopoiesis in non-competitive environment (without transplantation), a group of CD45.1 mice were irradiated and maintained for 2 months. Mice were sacrificed at different time intervals, and BM cellularity and total LSK cells were determined. About 120-fold increase of LSK cells between 3rd and 30th day of irradiation was observed (Figure 3A). In contrast, no expansion of host LSK cells was noticed (with transplantation) after 5th day of transplantation. The expansion of these cells was prominent only between 1st and 5th day of transplantation, with 15-fold increase in number. About 1000-fold amplification was observed in the donor-derived LSK cells which steadily increased up to 15th day of transplantation (Figure 3B). Interestingly, in case when purified donor LSK cells were transplanted, the donor cells proliferated till 15th day of transplantation (Figure 3C).
Results and discussion

Figure 1: Recovery of LSK cells 24 hrs after transplantation of different doses of BM cell

Three different doses (2.5, 5, and 10 x 10^6 per mice) of donor BM cells were transplanted in sub-lethally (700 cGy) irradiated recipient (CD45.1) mice through lateral tail vein injection. Mice were sacrificed after 24 hrs and BM cells were isolated from each mouse. Donor and recipient LSK cells were determined by flow-cytometry. Bar diagram represents the results of 5 animals in each group (n = 5).
Results and discussion

Figure 2A: Cross reactivity of CD45.1 Antibody

C57BL/6J-SJL (Recipient: CD45.1) mice were sacrificed and BM cells were isolated from them. Both CD45.1 and CD45.2 antibodies were used to stain the BMCs. Dot Plot shows no cross reactivity of the CD45.1 antibody.
Results and discussion

C57BL/6J (Donor: CD45.2) mice were sacrificed and BM cells were isolated from them. Both CD45.1 and CD45.2 antibodies were used to stain the BMCs. Dot Plot shows no cross reactivity of the CD45.2 antibody.

Figure 2B: Cross reactivity of CD45.2 Antibody
Mice irradiated and Transplanted with 30,000 Donor hematopoietic stem cells

Figure 2C: Cross reactivity of CD45.2 and CD45.1 antibody in the transplantated mouse model

C57Bl6/J-SJL (Recipient: CD45.1) mice were sacrificed post irradiation and transplantation. BM cells were isolated and both CD45.1 and CD45.2 antibodies were used to stain the BMCs obtained from the transplanted mouse model. Dot Plot shows no cross reactivity of the CD45.1 and CD45.2 antibody. Few double positive CD45.1 and CD45.2 cells were obtained but out of which 81.9% were positive for Mac1.
Results and discussion

A. W/o cells  
B. Crude cells  
C. LSK cells

Figure 3: Proliferation of LSK cells during marrow regeneration

A. Sub-lethally irradiated mice were maintained (without transplantation) for different time points. Mice were sacrificed and LSK cells were determined by flow-cytometry. Bar diagram shows proliferation kinetics of host BM-LSK cells (n = 6, each time point)

B. Each sub-lethally irradiated mouse received 10 x 10^6 crude BM cells of CD45.2 mouse. Mice were sacrificed and LSK cells were determined by flow-cytometry. Bar diagram shows proliferation kinetics of host and donor LSK cells (n = 6, each time point)

C. Each sub-lethally irradiated mouse received 3 x 10^4 CD45.2LSK cells. Mice were sacrificed and LSK cells were determined by flow-cytometry. Bar diagram shows proliferation kinetics of host and donor LSK cells (n = 3, each time point)
The distribution of donor and recipient HSCs and stromal cells are shown in transplantated (Figure 4A) and un-transplantated (Figure 4B) mouse model.

To ensure that LSK cells are indeed engraftable HSCs, a competitive marrow repopulation assay (MRA) was conducted for CD45.2\(^+\) cells, isolated from \(^1\)° recipient. Initially, CD45.2\(^+\) cells were harvested on 10th and 15th day post-transplantation from \(^1\)° recipients by positive selection method (Figure 5A). Four different doses of the sorted cells (10,000 to 300,000 cells per mouse) were transplanted in \(^2\)° hosts and CD45.2LSK cells were analyzed after a month of transplantation. It was found that marrow repopulation, in terms of donor LSK cells, was increased with cell dose (Figure 5B). Further, similar trend of results was observed in samples of both 10th and 15th day, and LSK cells were found to be several folds higher in the later time point in all 4 doses of cells. Combining these results, it has been concluded that donor-derived HSCs were more in number in 15th day than 10th day of engraftment.

6.2. Cell cycle activation of donor LSK cells is dynamically controlled

In the osteoblastic niche, HSCs are maintained in quiescent (\(G_0\)) state. Soon after irradiation, marrow HSCs enters into cell cycle to reconstitute bone marrow. To determine the kinetics of cell cycle activation of recipient and donor HSCs and competition between these cells, if any, respective LSK cells were stained with Ho and PY. Figure 6A shows the cell cycle status of crude BM cells of a donor mouse. It was observed that in the \(G_0\) state the cellular activity is minimum, which get increased in the metabolically active (\(G_1\)) state of the cells. Thus, the entire diploid LSK cells stained, were divided into two parts, one that stained low or no with PY (truly representing \(G_0\) cells) and the other that stained high PY
Results and discussion

Figure 4: Distribution of donor, recipient and stromal cells

Sub-lethally irradiated mice were left un-transplanted (A) or transplanted (B) with crude BM cells (10 x 10^6/mice).

A. Bar diagram shows distribution of hematopoietic and stromal compartment of cells. Hematopoietic (CD45.1^) cells and stromal (CD45.2^) cells were analyzed by flow-cytometry. The mean values were plotted (n = 6);

B. Bar diagram shows distribution of hematopoietic and stromal compartment of cells. Hematopoietic (CD45.1^, CD45.2^) and stromal (CD45.1^-CD45.2^-) cells were analyzed by flow-cytometry. The mean values were plotted (n = 6).
Results and discussion

A. Representative Experimental scheme for Competitive marrow repopulation assay

B. Donor (CD45.2⁺) cells of 10 and 15 days of transplantation were isolated from primary recipient. Four different doses of above sorted cells (10 x 10^3, 30 x 10^3, 100 x 10^3 and 300 x 10^3) were transplanted in 4 groups of mice, each consisting 4 mice. After 1 month of transplantation, BM cells were isolated from secondary recipient and analyzed for donor derived LSK cells. Proportional increase in the recovery of donor LSK cells with transplantation dose were observed.

Figure 5: Competitive marrow repopulation assay
Results and discussion

( representing G1 cells), as shown in the dot plot. It was observed that in irradiated mice without transplantation, G0 cells were significantly ($p < 0.05$) dropped to 25.85 ± 2.68% ($n = 6$) within 15 days, before it tend to regain the normal value (Figure 6 B). These results confirmed the entry of host LSK cells into the cycle for proliferation and reconstitution of hematopoietic system.

In irradiated mice with transplantation, the situation was different. The G0 fraction of donor LSK cells was observed to be declined and remained low up to 15th day, which later steadily increased till the end of the study (60 day) as shown in Figure 6C. The gating strategy in case of donor and recipient LSK cells for Ho and Py is shown in Figure 6D.

One most interesting observation made from this study was that, like host, the activity of donor LSK cells might have controlled by the stem cell niche. Soon after the partial recovery of BM, donor LSK cells gradually entered into quiescent state, perhaps by lodging on the osteoblastic niche. To confirm the location of the engrafted cells, GFP positive BM cells were transplanted in sub-lethally irradiated recipient mouse. Figure 7A shows the histological analysis of the transplanted cells in the bone sections. Immunohistochemical analysis of the trabicular bone showed a significant number of GFP positive BM cells which were present near osteoblastic niche area after 30 days of transplantation (Figure 7B). Further, to confirm the stemness of the donor GFP positive BM cells they were stained with stem cell specific antibody (Sea1). To confirm their presence in endosteal niche they were stained with (OPN antibody). It was observed that such transplanted GFP BM cells were indeed stem cells and were found to be properly lodged in the endosteal niche (Figure 7C).
Results and discussion

A. $A = B$.

R: recipient; D: donor

Irradiated w/o transplantation

Irradiated with transplantation

Figure 6: Cell cycle status of LSK cells during marrow regeneration

A. Bar diagram shows cell cycle status of LSK cells in healthy mice ($n = 4$);

B. Bar diagram shows cell cycle status of host LSK cells after irradiation ($n = 6$, each time point);

C. Bar diagram shows cell cycle status of recipient and donor LSK cells during marrow regeneration ($n = 6$, each time point).
Results and discussion

Donor

Recipient

Figure 6: Cell cycle status of LSK cells during marrow regeneration

D. Cells were stained for LSK, followed by Ho + PY. A representative gating strategy of the donor and recipient LSK cells is shown. Donor-derived LSK cells were selected as follows: donor cells (CD45.2+) were first selected, followed by gating for Sca-1+c-Kit+ and Lin- Sca-1+ c-Kit+ (LSK) cells. Finally, CD45.2+LSK cells were analyzed for Ho and PY. For recipient cells (CD45.2-) were first selected, followed by gating for Sca-1+c-Kit+ and Lin- Sca-1+ c-Kit+ (LSK) cells. Finally, CD45.2- LSK cells were analyzed for Ho and PY.
Results and discussion

A. Histology of Bone Tissue: Hematoxylin and Eosin staining

**Osteoblastic cells**

**Endothelial cells**

B. GFP staining

Figure 7: Donor-derived hematopoietic progenitor cells are localized in the trabecular bone

A. Sub-lethally irradiated mice were transplanted with GFP-expressing crude BM cells. After a month, mice were sacrificed; decalcified longitudinal trabecular bone cryo-sections (5 mm) were obtained. Sections were stained for Hematoxylin and Eosin. Yellow arrow shows the osteoblastic and the vascular niche cells respectively. Magnification 200 x.

B. Sub-lethally irradiated mice were transplanted with GFP-expressing crude BM cells. After a month, mice were sacrificed; decalcified longitudinal trabecular bone cryo-sections (5 mm) were obtained. Sections were stained for localization of the Donor cells using GFP antibody and DAPI for nuclear stain. Yellow arrow shows the GFP⁺ donor cells and the white arrow shows the non GFP recipient cells. Magnification 200 x.
Sub-lethally irradiated mice were transplanted with GFP-expressing crude BM cells. After a month, mice were sacrificed; longitudinal trabecular bone cryo-sections (5 mm) were obtained. Sections were stained for osteopontin (AF594, red), GFP (AF488, green), Sca-1 (AF555, pink) and nuclei (DAPI, blue). Both immuno-fluorescence (composite) and bright filed confocal images are shown. Arrows indicating donor derived progenitor cells.

Magnification 630 x
6.3. Transplantation rescues host cells from apoptosis and induces their proliferation

To decipher why the number of host LSK cells were high after transplantation as compared to the irradiated un-transplanted animal, *in vitro* studies were conducted. Irradiated host cells were cultured with direct and indirect contact of CD45+ donor cells. It was seen that the proliferation of host cells was significantly \((p < 0.001)\) increased on day 2 of culture (Figure 8A) in the presence of donor cells, as more cells stained for nuclear protein (Ki67) indicating that the cells were more proliferating as compared to the culture without donor cells where proliferating cell number were low. Similarly, apoptotic cells in the host compartment was significantly \((p < 0.05)\) declined on day 1 of culture in the presence of donor cells, as fewer cells stained for Annexin V (Figure 8B and Table 1).

Result showed that percentage of apoptotic and necrotic cells was significantly \((p < 0.001)\) declined in case of mice received transplantation as compared to that of un-transplanted recipient mice (Figure 9A, 9B and Table 2). This data was also supported by the expression of two critical cell cycle regulators cyclin A and p21. The expression of cyclin A in the larger population of host LSK cells, recovered from the mouse received transplantation, ensures that donor cells enhanced cell cycle activation as shown in Figure 10A and 10B.

6.4. Donor LSK cells retain high proportion of BrdU at 30 days of pulse-chase

To confirm that donor stem cells entered much faster in cell cycle than host, we performed BrdU pulse-chase experiments. Mice were given long pulse of BrdU for 10 days followed by 20 days of chase. The duration of pulse was maintained for long as LT-HSCs are known to be slowly entered into cell cycle. Results during pulse showed 68 ± 9% donor LSK cells incorporated BrdU, whereas in the same time only 20 ± 4.5% host LSK cells incorporated.
Results and discussion

Figure 8 A: In vitro proliferation of irradiated host cells

Sub-lethally irradiated host (CD45.1) cells were cultured in the absence (control) or in the presence (test) of un-irradiated CD45.2\(^+\) cells in contact or without contact. The host cells were analyzed for Ki67 staining by flow-cytometry. \(p < 0.001\) when compared to control.
Annexin V staining of cells

Control (culture without donor cells)

Test (co-culture with donor cells)

Control (culture without donor cells)

Test (non-contact culture with donor cells)

Figure 8 B: *In vitro* cyto-protection of irradiated host cells

Sub-lethally irradiated host (CD45.1) cells were cultured in the absence (control) or in the presence (test) of un-irradiated CD45.2+ cells in contact or without contact. The host cells were analyzed for Annexin V staining by flow-cytometry. p < 0.005 when compared to control.
Table 1: Effect of un-irradiated hematopoietic cells (contact and w/o contact) on proliferation and anti-apoptotic effects in irradiated bone marrow cells in culture

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Proliferation (Ki67(^+) cells %), M ± SEM</th>
<th>Apoptosis (Annexin V(^+) cells %), M ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct contact</td>
<td>Indirect contact</td>
</tr>
<tr>
<td></td>
<td>W/o donor cells</td>
<td>With donor cells</td>
</tr>
<tr>
<td>0</td>
<td>2.6 ± 0.10</td>
<td>2.6 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>2.4 ± 0.05</td>
<td>9.1 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>10.2 ± 0.15(^*)</td>
<td>20.2 ± 0.06(^*)</td>
</tr>
<tr>
<td>Significance</td>
<td>(^*p&lt;) 0.001</td>
<td>(^\ast p&lt;) 0.001</td>
</tr>
</tbody>
</table>

\(^*\)Represents the p-value between w/o and with donor cells in direct contact experiment
\(^\ast\)Represents the p-value between w/o and with donor cells in indirect contact experiment
Results and discussion

a  Host LSK cells without transplantation: Test for necrotic cells

b  Host LSK cells with transplantation: Test for necrotic cells

Figure 9A: In vivo cyto-protection of irradiated host cells.

Sub-lethally irradiated mice were left un-transplanted (a) or transplanted (b) with \(3 \times 10^4\) CD45.2LSK cells. Mice were sacrificed 3, 5 and 10 days of transplantation and host LSK cells were analyzed for necrotic cells by staining with PI. Representative dot-plots are shown. Means were significantly different (\(p < 0.001\)) when compared to without transplantated mouse Host LSK cells (\(n = 3\)).
Results and discussion

a. Host LSK cells with transplantation: Test for apoptotic cells

Sub-lethally irradiated mice were left un-transplanted (a) or transplanted (b) with $3 \times 10^4$ CD45.2LSK cells. Mice were sacrificed 3, 5 and 10 days of transplantation and host LSK cells were analyzed for apoptotic cells by staining with Annexin V. Representative dot-plots are shown. Means were significantly different ($p < 0.001$) when compared to without transplanted mouse Host LSK cells ($n = 3$).

Figure 9B: In vivo cyto-protection of irradiated host cells.
Table 2: Anti-apoptotic effect of graft on irradiated host cells

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Apoptotic CD45.1LSK cells (Annexin V⁺ in %), M ± SEM</th>
<th>Dead CD45.1LSK cells (PI⁺ in %), M ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without transplantation</td>
<td>With transplantation</td>
</tr>
<tr>
<td>3</td>
<td>26.60 ± 2.40*</td>
<td>8.20 ± 0.36*</td>
</tr>
<tr>
<td>5</td>
<td>18.06 ± 1.30</td>
<td>3.40 ± 0.37</td>
</tr>
<tr>
<td>10</td>
<td>10.90 ± 0.58</td>
<td>4.10 ± 0.75</td>
</tr>
<tr>
<td>Significance</td>
<td>*p &lt; 0.001</td>
<td>*p &lt; 0.001</td>
</tr>
</tbody>
</table>
Results and discussion

A. w/o transplantation with transplantation

Figure 10: Expression of cyclin A and p21cip1/waf1 in CD45.1LSK cells

A. Sub-lethally irradiated CD45.1 mice were transplanted with or without CD45.2LSK Cells. Mice were sacrificed after 3 days of transplantation and host LSK cells were sorted and immuno-stained for cyclin A protein. Representative images show that a few cells expressed cyclin A (arrow) in un-transplanted mouse than transplanted (magnification 600 x)

B. Host LSK cells of above two groups were compared for the expression of p21 gene by real time PCR. Bar diagram shows relative ΔCt values in two different conditions (n = 3).
the same analogue (Figure 11 top). These results suggest that relatively a small fraction of host LSK cells were in the cell cycle during the period of pulse. To know the proportion of BrdU retaining cells in both the compartments, a 20 days chase was conducted. Interestingly, all host and majority of the donor-derived LSK cells retained BrdU, though a modest dilution effect was noticed in case of donor LSK cells (Figure 11 bottom).

6.5. Donor LSK cells maintain high marrow turnover efficiency

In order to check whether donor-derived HSCs can maintain marrow regeneration capacity even after irradiation, we transplanted donor cells into sub-lethally irradiated mouse. Donor cells chimerism was routinely analyzed. After 7 months of transplantation (n=3), it was observed that donor cells was 76.4 ± 6.1% (Figure 12A). Further, to confirm the capability of the donor cells for marrow regeneration, these mice were exposed to radiation for the second round. After a month of re-irradiation, each mouse was examined for BM chimerism with respect to donor (CD45.2) cells. All mice showed high degree (76.8 ± 7.4%) of donor cells chimerism (Figure 12B). BM cells were further analyzed for LT-HSCs and ST-HSCs, results suggested no significant difference (percent) between donor and host in these two classes of stem cells; whereas in terms of absolute stem cell number, donor LT and ST- HSCs was much higher (Table 3).

6.6. Hematopoietic growth factor genes are highly expressed in early stromal compartment

Different hematopoietic growth factors/cytokines that support self-renewal of HSCs were studied. Since stromal cells express these factors, CD45+ cells were sorted from mice at different days of transplantation for real-time RT-PCR analyses of stem cell factor (SCF), fetal liver tyrosine kinase ligand (Flt3L), stem cell growth factor (SCGF), Jagged 2 (Jag2),
Results and discussion

Figure 11: Pulse-chase experiment for nuclear incorporation of BrdU

After transplantation as above, a group of mice were given BrdU pulse for 10 days, which was followed by chase for 20 days. First LSK cells were sorted and then stained for BrdU. The representative histogram (top) show 70.5% of donor (D) and 17.1% of recipient (R) LSK cells were labelled with BrdU. The representative histogram (bottom) shows 98% of recipient and 89% of donor cells retained BrdU. C: Control LSK cells (n = 3).
Results and discussion

Recipient

Donor

Mouse 1

Mouse 2

Mouse 3

Figure 12A: Donor cells chimerism

Mice were transplanted with $10 \times 10^6$ crude donor cells (CD45.2$^+$). Seven months of transplantation chimerism was determined. Dot-plot analyses show chimerism of donor (CD45.2) and host (CD45.1) cells for three mice ($n = 3$).
Experiment was initiated as before, after 7 months of transplantation mice were subjected to second cycle of irradiation and maintained for 1 month. Mice were sacrificed and BM cells were analyzed for chimerism. Representative dot-plot show 78% donor (CD45.2) cells chimerism (n = 3).
Table 3: HSC populations in bone marrow after second irradiation
The absolute numbers of HSCs were calculated by multiplying % stem cells with the total cell recovered.

<table>
<thead>
<tr>
<th>HSCs</th>
<th>Host LSKFlk2+ cells</th>
<th>Host LSKFlk2- cells</th>
<th>Donor LSKFlk2+ cells</th>
<th>Donor LSKFlk2- cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>0.033</td>
<td>0.006</td>
<td>0.008</td>
<td>0.003</td>
</tr>
<tr>
<td>Absolute number</td>
<td>1464</td>
<td>309</td>
<td>331</td>
<td>125</td>
</tr>
</tbody>
</table>

*Significantly (p < 0.01) higher as compared to same phenotype of the host cells.
Results and discussion

wingless-type MMTV integration site family members 3A (Wnt3a) ligand, vascular endothelial growth factor (VEGF), interleukin-3/6 (IL-3/6) genes. The results showed similar pattern of expression for above genes, having peak levels at day 2 and 3 (Figure 13). The expressions of these genes were significantly ($p < 0.05$) reduced from day 5 onwards. It was also observed that transient amplification of LSK cells continued till day 15 of transplantation despite of stiff decrease of gene expression from day 3 (Figure 3B and Figure 13).

6.7. Differential gene expression in microarray experiment

To study the differential expression of genes in stromal compartment at different times of marrow regeneration microarray experiments were conducted. The stromal cells (CD45<sup>-</sup>) obtained from day 0 (un-irradiated normal BL6/J mouse) was used as a control for normalizing gene expressions obtained from day 3, 5, 10, 15 and 30 of post transplantation. These results were compared amongst each other to identify genes, which were similarly up-regulated or down-regulated in all the time points with respect to the un-irradiated mouse. The whole study was done using Gene-spring GX software and R programming language package (Shinya Yamanaka et al 2007). In log<sub>2</sub> scale, genes expressed greater than 2 folds and genes expressed lower than 2 folds were designated as up-regulated and down regulated genes, respectively. In the microarray experiment, 175 genes were found to be up-regulated and 602 genes were down-regulated in day 5, 10 and 15 with respect to un-irradiated control mouse (Figure 14A).

6.8. Validation of microarray data by q-PCR with respect to few genes

The microarray result showed common up-regulation of 175 genes and common down regulation of 602 genes as shown in Figure 14A.
Results and discussion

Figure 13: Kinetics of gene expression by real time RT-PCR

BM cells were harvested at different time points following irradiation and transplantation; stromal cells (CD45−) were sorted and analyzed for relative (fold) expression of the hematopoietic growth factor genes compared to that in un-irradiated and un-transplanted mice. Bar diagrams show fold expressions of mRNAs for different growth factors at days 1, 2, 3, 5 and 15 of transplantation (n = 3). The expressions of SCF, SCGF, Jag2, Wnt3a, VEGF, Flt3, IL3 and IL6 genes were significantly reduced from day 5 onwards (p < 0.05)
RNA was isolated from the stromal cells (CD45') at day 0 (un-irradiated normal BL6/J mouse) and various days post transplantation (i.e. day 3, 5, 10, 15 and 30). Several genes were validated by q-PCR analysis. Majority of the genes showed expression trends similar to levels observed in microarray experiment, while certain genes did not exhibit expression levels corresponding to microarray experiment results (Figure 14B).

The overall list of genes was narrowed down by filtering the data using a cut off of +/- 2. From them, the up-regulated genes were selected. The genes which are key regulators of pathways involved in bone marrow cellular differentiation and proliferation were identified.

Interaction between the identified genes with other genes was analyzed using string database as shown in Figure 15. “STRING” is a biological database and web resource of known and predicted protein-protein interactions. The STRING database contains information from numerous sources, including experimental data, computational prediction methods and public text collections.

The genes which are novel and had no previous information present for the role in hematopoiesis were identified. The study was generally focused on the ligands or secretary growth factors which upon binding with its specific receptors may activate a pathway in the hematopoietic system and aid in HSC proliferation and maintenance.

Areg, an important ligand of the ErbB pathway and SCGF (also known as SCGF) of the Wnt Pathway were found to be highly up-regulated and were chosen to study in relation to HSC microenvironment.
Results and discussion

A. 

Figure 14: Global Gene expression analysis to identify genes of the stromal compartment (CD45) obtained from different time points of a physiologically compromised mouse model

Mice were transplanted with $10 \times 10^6$ crude donor cells (CD45.2^+). In various time points (5, 10, 15, 30 day) of transplantation CD45^- cells were sorted by FACS. Microarray was done on an agilent platform and various genes that were up-regulated and down-regulated are shown in A. Real time PCR analysis was done in some genes to validate the gene expression as shown in B (n = 3). Mean ± SEM of 3 independent experiments of RT-PCR is shown.
Results and discussion

Figure 15: String Database Analysis to see the molecular partners of Areg, Rac1 and SCGF

After identification of Areg, SCGF and Rac1 gene by microarray, string database analysis was done to study the interaction of these genes with other molecules.
6.9. Cloning of Areg ShRNA in pLKO.1-puro vector

Areg Sh-RNA oligos were heat annealed and ligated in pLKO.1-puro vector. The positive clones showed a sharp band on 1% agarose gel at 7 kb (desired size). The clones were confirmed by restriction digestion using the Sail restriction endonuclease. The positive clones were also validated by PCR using specific primers (Figure 16A).

6.10. Cloning of SCGF ShRNA in pLKO.1-puro vector

SCGF Sh-RNA oligos were also heat annealed and ligated in pLKO.1-puro vector. The positive clones showed a sharp band on 1% agarose gel at 7 kb (desired size). The clones were confirmed by restriction digestion using the Sail restriction endonuclease. The positive clones were also validated by PCR using specific primers (Figure 16B).

6.11. Cloning of Rac1 shRNA in pLKO.1-puro vector

Rac1 Sh-RNA clones were procured from sigma Aldrich, USA. Knockdown of Rac1 were generated in M210B4 cell line. These cell lines showed defects in the cell adhesion; there no further studies were carried out using these cell lines.

6.12. Knock down of Areg and SCGF in the stromal cells line (M210B4)

Sh RNA for Areg and SCGF were cloned into pLkO.1-puro vector. These construct was used to generate lentiviral particles which was further used for transduction experiments in M210B4 cell line. Optimum puromycin dose to kill the wild type cells were determined as shown in Figure 17A. M210B4 cells were exposed to different doses of puromycin (1μg/ml, 2μg/ml, 3μg/ml) and the dose at which all wild type cells died were selected as the optimum dose (3μg/ml). M210B4 cells are fibroblastic in nature as shown in Figure 17B when no puromycin was added to the culture.
Results and discussion

Figure 16: Positive selection of the sh-RNA lentiviral construct of Areg (A) and SCGF (B)

SalI (Unique Restriction Enzyme) was used for the restriction digestion of the Areg and SCGF Sh-RNA lentiviral construct. The desired 7 kb band size was obtained on 1% Agarose gel confirming the successful integration of the sh-RNA lentiviral construct in the pLkO.1-puro vector.
Results and discussion

A. Optimum puromycin dose to kill the wild type cells; M210B4 cells were exposed to different doses of puromycin (1µg/ml, 2µg/ml, 3µg/ml). The dose at which all cells died were selected as the optimum dose. Magnification 200 X.

B. Morphology of M210B4 cells was retained when no puromycin was added to the culture. All wild type cells (M210B4) died by puromycin selection while in case of empty vector only those cells carrying the puromycin resistance gene survived. Cells carrying transfer vector Areg, Rac1 and SCGF genes also survived separately in culture. However it was observed that cells with Rac1 gene lost adhesion property after 3-4 days in culture. Magnification 100 X.

Figure 17: Selection of Sh-RNA knockdown construct using puromycin (3µg/ml)
All wild type cells (M210B4) died by puromycin selection while in case of empty vector only those cells carrying the puromycin resistance gene survived. Cells carrying transfer vector Areg, Rac1 and SCGF genes also survived separately in culture. However it was observed that cells with Rac1 gene lost adhesion property after 3-4 days in culture.

The knockdown of Rac1, Areg and SCGF were confirmed by RT-PCR (n=3) from the c-DNA obtained from the transfected M210B4 cell line using gene specific primers (Figure 17C).

6.13. Co-culture studies of transduced and un-transduced M210B4 cells with HSCs (LSK)

Hematopoietic stem cells (LSK) were sorted from a GFP -BL6/J mouse having 98% purity in post sort. Fifteen thousand HSC cells were co-cultured with the transduced and un-transduced M210B4 cell line respectively.

6.14. HSCs grown in Areg and SCGF knockdown stroma effect total HSC cell recovery

Cells obtained from co-cultures of HSCs grown on Areg and SCGF knockdown stroma was counted by haemocytometer. It was observed that the total cell count obtained after day 5 and 10 post co-culture declined as that of HSCs grown on un-transduced cell line after day 5 and day 10 of co-culture. Percentage of GFP+ HSCs was found to be more in the wild type M210B4 cell line as compared to that in Areg and SCGF gene knockdown cell line on day 10 and day 5 of co-culture, as observed by microscopy (Figure 18A) and FACS (Figure 18B). A bar graph as shown in Figure 18C also depicts the same thing.
Figure 17: Lentiviral transduction for the generation of stable knockdown of Areg, Rac1 and SCGF separately in M210B4 cells

C. Real time PCR analysis was performed to detect the expression of Areg, Rac1 and SCGF in the generated knockdown stroma. Wild type M210B4 cells were used as normalizing control. Mean ± SEM of 3 independent experiments of RT-PCR is shown.
Results and discussion

Figure 18: Reduction in percentage of GFP+ cells in the co-culture of GFP+ HSCs in the wild type and knockdown (Areg and SCGF) M210B4 stromal cells

Percentage of GFP+ HSCs was found to be more in the wild type M210B4 cell line as compared to that in Areg and SCGF gene knockdown cell line on day 10 and day 5 of culture, as observed by (A) microscopy; Magnification 200X and (B) FACS. (C) A significant increase was observed in the GFP+ expression on Day 5 as well as Day 10, as compared with the HSCs cultured on the type stromal cells. Mean SEM of 3 independent experiments.
6.15. Evaluation of apoptosis and necrosis of hematopoietic stem cells when cultured separately on Areg and SCGF knock-down stroma

Knock down of the novel molecules Areg and SCGF, increases apoptosis of HSCs and thereby inhibits proliferation as compared to the wild type M210B4 cell line. In order to understand the role of Areg and SCGF in promoting HSC division, we used shRNA approach to knock down their expression independently in M210B4 cell line. The knock-down cell lines generated were irradiated (100 cGy) individually and cultured in a 6 well plate till 75% confluency. After 24 hrs, HSCs were sorted and fifteen thousand cells were co-cultured onto the irradiated Areg and SCGF knockdown stromal cell lines.

It was observed that in co-culture experiment of HSC with Areg knockdown stroma (cultured with and without SCF) apoptosis and necrosis in HSCs starts as early as two days of co-culture as compared to HSC cultured on normal stroma. Similar observation was noted when HSC were cultured on SCGF knockdown stroma (in presence and absence of SCF). When HSC were co-cultured with Areg knockdown stroma the percentage of apoptotic HSC was about five fold higher than the percentage apoptosis seen in HSC cultured with normal stroma ($p < 0.005$). Similarly the percentage apoptosis was a eight fold ($p < 0.005$) higher when HSC were cultured with SCGF knockdown stroma (Figure 19A and Figure 19B).

Similarly, it was observed that when HSCs were co-cultured with Areg knockdown stroma the percentage necrotic HSC was about three fold more when compared to the HSCs cultured with wild type stroma. In case of the HSCs cultured with SCGF knockdown stroma; the percentage necrotic was found to be around sixteen fold as compared to the HSCs on the wild type stroma as shown in Figure 19C and Figure 19D.
Results and discussion

Figure 19: The co-cultured HSCs grown in the knockdown stroma show increased apoptosis grown with and without SCF.

A. Representative histogram showing the Annexin V positive LSK cells grown on the wild type and the knockdown stroma after 2 days of culture with and without SCF.

B. A significant increase in percentage apoptosis was observed in the HSCs cultured in the knockdown stroma (Areg and SCGF separately) as compared to that in the wild type (p < 0.001). Mean ± SEM of 3 independent experiments.
Results and discussion

C. Representative histogram showing the Sytox Red positive LSK cells when grown on the wild type and the knockdown stroma after 2 days of culture with and without SCF.

D. A significant increase in percentage necrosis was observed in the HSCs cultured in the knockdown stroma (Areg and SCGF separately) as compared to that in the wild type (p < 0.001). Mean ± SEM of 3 independent experiments.
6.16. HSCs cultured on Areg and SCGF knock down M210B4 cell line inhibits hematopoietic colony forming potential

HSC have the ability to differentiate into erythrocytes, granulocytes, monocytes, platelets etc. This ability is functionally analyzed by *in vitro* hematopoietic colony forming assays (CFUs or CFCs). It was observed that when HSCs were cultured separately on the Areg knockdown and SCGF knockdown M210B4 cell line, it did not result in formation of hematopoietic colonies. However, when HSCs were cultured on the normal M210B4 cell line majority of them differentiated into macrophage and granulocyte (Figure 20A). Bar graph shows a significant reduction in the colony forming potential in the HSCs grown on separately on the gene knockdown stromal cells (Areg and SCGF) as compared to the wild type stromal cells (Figure 20B).

6.17. Determination of hematopoietic lineage markers by FACS

FACS analysis of HSCs co-cultured with Areg and SCGF knockdown stroma separately showed that there is no population of any differentiated cells as compared to the normal co-culture experiment. It was observed that in normal co-culture experiment about 25% HSCs got differentiated in Gr1 and Mac1 and 5-10% as B-cells. This result was in accordance with the colony assay data (Figure 21A). Bar graph shows no expression of myeloid and lymphoid lineages in the HSCs grown on gene knockdown stromal cells (Areg as well as SCGF) as compared to the wild type stromal cells; shown in Figure 21 B.
Results and discussion

A. HSCs cultured on wild type stromal cell show different hematopoietic stem cell colonies like Macrophage (M), Granulocyte (G), erythrocytes (E) and mixed colonies (GEMM) while the HSCs cultured on Areg or SCGF knockdown stroma show no colony forming potential.

B. A significant reduction in the colony forming potential in the HSCs grown on gene knockdown stromal cells (* Areg) as compared to the wild type stromal cells. Similar result was obtained for the HSCs cultured on the SCGF knockdown stroma.

(W/T: wild type; KD: knockdown). Mean ± SEM of 3 independent experiments.
Results and discussion

A.

i. Wild Type Stromal cells

ii. Knockdown Areg Stromal cells

B. The Bar graph shows no expression of myeloid and lymphoid lineages in the HSCs grown on Areg knockdown stromal cells as compared to the wild type stromal cells. Similar results were also observed for the HSCs grown on SCGF knockdown stromal cells. Mean ± SEM of 3 independent experiments.

Figure 21: SCGF and Areg knockdown in stroma influences myeloid and lymphoid expression in the HSCs

A. Representative contour plots shows the myeloid markers Mac-1 and Gr-1; lymphoid markers B220 for B cells and CD3 for T cells in the HSCs grown on (i) wild type and gene knockdown (ii) Areg and SCGF stromal cells.
7. Discussion

Bone marrow transplantation (BMT) is generally practiced in cases of non-malignant and malignant hematological disorders (Conrad et al., 1998). The purpose of transplantation is to reconstitute/rejuvenate hematopoietic system for continuous supply of blood lineages and to induce graft versus leukemic (GVL) reaction (in case of leukemia). Though HSC is the major player to induce physiological changes in the recipients, the cellular microenvironment is found to play a critical role for engraftment, proliferation and differentiation of the cells. In the present study it was shown that, under a competitive environment, donor LSK cells proliferate much rapidly than the host during marrow regeneration. Cell cycle status suggested that there exists a reversible switch in regenerating marrow, which induces quiescent stem cells into an actively proliferating state and then bring back some of them into the quiescent state. Most of the clinical transplantation protocols use high-dose total body irradiation (TBI) to prevent rejection of the donor graft (Moore et al., 1997) and create a condition (by increasing vascular permeability) in which stem cells are able to home to the BM niches (Cui et al., 1999; Shirota et al., 1992).

Further, high dose of TBI allows engraftment of cells by creating more space in the bone marrow or minimizing competition between remnant host stem cells and infused donor stem cells (Stewart et al., 1998; Quesenberry et al., 1994). Earlier study showed that degeneration of stroma can cause poor retention of donor cells in BM of lethally irradiated mice (Madhusudhan et al., 2004).

In this study mice were conditioned by sub-lethal dose of irradiation. In order to examine the proliferation kinetics of donor-derived LSK cells and possible competition with that of
host, crude BM cells (CD45.2) were transplanted in sub-lethally irradiated hosts (CD45.1). A concomitant increase of LSK cells in BM with the increase of cell dose was observed. However, early detection of donor LSK cells (within 24 h of transplantation) was observed at a dose of $10 \times 10^6$ cells. Henceforth, all experiments using crude cells were conducted using the same dose of $10 \times 10^6$ cells.

A transient amplification of host-derived LSK cells within first few days of transplantation was seen and was significantly higher than the LSK population in control mice which received no graft. In contrast, at later time points a suppressive effect was seen in host compartment, mainly due to competition between two classes of cells for the niche, one that was exposed to radiation and the other not. Interestingly, we showed that soluble factors, secreted by donor hematopoietic cells were able to rescue some host cells undergoing apoptosis and also induce their proliferation. As a result of which, the net effect was increase in cell number. This was supported by the expression of critical cell cycle regulators, cyclin A which accumulates at the G1/S check point entry and associated with cdk2/cdk1 cyclin-dependent kinases (Girard et al., 1991). It's replication suggesting active involvement in DNA synthesis. The expression of cyclin A in the larger population of host LSK cells, recovered from the mouse which received transplantation implies that donor cells enhanced cell cycle activation. Irradiation, like several therapeutic agents, induces DNA damage resulting growth arrest of the cells. In response to DNA damage, p53 transcriptionally activates the cyclin-dependent kinase inhibitor p21 and in turn inhibits cell proliferation for allowing DNA repair (Walker et al., 1991). In HSCs, p21 maintains quiescence; conversely decline/absence of p21 has been marked with the proliferation and increase in absolute number of cells (Cheng et al., 2000). The stiff decline in p21 expression suggested that radiation-exposed host LSK cells might have recovered much.
early from DNA repair path and thus experienced faster proliferation in the presence of donor cells than the absence. We propose that early entry in cell cycle was induced by some soluble factors secreted by donor cells. At present the identity of these soluble factors is not known, we speculate that they are different from the factors secreted by the stromal cells. It is needless to mention that at least at transcription level stromal-derived factors were abundant in host compartment. Earlier, it was shown that human peripheral blood leucocytes conditioned media can provide protection to rat cardiomyocytes in culture against necrosis and apoptosis, and a down-stream regulator of Wnt pathway ‘WISP1’ expression is higher was one of the factors expressed by the cells (Cheng et al., 2000). It was discernible from our preliminary experiments that WISP1 expression is higher in non-irradiated than irradiated cells. IL-12 secreted by non hematopoietic cells is known to confer cyto-protective effect to HSCs against irradiation (Chena et al., 2007). Other interesting features of this study were enormous expansion of donor-derived LSK cells and to achieve normal BM cellularity. Previous reports show that treatment of mice with CY and G-CSF leads to 10-fold proliferation of endogenous HSCs, prior to the mobilization of cells in the peripheral blood (Morrison et al., 1997; Wright et al., 2001). This study doesn’t give any evidence of mobilization of cells in the peripheral blood, despite massive expansion of stem and progenitor cells. However, the present study deals with marrow regeneration by the engrafted cells, whereas the above reports (Morrison et al., 1997; Wright et al., 2001) were concerned with egression and mobilization of cells in the peripheral blood.

A competitive marrow repopulation assay (MRA) was used for assessment of the functional HSCs in the graft (Harrison et al., 1993). The results of MRA not only ensured that HSCs amplified in the primary recipient, but also indicated that they were enriched in
Results and discussion

terms of stemness and/or engraftability. For same dose of transplantation, the recovery of donor’s LSK cells was 3 to 5 folds higher at day 15 than day 10. This is the first experimental evidence to demonstrate that symmetric self-renewal divisions of some donor HSCs might have occurred to regenerate depleted marrow cells following radiation injury. Symmetric division of stem cells was reported in other tissues. For example, in rodent forebrain, increase in symmetric cell divisions was observed after the stroke (Zhang et al., 2004). However, it is generally believed that under steady-state conditions (during normal physiological processes), stem cells divide asymmetrically.

Quiescent HSCs are maintained in the osteoblastic niche (Gong et al., 1978; Nilsson et al., 2001). During the course of asymmetric division they are believed to move out from the niche, and once the division process is completed the daughter stem cells return to the niche (Suda et al., 2000). The stiff drop in quiescent (G0) cells with concomitant increase in G1 and SG2M phase cells suggested that most of the donor LSK cells were actively proliferating in first 15 days of transplantation. Later, with increase in BM cellularity, these cells gradually withdrew from cell cycle and entered the quiescent state. The presence of donor-derived stem/progenitor cells in the trabicular bone area corroborated this observation. Earlier it has been shown that host-derived HSCs can enter into active state from dormancy and vice versa, under the influence of hematopoietic stress (Arai et al., 2007). BrdU is incorporated during DNA replication; un-dividing cells consistently retain BrdU in the nuclei. Since under steady-state conditions stem cells rarely divide, nuclear retention of BrdU confirms quiescence of the cells. The results of pulse-chase experiments supported our earlier notion that majority of the donor-derived LSK cells were in active proliferation state. Further, it was revealed that both in the donor and in the recipient compartment a commendable fraction of LSK cells retained BrdU; these results did not
conclude that all labeled cells were in quiescence state. Despite a greater fraction of label retaining LSK cells were present in the host compartment, as compared to that in donor compartment. The results after the second exposure of irradiation confirmed that donor LSK cells can confer high marrow turnover efficiency in this irradiation model. Marrow regeneration by donor and host stem cells following irradiation not only depends on the number of short-term and long-term HSCs, micro-environmental cues also have a pivotal role (Veiby et al., 1996). On the basis of microarray results, we selected eight hematopoietic growth factors and cytokines for their gene expressions in early stage of marrow regeneration. *In vitro* culture studies showed that these factors alone or in combination induces self-renewal of mouse and human primitive stem and progenitor cells, and also prevents apoptosis (Brandt et al., 1992; Lemoli et al., 1993; Kobayashi et al., 1996; Shah et al., 1996). Although the expression of gene does not always correlate with the synthesis of corresponding protein, we assumed that there may be transient expression of growth factors by the stromal cells. These results suggest that the presence of these growth factors at higher concentrations is required for activation of stem and progenitor cells. Once the cells are activated, their fate is decided by the intrinsic factors.

We have also shown here a particularly interesting observation about stem cell growth factor (SCGF), also known as Clec11a, which was found to be highly expressed at the initial phase of marrow regeneration along with other hematopoietic growth factor genes. Earlier studies have shown that the SCGF gene was highly expressed in human, rat and mouse stromal cells (Mio et al., 1998). Whether SCGF can independently act on primitive stem cells or not is not clear, but this and previous (Mio et al., 1998) results indicate that it may have a link with the primitive HSCs. *In vitro* experiments showed that SCGF can
potentiate the effect of many cytokines for expansion of human CD34*Lin* or CD34*CD38* cells (Hiraoka et al., 2001).

Our microarray data showed that various genes were up-regulated and down-regulated in the stromal compartment. Few genes were validated by real time PCR and out of which Areg and SCGF were chosen to study their potential role in this hematopoiesis. Knockdown studies were carried out in the stromal compartment (M210B4 cell line) to know the importance of these genes.

M210B4 cells were stably transfected with short hairpin RNA (shRNAs) against the cloned Areg and SCGF genes. Successful knock-down of these genes was achieved with 75% reduction in expression 24 hrs after transfection. To test the hypothesis that knockdown of Areg and SCGF in M210B4 cell line would lead to changes in expression of HSCs upon co-culture, we analyzed the expression of LSK cells (with GFP as a reporter) and apoptotic cells after 10 days. Knockdown of Areg and SCGF in M210B4 cells resulted in higher apoptosis of the co-cultured HSCs as compared to HSCS cultured on the wild type M210B4 cells.

Areg is a ligand for EBRR pathway. According to Nickerson et al. (2012) osteoblasts cells and breast cancer cells express the epidermal growth factor receptor (EGFR) and produce ErbB family ligands, suggesting participation of Areg in autocrine and paracrine signaling within the bone microenvironment. Inhibiting autocrine EGFR signaling in breast cancer cells may provide a means for reducing paracrine factor production that facilitates microenvironment support in the bone and mammary gland (Nickerson et al., 2012).

While stem cell growth factor (SCGF; also called C-type lectin domain family member 11A [Clec11a]), a hematopoietic growth factor important for development of erythroid and myeloid progenitors, was one of the most differentially expressed genes (Mio et al., 1998).